The Ubiquitin-Specific Protease Family from Arabidopsis. *At*UBP1 and 2 Are Required for the Resistance to the Amino Acid Analog Canavanine[1]

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Ubiquitin-specific proteases (UBPs) are a family of unique hydrolases that specifically remove polypeptides covalently linked via peptide or isopeptide bonds to the C-terminal glycine of ubiquitin. UBPs help regulate the ubiquitin/26S proteolytic pathway by generating free ubiquitin monomers from their initial translational products, recycling ubiquitins during the breakdown of ubiquitin-protein conjugates, and/or by removing ubiquitin from specific targets and thus presumably preventing target degradation. Here, we describe a family of 27 *UBP* genes from Arabidopsis that contain both the conserved cysteine (Cys) and histidine boxes essential for catalysis. They can be clustered into 14 subfamilies based on sequence similarity, genomic organization, and alignments with their closest relatives from other organisms, with seven subfamilies having two or more members. Recombinant *At*UBP2 functions as a bona fide UBP: It can release polypeptides attached to ubiquitins via either α- or ϵ-amino linkages by an activity that requires the predicted active-site Cys within the Cys box. From the analysis of T-DNA insertion mutants, we demonstrate that the *AtUBP1* and 2 subfamily helps confer resistance to the arginine analog canavanine. This phenotype suggests that the *At*UBP1 and 2 enzymes are needed for abnormal protein turnover in Arabidopsis.

The ubiquitin/26S proteasome proteolytic pathway plays an important role in eukaryotic cell growth, development, stress responses, and environmental adaptation by degrading short-lived and abnormal proteins (Hershko and Ciechanover, 1998; Vierstra, 1996; Callis and Vierstra, 2000). In this pathway, ubiquitin functions as a reusable tag to target specific proteins for breakdown. Via an ATP-dependent reaction cascade involving the sequential action of three classes of enzymes, E1s, E2s, and/or E3s, chains of ubiquitins become attached to proteolytic substrates through an isopeptide bond between the C-terminal Gly-76 of ubiquitin and a Lys residue in the target. These chains then serve as degradation signals for the 26S proteasome, a 2-MDa multisubunit protease that breaks down the protein into small peptides and amino acids but releases the ubiquitins intact.

Both the characterization of ubiquitin/26S proteasome pathway mutants and the analysis of individual substrates indicate that the pathway degrades a wide variety of short-lived proteins (Hershko and Ciechanover, 1998; Callis and Vierstra, 2000). To define how these substrates are chosen, most studies have focused on the E2/E3 enzymes that direct ubiquitin attachment. However, recent data indicate that the steps that release ubiquitins from targets and generate free monomers can also affect the selectivity of the pathway and the half-life of a substrate (Wilkinson, 1997; D'Andrea and Pellman, 1998; Chung and Baek, 1999). These steps are performed by a unique group of deubiquitinating enzymes (DUBs); they are thiol proteases that specifically cleave the peptide bond between the C-terminal Gly of ubiquitin and covalently attached polypeptides. Yeasts and animals contain a number of DUBs that vary substantially in sequence, suggesting that they recognize distinct substrates and/or have discrete functions (Wilkinson, 1997; D'Andrea and Pellman, 1998). Mutant analyses have implicated specific DUBs in numerous cellular processes, including cell growth (Papa and Hochstrasser, 1993; Zhu et al., 1996; Naviglio et al., 1998), cell differentiation (Chung et al., 1998; Lindsey et al., 1998; Liu et al., 1999), eye development (Huang et al., 1995; Taya et al., 1998; Taya et al., 1999), neural function (Wilkinson et al., 1989; Hegde et al., 1997; Leroy et al., 1998), coordinated DNA replication (Singer et al., 1996), gene silencing (Moazed and Johnson, 1996; Kahana and Gottschling, 1999), endocytosis (Galan and Haguenauer-Tsapis,

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1997), oncogenesis (Nakamura et al., 1992; Papa and Hochstrasser, 1993; Gray et al., 1995), heat shock (Baxter and Craig, 1998), and the breakdown of abnormal proteins (Papa and Hochstrasser, 1993; Amerik et al., 1997).

Enzymatic analyses indicate that DUBs have three general roles in the ubiquitin/26S proteasome pathway, each of which can profoundly influence the overall activity and/or specificity of the pathway (Fig. 1). One role is to generate ubiquitin monomers from the initial translation products of ubiquitin genes (Callis and Vierstra, 1989; Eytan et al., 1993; Baek et al., 1998). Ubiquitin is unusual in that it is naturally synthesized as a translational fusion. These fusions contain either a single ubiquitin fused to the N terminus of an unrelated protein (ubiquitin extension) or tandem repeats of ubiquitin linked head-to-tail and capped at the C terminus with one or more additional amino acids (polyubiquitin). DUBs are essential for releasing ubiquitin monomers from these fusions by cleaving the α-amino peptide bond after each ubiquitin moiety. The second role of DUBs is to regenerate free ubiquitin monomers during the breakdown of ubiquitin-protein conjugates by the 26S proteosome. In this case, DUBs remove peptide fragments that remain attached following hydrolysis of the target and disassemble the multiubiquitin chain (Hadari et al., 1992; Papa and Hochstrasser, 1993; Amerik et al., 1997). The third role is to deubiquitinate conjugates; in this case both the target and the attached ubiquitins are released intact (Chung et al., 1998; Taya et al., 1998; Taya et al., 1999). The last two roles are accomplished by cleaving isopeptide bonds in which the C-terminal Gly of ubiquitin is attached to lysyl ϵ-amino groups.

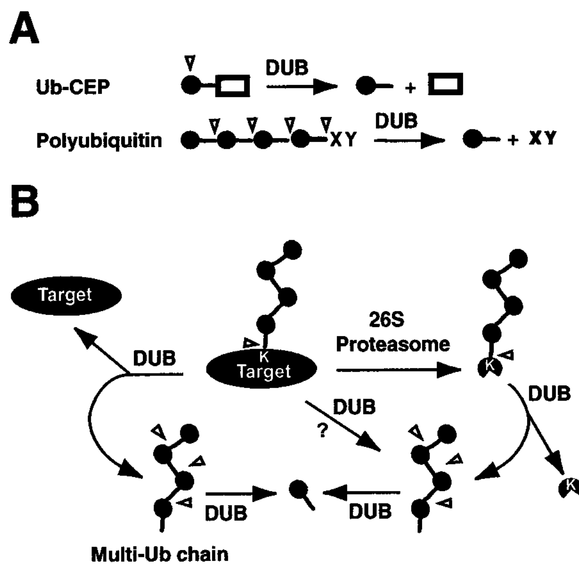

Figure 1. Possible functions of DUBs in the ubiquitin/26S proteasome proteolytic pathway. A, Generating free ubiquitin monomers from the translation products of polyubiquitin and ubiquitin-extension genes by cleaving the α-amino peptide bonds following each ubiquitin moiety. XY represent additional amino acids appended to the C-terminal Gly of the last ubiquitin repeat. B, Cleaving ubiquitin linked either to itself or to other proteins through ϵ-amino isopeptide bonds. Two routes are possible. DUBs could remove multiubiquitin chains bound to proteins, releasing both intact, and then the free ubiquitin chains would be disassembled into free ubiquitin monomers. During or following degradation of the target, free multiubiquitin chains could be released from peptide fragments of the target and then would be disassembled into free ubiquitin monomers. White arrowheads indicate the various bonds cleaved by DUBs.

Substrate recognition by all DUBs is highly dependent on the ubiquitin moiety, especially the C-terminal Gly (Wilkinson, 1997). However, UBPs are remarkably non-specific with respect to the appended polypeptide. For those cleaving isopeptide bonds, all attached polypeptides appear to be acceptable. For those cleaving peptide bonds, all translational fusions can be tolerated with the exceptions of appended sequences beginning with Pro, which are often resistant to cleavage. DUBs are divided into two general groups based on their amino acid sequence and substrate specificity (Wilkinson, 1997). One group called ubiquitin C-terminal hydrolases (UCHs) is composed of relatively small proteins (20–30 kD) that are structurally defined by the presence of a catalytic triad consisting of a positionally conserved Cys, His, and Asp (Johnston et al., 1997). In vitro, UCHs can remove small molecules (e.g. ester adducts and lysines) and peptides (Wilkinson, 1997), some of which attach non-specifically by reacting with activated ubiquitin during its conjugation cascade. UCHs can also process short multiubiquitin chains (Lam et al., 1997) and ubiquitin precursors (Pickart and Rose, 1985), suggesting a role in the production of free ubiquitin monomers.

The second group of DUBs, called ubiquitin-specific proteases (UBPs), cleaves ubiquitins linked to larger proteins by either peptide or isopeptide bonds. Enzymes in this group vary greatly in size but can be easily identified by the presence of two conserved catalytic motifs; one contains an essential Cys (Cys box) and the other contains two essential histidines (His box; Huang et al., 1995; Wilkinson et al., 1995; Wilkinson, 1997). Outside of these domains, their amino acid sequences diverge, possibly reflecting specific targets and/or cellular functions. Large families of UBPs have been identified in a variety of organisms. For example, whereas yeast (*Saccharomyces cerevisae*) has a single UCH, it contains 16 different UBPs (Wilkinson, 1997).

Despite their potential importance, little is known about UBPs in plants. Prior to this study, only three have been described, *AtUBP3*, *4*, and *5* from Arabidopsis (Chandler et al., 1997; Rao-Naik et al., 2000), and no *UBP* mutants had been reported. Using the conserved Cys and His boxes as queries, we have identified 24 additional *UBP* genes in the near finished Arabidopsis genome. Protein sequence comparisons cluster the complete gene collection into 14

possible *AtUBP* subfamilies, with seven having two or more members. Substantial diversity among the subfamilies suggests that each may have unique roles in Arabidopsis physiology, growth, and development. Analysis of a subfamily encoded by *AtUBP1* and *2* indicates that these proteins are bona fide UBP enzymes, capable of cleaving ubiquitin linked via peptide or isopeptide bonds. T-DNA insertion mutants of *AtUBP1* and *2* are phenotypically normal under standard growth conditions. However, the single and double homozygous plants are hypersensitive to the amino acid analog canavanine (CAN), supporting a role for these UBPs in particular, and the ubiquitin/26S proteasome pathway in general in aberrant protein turnover in plants.

RESULTS

Identification of UBPs in Arabidopsis

Sullivan et al. (1990) first reported that plants have UBP-like activities capable of cleaving ubiquitin attached to other proteins via peptide or isopeptide linkages. To identify the responsible enzymes, we used the sequence of yeast UBP4 (Papa and Hochstrasser, 1993) as the query to search the Arabidopsis expressed sequence tag (EST) database for related proteins. Various yeast and Arabidopsis UBP sequences subsequently were used to examine the Arabidopsis bacteria artificial chromosome (BAC) and EST databases for additional candidate genes. This extensive search (last completed on September 26, 2000) ultimately identified 27 distinct genes that encode proteins with both the Cys- and His-box signature motifs (Wilkinson, 1997). Three of these Arabidopsis genes (*AtUBP3-5*) have been described recently (Chandler et al., 1997; Rao-Naik et al., 2000). Partial or complete cDNAs have been identified for 21 of the additional 24 genes (the exceptions being *AtUBP11*, *18*, and *19*), indicating that most *AtUBP* family members (at least 24 of the 27) are actively expressed. By comparing the genomic sequences with their corresponding cDNAs, or by deducing intron/exon boundaries using alignments with possible paralogs, the complete coding regions were predicted for all 27 (Chandler et al., 1997; Rao-Naik et al., 2000; data not shown). In many cases, these coding sequences disagreed with those annotated in the AGI database.

Figure 2 shows the organization of the *At*UBP protein family and the relationship of some members to possible orthologs from other species. As found in other organisms, Arabidopsis UBPs vary considerably in size with lengths ranging from 365 to 1,116 amino acids. As expected, each contains the Cys and His boxes essential for catalysis. Within these boxes are the positionally conserved Cys and His residues that comprise parts of the active-site (Fig. 3). The Cys boxes of the *At*UBP family show high conservation both in sequence (60%–100% similarity) and length (all are 18 residues). In contrast, the His boxes are more diverse in sequence (40%–100% similarity) and show substantial differences in length (55–100 residues) as a result of several insertions/extensions (Fig. 3). In addition, the collection of *At*UBPs contains several less conserved motifs common among UBPs (Wilkinson, 1999); these include the Q, G, L, and F boxes, defined by the presence of one or more of these amino acids in their respective domains (Fig. 2). The function(s) of these domains are unknown at present.

Using overall amino acid sequence homology, the positions of the Cys, His, Q, G, L, and F boxes, the presence of additional protein sequence motifs, and the positions of known or predicted introns/exons, the family of *AtUBP* genes was tentatively clustered into 14 subfamilies. In all cases, these four criteria were in agreement, supporting our subfamily classification (Figs. 2 and 3; data not shown). Percent amino acid sequence similarity among members of the predicted subfamilies ranged from 95% (for the *At*UBP3 and 4 subfamily) to 55% (for the *At*UBP20 and 21 subfamily) but dropped to <40% when nonfamily members were compared. As can be seen in Figure 2, discriminating features of each subfamily include the presence of N- and/or C-terminal extensions beyond the Cys and His boxes and insertions that often separate the G and L motifs. Seven of the subfamilies have two or more members, including the *AtUBP5, 8-11* subfamily, and the *AtUBP15-19* subfamily that contain five members each. The remaining seven subfamilies contain only a single gene (*AtUBP14* and *22-27*). Outside of the six conserved regions, the *At*UBP proteins display little similarity outside of their subfamilies, suggesting that most if not all subfamilies have unique functions and/or recognize distinct substrates.

A number of the UBP subfamilies are predicted to contain additional sequence motifs, including zinc fingers, MATH, ubiquitin-like, and ubiquitin-associated (UBA) domains that may insinuate function (Fig. 2). Potential zinc fingers were found near the N terminus of the *At*UBP1-2, *At*UBP14, and the *At*UBP15-19 subfamilies. These fingers often mediate direct protein-protein interactions following chelation of a zinc ion by positionally conserved Cys and His residues (Freemont, 1993; Takatsuji, 1998; Tyers and Jorgensen, 2000). The *At*UBP12-13 subfamily contains a MATH domain common among a number of proteins, including TRAF-related proteins and the meprin family of metalloproteases, and may be involved in dimerization or protein-protein interactions (Uren and Vaux, 1996). Ubiquitin-like domains were detected in *At*UBP6-7 and *At*UBP26. Similar to their yeast ortholog *Sc*UBP6 (Wyndham et al., 1999), this domain is near the N terminus of *At*UBP6 and 7. However for *At*UBP26, the ubiquitin-like domain is near its C terminus. The ubiquitin-like domain could help these UBPs bind to ubiquitin-interacting pro-

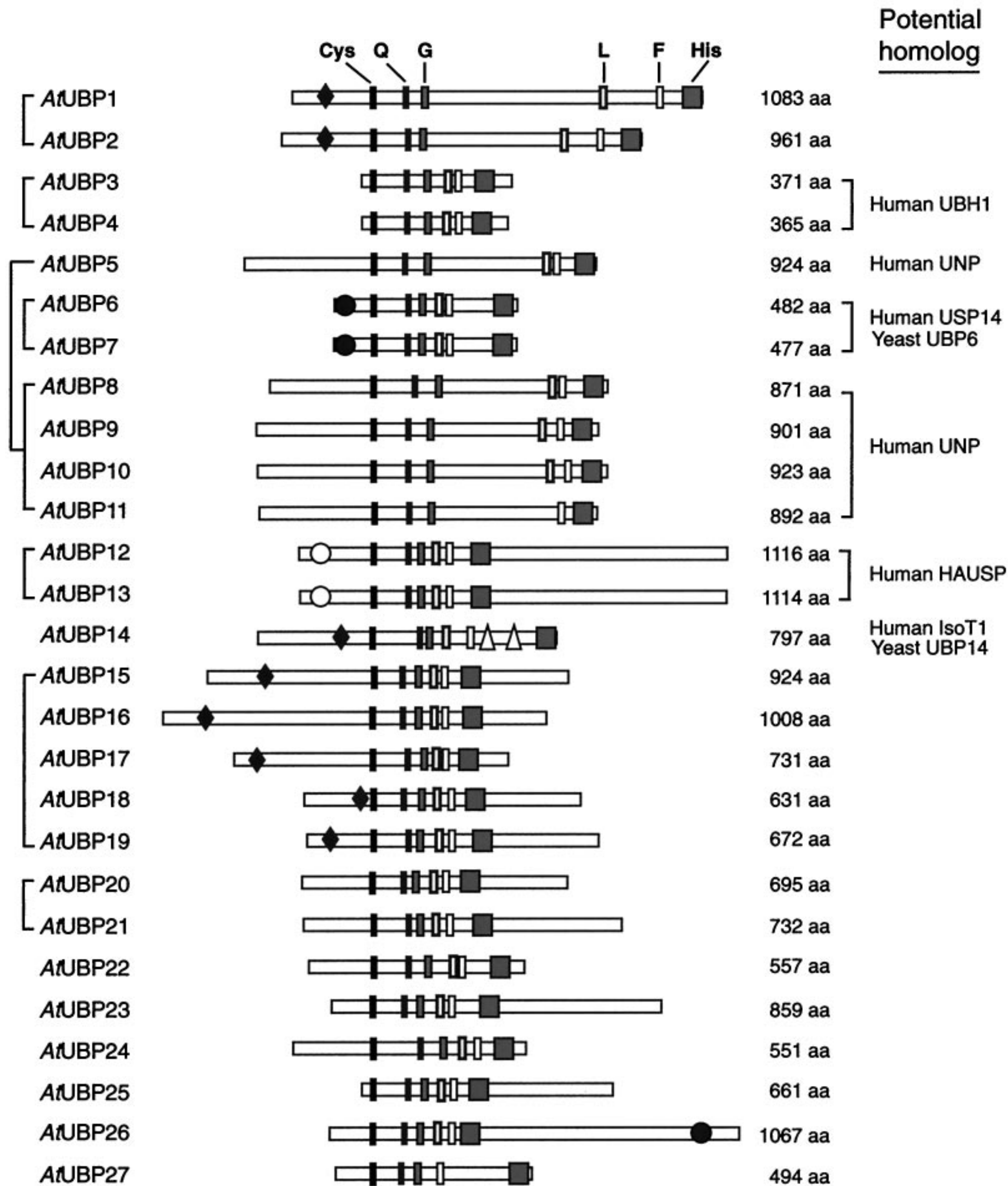

Figure 2. Structure of the members of the Arabidopsis UBP family. Locations of the Cys, Q, G, L, F, and His boxes are indicated. *At*UBP proteins with similar structures are grouped by brackets. Predicted amino acid lengths are shown on the right. Potential orthologs in yeast and animals are indicated if available. Amino acid sequence alignments of the Cys and His boxes are shown in Figure 3. *At*UBP3, 4, and 5 were recently described by Chandler and Callis (1997) and Rao-Naik et al. (2000). Accession number for the other *At*UBP sequences are: *At*UBP1 (AF302658), *At*UBP2 (AF302659), *At*UBP6 (AF302660), *At*UBP7 (AF302661), *At*UBP8 (AF302662), *At*UBP12 (AF302663), *At*UBP14 (AF302664), *At*UBP15 (AF302665), *At*UBP16 (AF302666), *At*UBP17 (AF302667), *At*UBP20 (AF302668), *At*UBP21 (AF302669), *At*UBP22 (AF302670), *At*UBP23 (AF302671), *At*UBP24 (AF302672), *At*UBP25 (AF302673), *At*UBP26 (AF302674), and *At*UBP27 (AF302675). The remaining *At*UBP proteins can be located in various BAC clones annotated in the AGI database: *At*UBP9 (AF118222), *At*UBP10 (AF118222), *At*UBP11 (AC006424), *At*UBP13 (AC0016795), *At*UBP18 (AL031004), and *At*UBP19 (AC006954). ♦, Indicates the presence of potential zinc finger; ●, indicates the ubiquitin-like domains; ○, indicates the MATH domains; △, indicates the UBA domains.

teins; for *Sc*UBP6, it is dispensable for catalytic activity (Wyndham et al., 1999). Two consensus UBA domains are located near the C terminus of *At*UBP14. UBA domains have been found in a number of proteins related to ubiquitin metabolism, including E2s, E3s, and other UBPs (Amerik et al., 1997; Bates and Vierstra, 1999; Hofmann and Pickart, 1999). It has been proposed that this motif binds ubiquitin noncovalently but its function is unknown (Hofmann and Pickart, 1999).

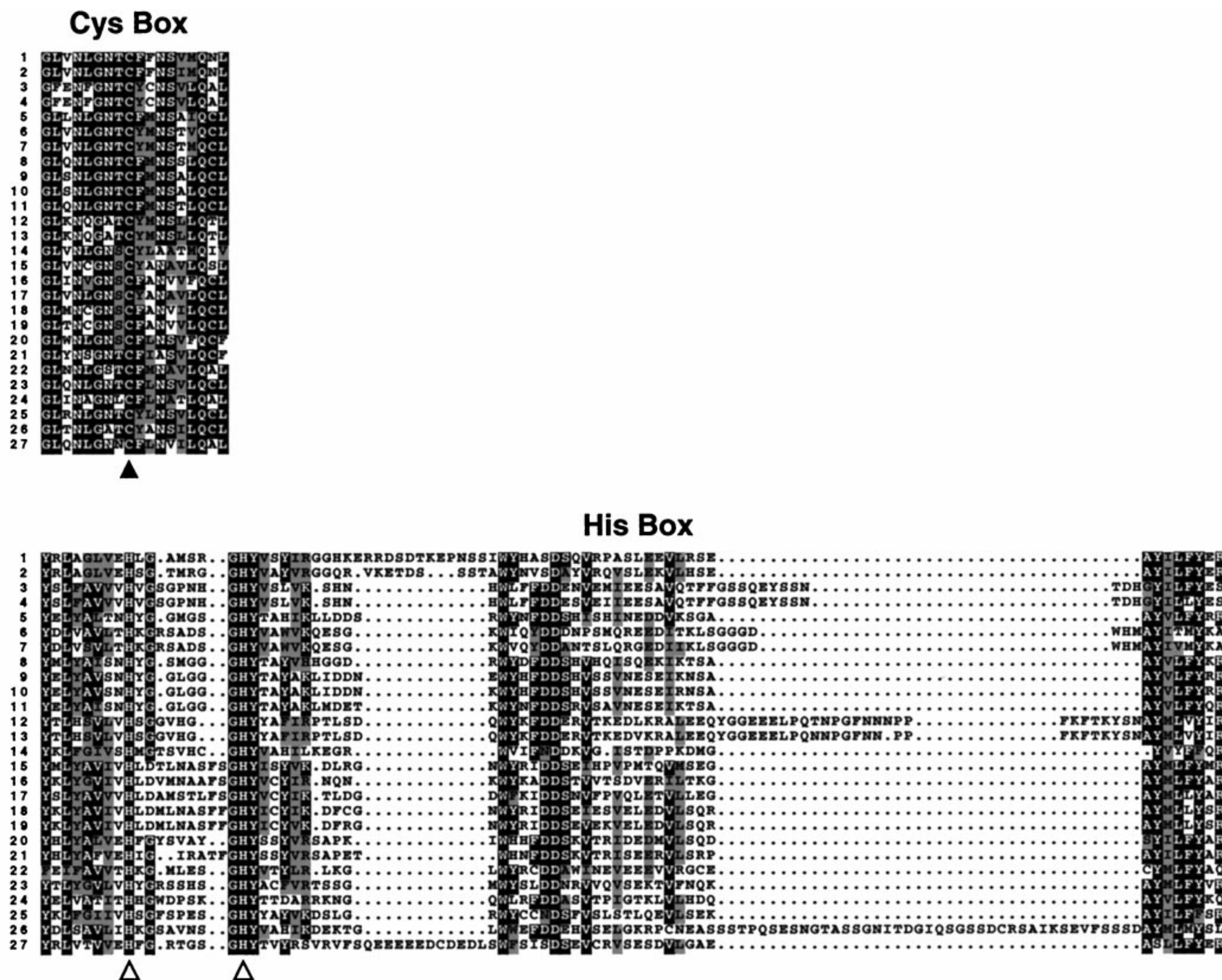

Figure 3. Alignment of the Cys and His boxes from the members of the Arabidopsis UBP family. Black and white arrowheads indicate the positions of the essential Cys and His residues, respectively. Reverse type and gray boxes denote identical and similar amino acids, respectively. Dots indicate gaps. Comparisons were made with the University of Wisconsin-Genetics Computer Group program Pileup and displayed by MacBoxshade 2.7.

*At*UBP1 and 2 Subfamily

To further define the functions of the Arabidopsis UBPs, we continued an in-depth characterization of the *At*UBP 1 and 2 subfamily. To confirm that these two genes represent the entire subfamily, genomic DNA from the ecotype Wassilewskija (WS) of Arabidopsis was subjected to DNA gel-blot analysis using either *AtUBP1* or *2* as the probe. As can be seen in Figure 4, only *AtUBP1*- and *2*-derived fragments could be detected following either high- or low-stringency washes after digestion of the genomic DNA with three different restriction enzymes. These results indicate that *AtUBP1* and *2* are the only members in this subfamily. By sequence analysis of genomic and cDNA clones, the partial organization for *AtUBP1* and the complete organization of *AtUBP2* was determined (Fig. 5A). Each contains a positionally conserved intron between the sequences for the F and His boxes, whereas *AtUBP1* is predicted to contain a second intron following the sequence for the G box. A 531-bp intron was detected upstream of the Met start codon in *AtUBP2*; a similarly positioned intron may be present in *AtUBP1* but could not be identified without an available cDNA sequence in that region.

The encoded *At*UBP1 and 2 proteins are 120 and 106 kD, respectively, and contain all six of the conserved UBP motifs (Fig. 5A). Sequence comparisons revealed that *At*UBP1 and *At*UBP2 are more related to each other than to any of the other UBPs in Arabidopsis, sharing 62% amino acid sequence similarity. Dotplot comparisons show that this homology is evident even outside the six conserved domains, where most other Arabidopsis UBPs show little relatedness (Fig. 5B). No orthologs have been detected thus far in any other plant species. Their closest

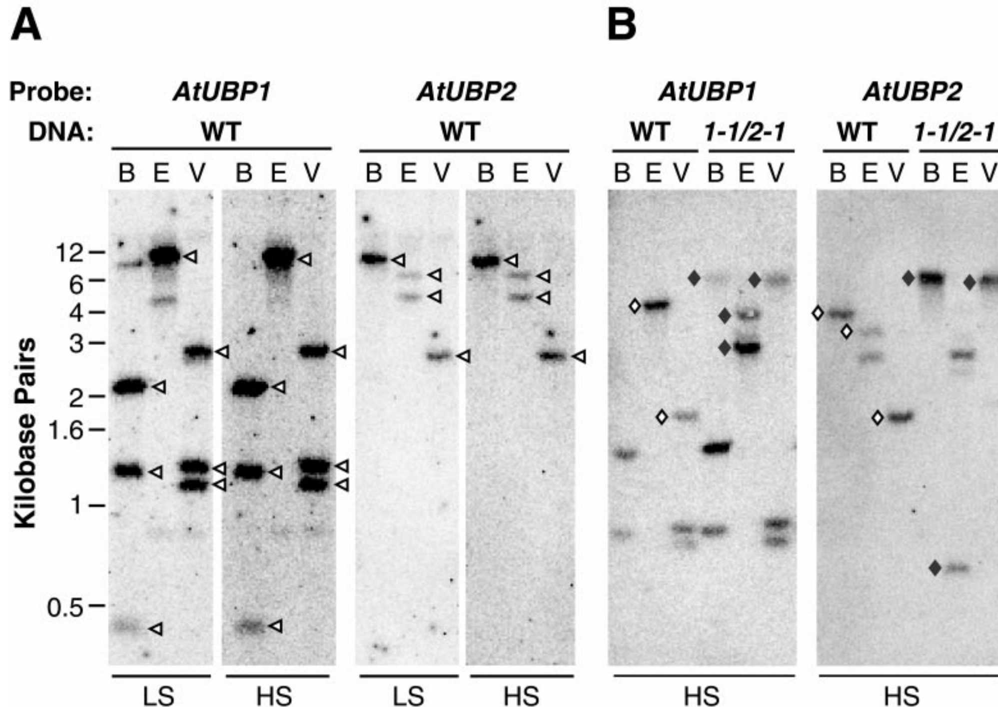

Figure 4. DNA gel-blot analysis of *AtUBP1* and *2* from WT Arabidopsis (WT) and *ubp1-1*/*ubp2-1* mutant plants. Arabidopsis genomic DNA was isolated from the ecotype WS and the double homozygous *ubp1-1*/*ubp2-1* line, digested with *Bgl*II (B), *Eco*RI (E), or *Eco*RV (V) and then probed with either an *AtUBP1* or *2* gene-specific probe. A, Analysis of WT Arabidopsis genomic DNA following washes at either low stringency (LS) or high stringency (HS). Each band marked by a white arrowhead represents a genomic fragment that corresponds to the gene-specific probe used in that blot. B, Analysis of genomic DNA from WT or the *ubp1-1*/*ubp2-1* (*1-1/2-1*) double mutant. Blots were washed at high stringency. ◇, Indicates fragments only detected in DNA from WT and not the mutant plants. ◆, Denotes fragments present in DNA from the mutant and not WT plants.

homologs outside of plants are human UBP-M (Cai et al., 1999), UBPY (Naviglio et al., 1998), and the onco-protein tre-2 (Papa and Hochstrasser, 1993) (49%, 49%, and 45% similar to *At*UBP1, respectively). However, Dotplot comparisons of UBPM, UBPY, and tre-2 versus *At*UBP1 or 2 showed that this similarity is restricted to the six conserved motifs, suggesting that these human UBPs are not functional orthologs (Fig. 5C; data not shown). The absence of possible orthologs suggests that *At*UBP1 and 2 are unique to plants.

*At*UBP2 Is Active in Vivo and in Vitro

UBPs are best defined by their ability to cleave ubiquitin attached via peptide (α-amino) and/or isopeptide (ϵ-amino) bonds to other proteins. To confirm this activity for *At*UBP1 and 2 and to identify the nature of their preferred linkages, the recombinant *At*UBP2 protein was assayed against a variety of substrates both in vitro and in vivo. For ubiquitin linked via a peptide bond, three translational fusions of varying sizes were tested: the hexameric polyubiquitin protein *At*UBQ10 (Callis et al., 1995), the *At*UBQ1 ubiquitin-extension protein bearing the 52-amino acid ribosomal protein appended to a single ubiquitin moiety (Callis and Vierstra, 1989), and a fusion of ubiquitin and β-galactosidase (Ub-βgal) (Varshavsky, 1997). For ubiquitin linked via an isopeptide bond, a population of multiubiquitin chains linked through Lys-48 was the substrate (van Nocker and Vierstra, 1993). The wild-type (WT) *At*UBP2 was tested along with two mutant forms in which the active-site Cys at position 240 (Fig. 3) was substituted for either Ser (*At*UBP2$_{C240S}$) or Ala (*At*UBP2$_{C240A}$). All three proteins could be expressed to high levels as soluble proteins in *Escherichia coli*.

As can be seen in Figure 6, A and B, *At*UBP2 effectively cleaved ubiquitin attached via peptide linkages in vivo. When the recombinant protein was co-expressed with a hexameric polyubiquitin (*At*UBQ10) or a ubiquitin-extension protein (*At*UBQ1), free ubiquitin of the correct mobility was generated (Fig. 6, A and B). For the polyubiquitin reactions, the cleavage products were released as doublets. The species of higher mass in each doublet represented ubiquitin polymers containing an 11 amino acids N-terminal extension, which was added during the construction of the *AtUBQ10* vector for expression in *E. coli* (Fig. 6A). The activity of *At*UBP2 was similar to that of yeast *Sc*UBP1, which has been previously shown to cleave ubiquitin attached via

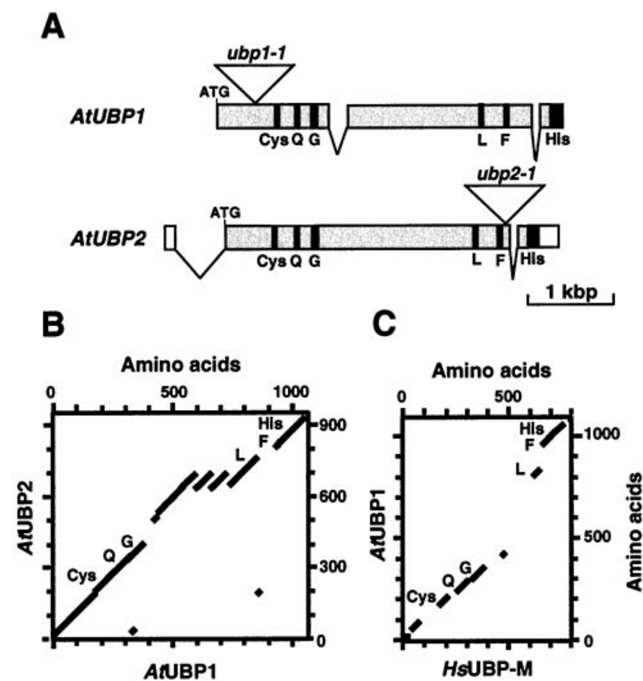

Figure 5. Structure and derived amino acid sequence alignments of the *AtUBP1* and *2* genes. A, Structure of *AtUBP1* and *2* genes. Lines indicate introns and boxes indicate exons; white boxes, untranslated regions; gray/black boxes, translated regions. The Cys, Q, G, L, F, and His boxes are indicated in black. The T-DNA insertion sites for the *ubp1-1* and *ubp2-1* mutants are indicated by the triangles. B, Dotplot comparison of the deduced amino acid sequence of *At*UBP1 with that of *At*UBP2 (left) or human UBP-M (AF12636; Cai et al., 1999) (right). The positions of the conserved Cys, Q, G, L F, and His domains are labeled. Axes denote amino acid position.

α-amino peptide linkages (Tobias and Varshavsky, 1991). As expected, the activity of *At*UBP2 was dependent on the active-site Cys; both the *At*UBP2$_{C240S}$ and *At*UBP2$_{C240A}$ mutants were inactive.

*At*UBP2 was also co-expressed with Ub-X-β-galactosidase in which either a Met (Ub-M-βgal) or a Leu (Ub-L-βgal) residue immediately followed the ubiquitin moiety. As shown by Papa et al. (1993), this combination of substrates helps confirm that cleavage occurred at the correct site, i.e. immediately following the C-terminal Gly of the ubiquitin moiety. Correct cleavage of Ub-M-βgal releases M-βgal, which is stable and accumulates to high levels in *E. coli*. In contrast, correct cleavage of Ub-L-βgal generates L-βgal, which is rapidly degraded by the N-end rule pathway, and thus accumulates to substantially lower levels (Varshavsky, 1997). For example, when yeast *Sc*UBP1 is used, loss of Ub-M-βgal and Ub-L-βgal was evident. However, whereas detectable levels of the Ub-M-βgal digestion product could be seen, the expected cleavage product of Ub-L-βgal was undetectable (Fig. 6C; Papa and Hochstrasser, 1993). A similar outcome was observed for *At*UBP2; whereas the M-βgal accumulated, the L-βgal product did not (Fig. 6C). Like the results obtained with the polyubiquitin and ubiquitin-extension protein substrates, the activity of *At*UBP2 on Ub-X-β-gal substrates was dependent on the active-site Cys.

Recombinant *At*UBP2 could also cleave in vitro ubiquitin attached via isopeptide (ϵ-amino) linkages. Similar to yeast UBP14 (Amerik et al., 1997), *At*UBP2 digested Lys-48-linked multiubiquitin chains and generated free ubiquitin monomers in a reaction that also required Cys-240 (data not shown).

Analysis of T-DNA Insertion Mutants of *AtUBP1* and *2*

To investigate the biological function(s) of the *At*UBP1 and 2 subfamily, we screened available T-DNA-transformed populations of Arabidopsis (Krysan et al., 1996; Krysan et al., 1999) for disruptional insertion(s) in the corresponding genes. Insertion mutants *ubp1-1* and *ubp2-1* were identified that contain a T-DNA insertion in the coding region, 703- and 2,539-bp downstream of the respective translation start site, with the T-DNA either upstream of the Cys box (*ubp1-1*) or between the F and His boxes (*ubp2-1*) (Fig. 5A). Both insertions were predicted to generate a truncated protein missing one or more domains essential for catalysis and hence should represent loss-of-function alleles. To eliminate potential second-site mutations, three back crosses of the mutants to the WT ecotype WS were performed before the homozygous *ubp1-1* and *ubp2-1* lines were crossed and a double homozygote was isolated.

To confirm that the *AtUBP1* and *2* genes were affected in the *ubp1-1*, *ubp2-1*, and *ubp1-1/ubp2-1* lines, genomic DNA was isolated from the homozygotes and analyzed by DNA gel-blot analysis. In each case, the banding patterns of the mutant differed as predicted from that of WT at the respective loci (Fig. 4B; data not shown). By using RT-PCR, we found that the T-DNA insertion also affected expression of the *AtUBP1* and *2* genes. Whereas, the *AtUBP1* and *2* mRNAs could be easily detected by RT-PCR, using as a template RNA isolated from WT plants treated with or without CAN, none could be detected using RNA from the corresponding mutants treated similarly (Fig. 7). As a result, we consider it likely that *ubp1-1* and *ubp2-1* represent null alleles.

To assess the phenotypic functions of the *At*UBP1 and 2 subfamily, the *ubp1-1* and *ubp2-1* mutant plants were examined under a variety of growth conditions. Under normal conditions, either on minimal agar media or in soil, the homozygous *ubp1-1*, *ubp2-1*, and *ubp1-1/ubp2-1* plants were phenotypically indistinguishable from WT plants, including time of germination, growth rate, flowering time, and overall development. To potentially reveal more subtle phenotypes, we also grew the plants under a variety of adverse conditions, including media that contained amino acid analogs, heavy metals, high concentrations of salts or hormones, and various environmental stresses, e.g. heat, cold, high, and low light (J.C. Young, personal communication). Several of these

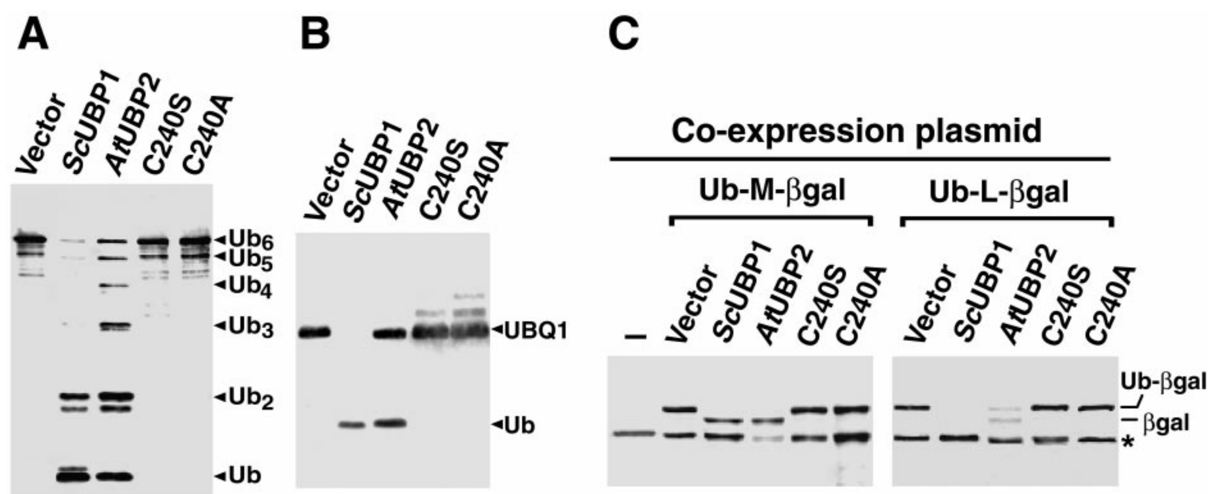

Figure 6. *At*UBP2 encodes a functional UBP protein capable of cleaving polypeptides linked by α-amino peptide bonds to the C terminus of ubiquitin. The substrates UBQ10 (hexameric polyubiquitin) (A), UBQ1 (ubiquitin-extension protein) (B), and Ub-X-β-galactosidase (C) (X = Met or Leu) were co-expressed in *E. coli* NovaBlue (DE3) strain (Novagen) with either a control vector, a vector expressing yeast *Sc*UBP1, or a vector expressing Arabidopsis *At*UBP2, *At*UBP2$_{C240S}$, or *At*UBP2$_{C240A}$. The intact polyubiquitin hexamer (A) and the ubiquitin-extension protein (B) and their cleavage products were detected by immunoblot analyses with anti-ubiquitin antibodies. Ub-X-β-gal and X-β-gal (C) were detected using anti-β-gal antibodies. The positions of the relevant products are indicated, Ub$_{1-6}$ = polyubiquitins of the indicated lengths. The asterisk in C denotes the position of the ω fragment of β-gal expressed constitutively in the NovaBlue (DE3) strain.

conditions were chosen based on the reported involvement of the ubiquitin/26S proteasome in the response of plants to hormones (Ruegger et al., 1998; Xie et al., 1998; Girod et al., 1999), light (Jabben et al., 1989), drought (Kiyosue et al., 1994), and exposure to amino acid analogs (Bachmair et al., 1990; Girod et al., 1999). For almost all conditions, the mutant plants responded similar to WT.

The only exception was growth of the mutants on media containing the Arg analog, CAN. Whereas WT plants were mildly affected by concentrations of CAN greater than 5.5 μM, the *ubp1-1*, *ubp2-1*, and *ubp1-1/ubp2-1* homozygous plants were severely stunted and had shorter roots and chlorotic leaves (Fig. 8). Comparisons of seedling fresh weight indicated that the greatest differences occurred when CAN concentrations were between 11 and 16 μM; at these levels, the fresh weights of mutant plants were 19% to 23% of those for WT plants (Fig. 9A). That the two single mutants and the double mutant showed similar CAN-sensitivity indicates that both *At*UBP1 and 2 are necessary for optimal resistance to this amino acid analog (Figs. 8 and 9A).

To confirm that the sensitivity to CAN was a direct result of the T-DNA disruption of either the *AtUBP1* or 2 genes, we attempted to rescue the mutant phenotype of *ubp1-1* by complementation with the WT *AtUBP1* gene and the active-site Cys mutant *AtUBP1*$_{C211S}$ (Fig. 5C). These two genes were introduced into a homozygous *ubp1-1* line using an *Agrobacterium*-based pCAMBIA3300 vector and T_0 transformed seedlings were selected by BASTA herbicide resistance. T_1 plants (heterozygous for the transgene), containing either the *AtUBP1* or *AtUBP1*$_{C211S}$ transgene, were then self-pollinated. The progenies (T_2) from each independently transformed T_1 plant were grown on 11 μM CAN together with WT ecotype WS and the homozygous *ubp1-1* mutant. Those plants containing the *AtUBP1* transgene showed a 3:1 co-segregation of the CAN resistance with that of BASTA resistance, a marker for the *AtUBP1* transgene, whereas all those harboring *AtUBP1*$_{C211S}$ remained CAN-sensitive like the homozygous *ubp1-1* mutant (Fig. 8C; data not shown). The results collectively demonstrated that *AtUBP1* is required for optimal CAN resistance and that an active enzyme is required. We also tried to complement the homozygous *ubp2-1* line with *AtUBP1*. However, none of the BASTA-resistant plants regained resistance to CAN (data not shown), suggesting that the sensitivity is not simply caused by a lower dosage of the *At*UBP1/2 activity in the *ubp1-1* or *ubp2-1* plants.

CAN is naturally produced by certain legumes as an anti-herbivore compound. Its toxicity is a result of its ability to substitute for Arg during translation. Once incorporated into a protein, CAN can profoundly alter both protein charge and structure, often leading to the production of an abnormal protein (Rosenthal and Dahlman, 1991; Pazlarova et al., 1993). In plants and animals, such CAN-containing proteins are rapidly purged by the ubiquitin/26S proteasome system (Bachmair et al., 1990; Seufert and Jentsch, 1990; Girod et al., 1999). To help prove that the CAN toxicity was a direct result of its ability to substitute for Arg, we attempted to phenotypically

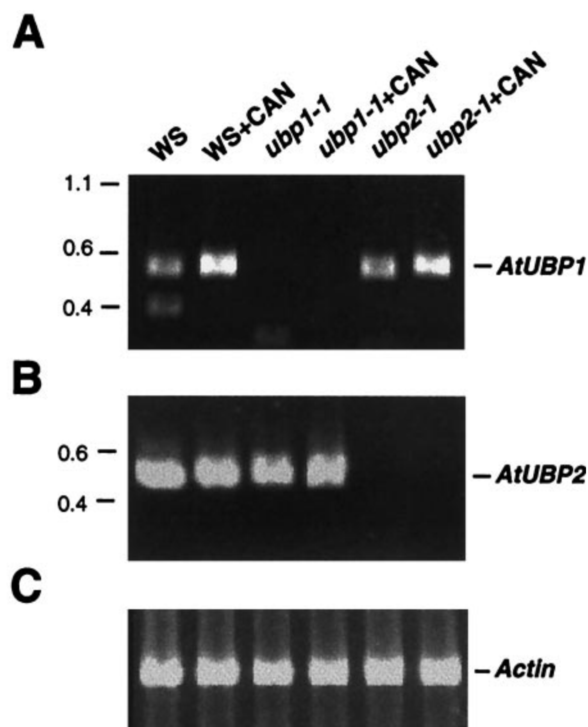

Figure 7. *AtUBP1* and *2* mRNAs are absent in the Arabidopsis *ubp1-1* and *ubp2-1* mutants, respectively. Total RNA was isolated WT ecotype WS, *ubp1-1*, and *ubp2-1* seedlings grown for 6 d on media without CAN followed by an additional 2 d on media with or without 44 μM CAN. RT-PCR was performed using 2 μg of RNA from the appropriate plant and gene-specific primers for *AtUBP1* (A), *AtUBP2* (B), or the Arabidopsis actin *ACT2* gene (C; as a positive control). Reaction products were resolved by agarose gel electrophoresis and stained with ethidium bromide.

rescue the mutant plants by adding increasing concentrations of Arg or other amino acids (Lys, His, Pro, or Glu) to the CAN-containing media. Lys was chosen because it is structurally similar to Arg; Pro was chosen because it shares a part of the Arg biosynthesis pathway (Arnstein, 1978); Glu was chosen because it is easily converted into Arg in vivo (Arnstein, 1978); and His was chosen because its biosynthesis is unrelated to that for Arg but carries a similar positive charge. We found that only Arg was able to partially restore CAN resistance. At a molar Arg/CAN ratio of 5:1, the *ubp1-1*, *ubp2-1*, and *ubp1-1*/*ubp2-1* seedling displayed substantially improved growth. The fresh weights of the mutants grown in the presence of Arg increased to 58% to 66% those for WT plants as compared with approximately 20% if Arg was omitted. None of the other amino acids showed beneficial effects even when added at molar ratios of 10:1 (Fig. 9 and data not shown).

We also tested other amino acid analogs to see if the sensitivity of the *ubp1-1* and *ubp2-1* mutants was a general effect. These included *p*-fluoro-Phe (14–136 μM), *S*-(β-aminoethyl) Cys (25–125 μM), 7-aza-Trp (5–122 μM), azetidine 2-carboxylic acid (2–247 μM), and *O*-methyl Thr (0.75–187 μM), analogs of Phe, Lys, Trp, Pro, and Ile, respectively (Busiello et al., 1979; Robinson and Ellis, 1985; Ghislain et al., 1995; van Nocker et al., 1996; Wong and Eftink, 1998). At these ranges of concentrations, all the analogs detectably

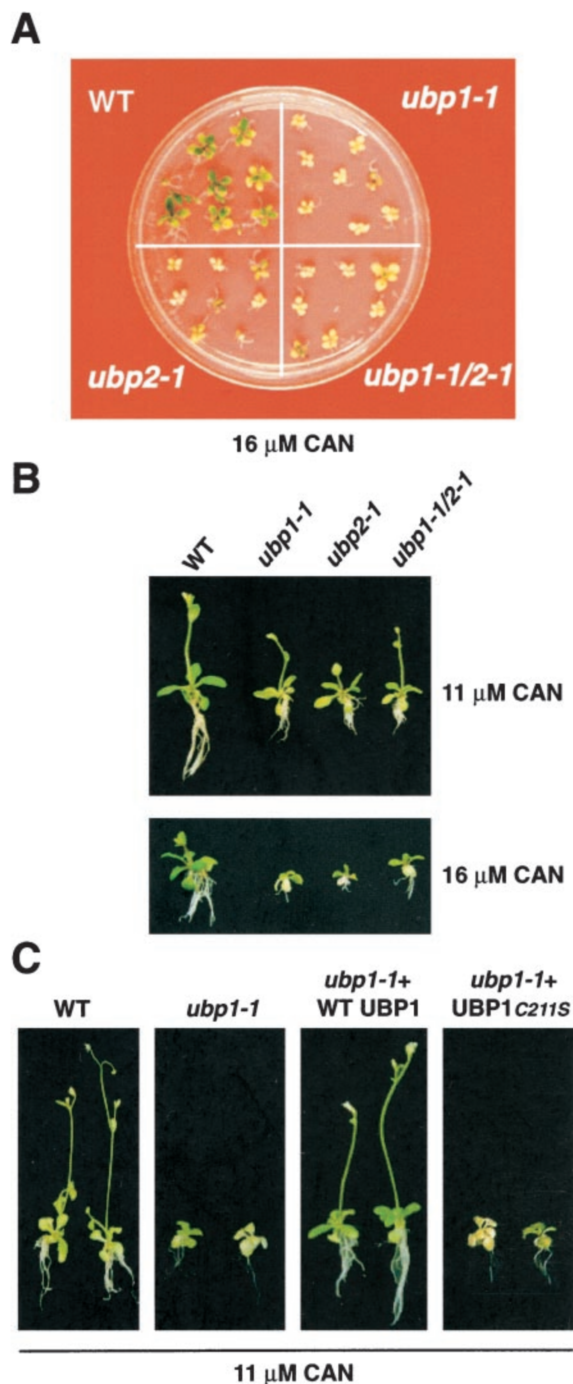

Figure 8. T-DNA insertion mutants of Arabidopsis *UBP1* and *2* are sensitive to the Arg analog CAN. Plants include WT ecotype WS and lines homozygous for *ubp1-1*, *ubp2-1*, and *ubp1-1*/*ubp2-1*. A, Plate of seedlings grown for 20 d on 16 μM CAN. B, Representative seedlings grown for 25 d on 11 or 16 μM CAN. C, Complementation of the *ubp1* CAN-sensitive phenotype by transformation with the WT *AtUBP1* gene and the active-site mutant $AtUBP1_{C211S}$. Plants were grown for 25 d on 11 μM CAN.

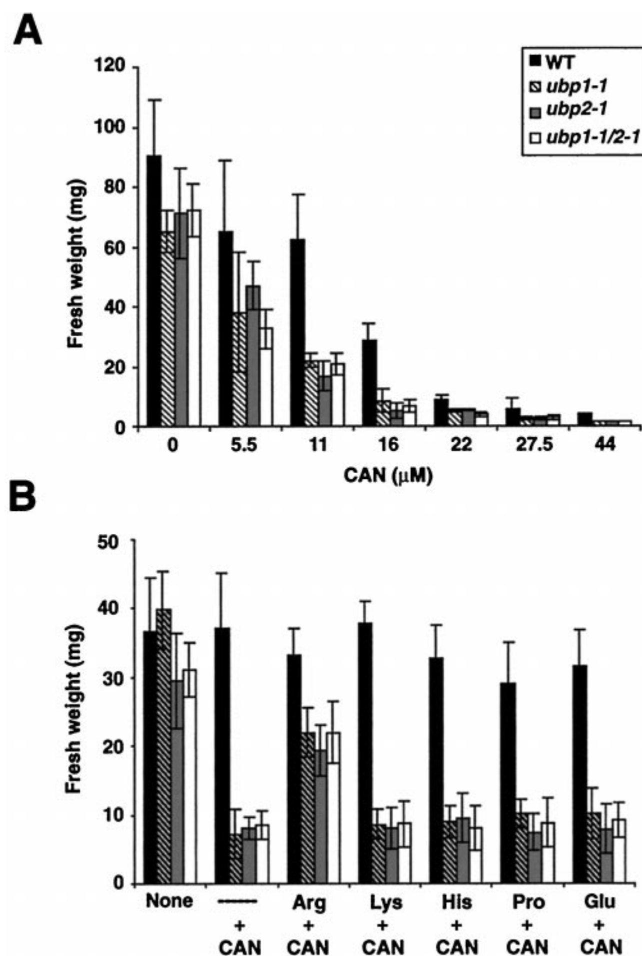

Figure 9. Partial rescue of the CAN growth inhibition of *ubp1-1* and *ubp2-1* plants by simultaneous addition of Arg. A, Dose response of Arabidopsis seedlings grown on various concentrations of CAN. WT seedlings and lines homozygous for the *ubp1-1*, *ubp2-1*, and *ubp1-1*/*ubp2-1* insertions were grown on various concentrations of CAN. After 25 d, the fresh weights of the plants were measured. B, Partial rescue of the growth inhibition by adding Arg to the growth media. Plants were grown for 25 d on 16 μM CAN with or without 80 μM Arg, Lys, His, Pro, or Glu. "None" represents plants grown without CAN or amino acids. Each bar represents the average of 10 plants ±SD

inhibited growth of WT Arabidopsis. However, no difference in sensitivity was observed between the WT and either the *ubp1-1*, *ubp2-1*, or *ubp1-1*/*ubp2-1* seedlings (data not shown). Likewise, the mutants were not hypersensitive to a number of other environmental stresses known to require the ubiquitin/26S proteasome pathway for resistance (Wilkinson, 1999), including heat shock and exposure to heavy metals such as cadmium and copper (data not shown). Taken together, it appeared that the sensitivity of the mutants was specific to CAN.

DISCUSSION

Arabidopsis like other eukaryotes contains a large family of UBPs capable of removing polypeptides attached via peptide and/or isopeptide bonds to the C-terminal Gly of ubiquitin. Here, we described a family of 27 UBPs from the near complete genomic sequence of Arabidopsis, which can be tentatively grouped into 14 subfamilies. All contain a consensus His and Cys box that constitutes part of an active site as well as several less conserved boxes of unknown importance. Both the number of genes and the variety of distinct subfamilies suggest that these proteases must have important roles in the ubiquitin/26S proteasome pathway. Likely roles include: (a) generating free ubiquitins from their translation products; (b) recycling ubiquitins during target degradation by removing ubiquitin from peptide fragments and disassembling the multiubiquitin chains; and/or (c) releasing ubiquitins from conjugates before the target can be degraded (Fig. 1).

What are the specific functions of each of the Arabidopsis UBP subfamilies? Enzymatic analyses have provided few clues except to demonstrate that each has UBP activities. For example, *At*UBP2 (and likely *At*UBP1) appears to have broad specificity, capable of cleaving ubiquitin attached to a variety of substrates by either peptide (α-amino) or isopeptide (ϵ-amino) linkages (Fig. 6). Likewise, Callis and coworkers have shown that *At*UBP3, 4, and 5 will cleave a variety of peptide linked substrates (Chandler et al., 1997; Rao-Naik et al., 2000). Sequence analysis has also been uninformative. Outside of the conserved regions (Cys, Q, G, L, F, and His boxes), the UBP subfamilies bear little relation to each other, suggesting that they perform specific role(s) and/or have distinct substrate specificities. Members of several subfamilies have additional sequence motifs that likely confer specific functions, e.g. zinc fingers, MATH, ubiquitin-like, and UBA domains, but their function(s) and interaction with potential substrates are unknown.

Cellular location may also determine the function of UBPs. In this regard, the *At*UBP3 and 4 proteins have been localized to the nucleus (Chandler et al., 1997) and a nuclear localization signal has been detected in *At*UBP5 (Rao-Naik et al., 2000). Given the likelihood that processing of the initial translation products of the polyubiquitin and ubiquitin-extension genes is co-translational (Baker et al., 1992; Hondred et al., 1999), this distribution would exclude involvement of *At*UBP3-5 in cleaving these ubiquitin precursors and potentially restrict them to isopeptide-linked substrates.

The relationship of the Arabidopsis UBPs with those from other species may help infer function. However, the functions of most UBPs from other species are unknown. Deletion of many of the 16 *UBP* genes in yeast is without phenotypic consequence, suggesting that they have overlapping functions. Notable exceptions are *Sc*UBP4 (DOA4) and *Sc*UBP14. *Scubp4*Δ mutants show a number of defects, including sensitivity to CAN and the accumulation of ubiquitin coupled to small peptides. These data suggest

that *Sc*UBP4 is required for the regeneration of ubiquitins during or following target degradation by removing peptide fragments that remain bound to ubiquitin during hydrolysis of the target by the 26S proteasome. *Sc*UBP14 (and its human ortholog IsoT) appears responsible for disassembling free multiubiquitin chains, thereby replenishing the supply of ubiquitin monomers following target degradation (Amerik et al., 1997). *Scubp14*Δ strains are hypersensitive to CAN and exhibit a strong sporulation defect, a common feature among many ubiquitin pathway mutants in yeast (Hochstrasser, 1996; van Nocker et al., 1996). They also accumulate higher levels of free multiubiquitin chains. Arabidopsis UBP14 could be an ortholog of *Sc*UBP14 (Fig. 2). It is interesting that disruptions of *AtUBP14* cause embryonic lethality, suggesting that multiubiquitin chain disassembly is vital during embryogenesis (unpublished data).

With regard to *At*UBP1 and 2, we show here that this subfamily is not essential in Arabidopsis. However, it is needed for optimal resistance to CAN, the Arg analog that can increase the production of abnormal proteins by substituting for Arg during translation. The fact that disruption of either *At*UBP1 or 2 individually generates a similar CAN sensitivity indicates that both members have non-overlapping roles in removing abnormal proteins. The failure of *AtUBP1* to complement the *ubp2-1* mutant would preclude the possibility that gene dosage is an important consideration. Whether the need for both enzymes reflects unique expression patterns, different cellular locations, and/or different substrates is unknown. Sequence comparisons of *At*UBP1 with 2 identified two patches of unrelated sequences (positioned at 393–414 and 869–929 in *At*UBP1) that could impart distinct functions. Using RT-PCR under semiquantitative conditions, we did not observe any changes in *AtUBP1* or *2* mRNA levels when WT seedlings were exposed to CAN (data not shown), indicating that the expression of neither gene is enhanced by the analog.

It was surprising that we did not find that the *ubp1-1* and *ubp2-1* mutants had increased sensitivity to other toxic amino acid analogs or other stressful conditions, which suggests that the corresponding UBP proteins do not contribute to a general stress response pathway. However, it is possible that the other amino acid analogs are detrimental to growth for reasons other than the increased production of abnormal proteins (e.g. inhibition of amino acid biosynthesis or transport).

How do *At*UBP1 and 2 help confer CAN resistance? The lack of an obvious ortholog in yeast or animals suggests a novel function. If these UBPs are required for generating ubiquitin monomers from their translation products or regenerating free ubiquitin by disassembling multiubiquitin chains (Fig. 1), *ubp1-1* and *ubp2-1* mutants should have substantially reduced levels of free ubiquitin, which in turn could impair overall protein turnover by the ubiquitin/26S proteasome pathway. Like the CAN-sensitive *ubp14*Δ mutant of yeast (Amerik et al., 1997), this effect can be observed by a reduction in the pool of free ubiquitin and by an increase in the pool of free multiubiquitin chains and ubiquitin-protein conjugates. However, when crude extracts from WT, *ubp1-1*, *ubp2-1*, and *ubp1-1/ubp2-1* plants grown with or without CAN were subjected to immunoblot analysis with anti-ubiquitin antibodies, indistinguishable profiles of ubiquitin, free multiubiquitin chains, and ubiquitinated proteins were observed (data not shown). This similarity implies that the levels and overall distribution of ubiquitin are unaffected by either mutation.

It is conversely possible that *At*UBP1 and 2 are involved in a more subtle change in ubiquitination patterns. One scenario is that they are required to regulate the ubiquitination levels of one or more specific proteins essential for CAN resistance. Deubiquitination of these factors by *At*UBP1 and 2 could save them from degradation by the 26S proteasome, leading to increased levels and a concomitant increase in CAN resistance. In a similar fashion, it has been proposed that the *Drosophila* UBP fat facets participates in eye development by deubiquitinating and thus stabilizing a negative regulator of facet development (Huang et al., 1995). Likewise, yeast UBP3 has been proposed to participate in the heat shock response by deubiquitinating a specific target, rather than affecting overall ubiquitination (Baxter and Craig, 1998). Certainly the nature of these specific substrates will help confirm this possibility. However, at present only three physiological substrates for UBPs have been identified, the ubiquitinated form of MEK kinase for *Dictyostelium* UbpB and ubiquitinated forms of AF-6 and β-catenin for the mouse UBP Fam (Chung et al., 1998; Taya et al., 1998; Taya et al., 1999). With the discovery of more natural substrates, the regulatory roles of UBPs will be better understood.

MATERIALS AND METHODS

Identification of Arabidopsis *UBP* Genes

The Arabidopsis ecotype Columbia genomic and EST databases (http://genome-www.stanford.edu/Arabidopsis/) were searched by BLAST (Altschul et al., 1990) for potential UBP sequences using the consensus Cys and His boxes of yeast UBPs as queries. cDNA sequence information was obtained for 18 of the UBP genes by various strategies. Full-length cDNAs (*AtUBP6*, *15*, and *22*) and partial cDNAs (*AtUBP8*, *12*, *16*, and *25*) were provided by the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH). Full-length cDNA clones for *AtUBP2* and *14* were identified in size-selected cDNA libraries (Kieber et al., 1993). RT-PCR was performed to amplify all or part of the predicted coding region for *AtUBP1*, *7*, *12*, *16*, *17*, *20*, *21*, *23*, *24*, *25*, and *27*. RACE was

used to obtain the 5′ and/or 3′ ends for the *AtUBP7*, *16*, *20*, *21*, and *26* cDNAs. The nucleotide sequence for each cDNA was determined by the PCR-based dideoxy method (Perkin-Elmer Applied Biosystems, Foster City, CA). Intron/exon boundaries were identified by comparing the genomic DNA and the corresponding cDNA sequences. For several genes (*AtUBP1*, 8–13, 18, 19, 25, and 26), all or part of the coding regions were predicted using the NetPlantGene program (The Arabidopsis Information Resource database [TAIR]) and by matching the genomic sequence with probable Arabidopsis paralogs. DNA and deduced amino acid sequences were analyzed by using the University of Wisconsin-Genetics Computer Group software packages (Madison, WI). Clustering of the proteins into subfamilies was performed by the programs Pileup and ClustalX. Amino acid sequence alignments were created using Pileup and displayed using MacBoxshade 2.7 (Institute for Animal Health, Pirbright Surrey, UK), and Dotplot analyses were performed using Dotplot with the window set at 30 and the stringency set at 30. Homologs of the *AtUBP* family from other species were identified by BLAST (Altschul et al., 1990) using all available DNA sequences in the GenBank database; possible orthologs were defined using a cut off of e-100.

The genomic sequences of *AtUBP1* and *2* were identified in the BAC clones F24L7 and F13M7, respectively. By comparison to its genomic sequence, the *AtUBP2* cDNA was discovered to contain a single nucleotide deletion at position +251, thus causing a frame shift at codon 84. This mutation was corrected by PCR amplifying a 1.4-kb fragment (from nucleotide +1 to +1,462) of *AtUBP2* from Arabidopsis genomic DNA (ecotype Columbia) using *Pfu* polymerase and a 5′ primer that introduced a *Bam*HI site at the native start codon. This PCR product was digested with *Bam*HI and *Xho*I to generate an approximately 1.2-kb fragment (from nucleotide +1 to +1,196), which was then used to replace the region containing the error in the *AtUBP2* cDNA harbored in pET32a (Novagen, Madison, WI). The *At*UBP2 active-site mutants (p*AtUBP2*$_{C240S}$ and p*AtUBP2*$_{C240A}$) were generated using the QuickChange site-directed mutagenesis method (Stratagene, La Jolla, CA). The primer pairs were CCTTGGGAACACATCGTTCTTTAATTCGATAATGCAG and CTGCATTATCGAATTAAAGAACGATGTGTTCCCAAGG for the Cys→Ser mutant, and CCTTGGGAACACAGCTTTCTTTAATTCGATAATGCAG and CTGCATTATCGAATTAAGAAAGCTGTGTTCCCAAGG for the Cys→Ala mutant (the mutated nucleotides are underlined).

Genomic DNA Gel-Blot Analysis and RACE

Total genomic DNA was isolated from WT and mutant Arabidopsis (ecotype WS) (Cone et al., 1989), digested with various restriction enzymes, and subjected to DNA gel-blot analysis as described (Fu et al., 1998). ^{32}P-labeled *AtUBP1* or *2* DNA probes were hybridized to the membrane-bound DNA at 65°C in 0.5 M sodium phosphate (pH 7.2), 7% (v/v) SDS, 1 mM Na_4EDTA. High-stringency wash conditions were 65°C in 0.5× SSC and 0.1% (v/v) SDS (20× SSC = 3 M NaCl and 0.3 M Na_3 citrate). Low-stringency wash conditions were 65°C in 3× SSC and 0.1% (v/v) SDS. Following the washes, the blots were subjected to autoradiography.

Total RNA was extracted from 2-week-old Arabidopsis (ecotype Columbia) seedlings grown on Gamborg B-5 agar medium (GIBCO-BRL, Gaithersburg, MD) and purified by LiCl precipitation (Rapp et al., 1992). Residual DNA was digested with DNAse RQ1 (Promega, Madison, WI). 5′ and 3′ RACE was performed according to the manufacturer's instructions (CLONTECH, Palo Alto, CA). For RT-PCR, first-strand cDNA was generated using 1 μg of RNA, 80 units Moloney murine leukemia virus reverse transcriptase, and 6 pmol of a 3′-gene-specific primer in a 25-μL reaction at 37°C for 1 h. One microliter of this reaction was then used as template DNA in a 25-μL PCR containing 6 pmol each of 5′- and 3′-gene-specific primers and 1 unit Ex-*Taq* polymerase (PanVera, Madison, WI).

UBP Activity Assays

The ability of UBPs to cleave ubiquitin linked via α-amino linkages was determined in vivo using the substrates polyubiquitin *At*UBQ10 (modified from p8190; Rao-Naik et al., 2000), ubiquitin-extension protein *At*UBQ1 (p8185; Chandler et al., 1997), and Ub-X-β-galactosidase (X = Met or Leu; Papa and Hochstrasser, 1993). To attenuate the expression of *AtUBQ10*, the first three nucleotides of the transcription start site were changed to unfavorable bases (Milligan and Uhlenbeck, 1989) by the QuickChange method (Stratagene) using the two degenerate oligonucleotides AATACGACTCACTATAC[A/C][A/C/G]AGACCACAACGGTTTC and GAAACCGTTGTGGTCT[C/G/T][G/T]GTATAGTGAGTCGTATT (substitutions are underlined; degenerate nucleotides are bracketed). A low-expressing clone (*pAtUBQ10-LE*) of *AtUBQ10* was identified by immunoblot analysis of individual colonies using anti-ubiquitin antibodies (van Nocker and Vierstra, 1993). All substrate constructs were pACYC184-based plasmids.

Each of the three α-amino substrates was co-expressed with WT or mutant versions of *AtUBP2* in pET32a (see above) in the *Escherichia coli* strain NovaBlue (DE3) using the standard conditions (Novagen). Lysates were subjected to SDS-PAGE, transferred to nitrocellulose (Millipore, Bedford, MA) for *At*UBQ10 or *At*UBQ1 substrates or Immobilon-P polyvinylidene difluoride (Millipore) for Ub-X-β-gal substrates, and probed with anti-ubiquitin antibodies or anti-β-gal antibodies (Promega). Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins (Kirkegaard and Perry Laboratories, Gaithersburg, MD), in conjunction with the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, were used for detection. Yeast UBP1, expressed from plasmid RB293 (Tobias and Varshavsky, 1991; RT Baker, unpublished data), was used as a positive control.

The in vitro cleavage assay for ubiquitin attached via an ϵ-amino isopeptide bond used Lys-48-linked multiubiquitin chains as substrates. These chains were synthesized in vitro using the wheat E2 *Ta*UBC7 as described (van Nocker and

Vierstra, 1993). Cell extracts containing recombinant *At*UBP2 or yeast UBP14 (Amerik et al., 1997) were obtained by sonicating packed cells expressing the corresponding proteins resuspended in 1/20 of the original culture volumes with 50 mM Tris-HCl, pH 8.0, 5% (v/v) glycerol, 1 mM dithiothreitol, and 1 mM Na_4 EDTA. Lysates (37.5 μL) were incubated for 2 h at 37°C with 2.5 μL of multiubiquitin chains (50 ng). The reactions were quenched by adding 5× SDS-PAGE sample buffer and heating for 5 min. Reaction products were subjected to SDS-PAGE and immunoblot analysis with anti-ubiquitin antibodies.

Isolation of T-DNA Insertion Mutants in *AtUBP1* and 2

Using the PCR-based method of Krysan et al. (1999), Arabidopsis lines containing a T-DNA insertion within either *AtUBP1* or *2* were identified. For the initial screens, DNA pools prepared from approximately 1,200 individually transformed plants were PCR amplified with a 3′-gene-specific primer of either *AtUBP1* (AAGATAT CAAGCTTCCGTGTTCTCAGATTC) or *AtUBP2* (ACCTC CTCTAACATACGCCACATAATGACC) in combination with either a left border or right border (RB) T-DNA-specific primer (Krysan et al., 1996). The PCR products were subjected to DNA gel-blot analysis using *AtUBP1* or *AtUBP2* gene-specific probes. The candidate PCR products were sequenced to confirm disruption of the correct gene and locate the exact insertion site of the T-DNA. DNA subpools from pools that tested positive were screened individually by PCR using the appropriate primer combinations. Individual mutant plants were identified by PCR and DNA gel blotting using the corresponding gene as the probe. *ubp1-1* and *ubp2-1* were found within the T-DNA insertion lines generated by Dr. K. Feldmann (obtained from the Arabidopsis Biological Resource Center and from E.I. du Pont de Nemours & Company, Wilmington, DE). Homozygous lines for each mutant were isolated following three backcrosses of the heterozygous mutants to WT ecotype WS and then one round of self pollination. The presence of the T-DNA (which carries the *NPTII* gene) was identified by both PCR and kanamycin resistance. The *ubp1-1/ubp2-1* double mutant was generated by crossing homozygous *ubp1-1* with homozygous *ubp2-1* plants; individuals homozygous for both insertions were identified in the F_2 generation.

To verify that *AtUBP1* and *2* are not expressed in the Arabidopsis *ubp1-1* and *ubp2-1* mutants, respectively, RNA was from each line was subjected to RT-PCR. Total RNA was isolated from the mutant and WT ecotype WS seedlings (see above; Rapp et al., 1992) grown for 6 d on minimal media and an additional 2 d with or without 44 μM CAN. RT-PCR was performed using 2 μg RNA from the appropriate plants and gene-specific primers for *AtUBP1* (GGCTTTTGATGAGTGTAGAGAC and CATT-GCCCCTAAATGTTCC), *AtUBP2* (ATCAAGCAACAC-CAGCAAC and GCCACATAATGACCTCCTC), or the Arabidopsis actin *ACT2* gene (GGTTTTGCTGGTGAT-GATG and ACCATAAGGTTCTAAAGAG). The conditions for RT-PCR were as described above.

Phenotypic Analysis of *AtUBP1* and 2 T-DNA Insertion Mutants

Sterilized seeds of WT (ecotype WS) and mutant plants were stratified for 4 d at 4°C and then spotted on agar plates containing 0.5× Murashige and Skoog media (GIBCO BRL) with or without supplements. Under most conditions, seedlings were grown at 21°C with a 19-h-light/5-h-dark photoperiod. The effects of the various supplements were assayed after 25 d of growth by measuring seedling fresh weight. For immunoblot analysis, leaves were collected and homogenized in extraction buffer (3 mL/g fresh weight) containing 50 mM Tris, pH 8.0, 1 mM Na_4EDTA, and 10 mM $Na_2S_2O_5$. Following clarification at 14,000*g* for 5 min, an appropriate volume of 5× SDS-PAGE sample buffer was added to each supernatant, and the total mixture was boiled for 5 min. Samples were assayed by SDS-PAGE and immunoblot analysis using anti-ubiquitin antibodies as described above.

Complementation of *ubp1-1*

For complementation of the *ubp1-1* mutation, a WT *AtUBP1* gene was reconstructed from a 5-kb *Xba*I/*Xho*I fragment from BAC F24L7 containing the 5′ region of *AtUBP1* and a 400-bp *Xho*I/*Eco*RI fragment from the *AtUBP1* cDNA containing the 3′ region. The resulting 5.4-kb sequence included the entire coding region of *AtUBP1* and 1.8 kb upstream of the predicted start codon. The Cys→Ser active-site mutant was prepared by converting the Cys_{211} codon in the *Xba*I/*Xho*I 5′ fragment to that for Ser by the QuickChange method. The WT and mutated genes were cloned into the binary vector pCAMBIA3300 (CAMBIA, Canberra, Australia). The vectors were introduced into the *Agrobacterium* strain GV3101, which then was used to infect the Arabidopsis *ubp1-1* mutant by the floral dip method (Clough and Bent, 1998). Transgenic plants harboring the *BAR* selection marker were identified by spraying T_1 seedlings from the original transformants with 200 mg/L of the herbicide BASTA (Casas et al., 1993).

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