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The ubiquitin-specific protease UBP14 is essential for early embryo development in *Arabidopsis thaliana*

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Summary

The ubiquitin/26S proteasome pathway is a major route for selectively degrading cytoplasmic and nuclear proteins in eukaryotes. In this pathway, chains of ubiquitins become attached to short-lived proteins, signalling recognition and breakdown of the modified protein by the 26S proteasome. During or following target degradation, the attached multi-ubiquitin chains are released and subsequently disassembled by ubiquitin-specific proteases (UBPs) to regenerate free ubiquitin monomers for re-use. Here, we describe *Arabidopsis thaliana* UBP14 that may participate in this recycling process. Its amino acid sequence is most similar to yeast UBP14 and its orthologues, human IsoT1-3 and *Dictyostelium* UbpA, and it can functionally replace yeast UBP14 in a *ubp14Δ* mutant. Like its orthologues, *At*UBP14 can disassemble multi-ubiquitin chains linked internally via ε-amino isopeptide bonds using Lys48 and can process some, but not all, translational fusions of ubiquitin linked via α-amino peptide bonds. However, unlike its yeast and *Dictyostelium* orthologues, *At*UBP14 is essential in *Arabidopsis*. T-DNA insertion mutations in the single gene that encodes *At*UBP14 cause an embryonic lethal phenotype, with the homozygous embryos arresting at the globular stage. The arrested seeds have substantially increased levels of multi-ubiquitin chains, indicative of a defect in ubiquitin recycling. Taken together, the data demonstrate an essential role for the ubiquitin/26S proteasome pathway in general and for *At*UBP14 in particular during early plant development.

Keywords: ubiquitin, ubiquitin-specific protease, protein degradation, *Arabidopsis*, 26S proteasome

Introduction

The covalent attachment of ubiquitin to a variety of cellular proteins is involved in a number of growth and developmental processes in both animals and plants. These include cell-cycle progression, activation of stress responses, signal transduction, DNA repair, modification of chromosome structure, organelle biogenesis, endocytosis, and programmed cell death (Callis and Vierstra, 2000; Hershko and Ciechanover, 1999; Vierstra, 1996). In most cases, ubiquitin fulfils these roles by serving as a reusable tag to target proteins for degradation by the 26S proteasome. Here, chains of multiple ubiquitins, linked internally via an isopeptide bond between the C-terminal glycine and Lys48, are attached to free lysl ε-amino groups within the target (van Nocker and Vierstra, 1993; Piotrowski *et al.*, 1997; Thrower *et al.*, 2000). Proteins bearing chains of four or more ubiquitins are selectively recognized and degraded by the 26S proteasome with the concomitant release of the ubiquitin moieties for re-use. In other cases, the attached ubiquitin(s) do not direct the protein to the 26S proteasome but may confer other functions. For example, proteins bearing a single ubiquitin or chains of ubiquitins linked through Lys63 have been detected (Ball *et al.*, 1987; Hofmann and Pickart, 1999; Spence *et al.*, 1995; Spence *et al.*, 2000). These modifications appear to affect the function of the tagged protein in a non-proteolytic manner, possibly via a ubiquitination/de-ubiquitination cycle.

Although the reactions that attach ubiquitins are clearly important for determining the functions and specificity of ubiquitination, it has become apparent that the reactions

that remove ubiquitins from proteins can also be influential. These reactions are directed by a special group of thiol proteases called **d**e-**ub**iquitinating enzymes (DUBs) (D'Andrea and Pellman, 1998; Wilkinson, 1997). They have high specificity for the ubiquitin moiety and will remove most adducts linked via peptide or isopeptide bonds to the C-terminal glycine. DUBs are proposed to have several essential roles in ubiquitin-dependent processes. (1) They are necessary for the initial synthesis of the 76-amino acid ubiquitin monomer by releasing the ubiquitin moieties linked via α-amino peptide bonds to their precursors: polyubiquitin and ubiquitin extension proteins (see Callis *et al.*, 1995). (2) DUBs can repair ubiquitins that inadvertently react with small nucleophiles by removing adducts linked to the free C-terminal glycyl carboxyl group. (3) DUBs can de-ubiquitinate proteins, thus reversing the effects caused by ubiquitin modification. In the case of Lys48-linked multi-ubiquitinated proteins, this release presumably prevents degradation of the protein by the 26S proteasome. (4) DUBs can recycle ubiquitins during or following the degradation of target proteins. This involves removing small peptide fragments that remain bound to the ubiquitins during target degradation and disassembling the multi-ubiquitin chain to release the free ubiquitin monomers for re-use (Amerik *et al.*, 1997; Papa and Hochstrasser, 1993).

Plants, animals and fungi contain numerous DUBs. One large class is the ubiquitin-specific proteases (UBPs). They are a family of divergent proteins grouped together by the presence of six conserved regions, the most important being the Cys and His boxes that are essential for catalysis (D'Andrea and Pellman, 1998; Wilkinson, 1997). Whereas yeast (*Saccharomyces cerevisae*) contains 16 distinct UBP genes (Hochstrasser, 1996), the higher plant *Arabidopsis thaliana* contains 27 UBP genes, which can be clustered into 14 subfamilies (Chandler *et al.*, 1997; Rao-Naik *et al.*, 2000; Yan *et al.*, 2000). Mutant analyses have implicated specific UBPs in a number of cellular processes, including growth/oncogenesis, differentiation, eye development, neural function, endocytosis, coordinated DNA replication, gene silencing, and stress responses (Amerik *et al.*, 1997; Baxter and Craig, 1998; Cadavid *et al.*, 2000; Chung and Baek, 1999; Papa and Hochstrasser, 1993; Wilkinson, 1997; Yan *et al.*, 2000). However, in most cases, the roles of UBPs in these processes and the identity (or identities) of their natural substrates remains unknown. Systematic disruptions of the yeast *UBP* genes showed that none are essential for viability, suggesting that members of the family perform overlapping functions (Hochstrasser, 1996).

One subfamily of UBPs that has attracted considerable interest includes yeast UBP14 and its human IsoT and *Dictyostelium* UbpA orthologues (Amerik *et al.*, 1997; Hadari *et al.*, 1992; Lindsey *et al.*, 1998; Wilkinson *et al.*, 1995). Their proposed function is to recycle ubiquitins by disassembling free multi-ubiquitin chains released from proteins during or following their breakdown by the 26S proteasome. This recycling helps to maintain a sufficient supply of free ubiquitin and removes free chains that presumably would compete with ubiquitinated proteins for binding to the 26S proteasome. Whereas yeast *ubp14Δ* mutants are mildly compromised, showing a sporulation defect and a hypersensitivity to the arginine analogue canavanine (CAN) (Amerik *et al.*, 1997), *Dictyostelium ubpA* mutants are more affected. The vegetative growth of *ubpA* mutants is normal but, during the sporulation phase, they are defective in aggregation, chemotaxis, cAMP relay and cell adhesion, and ultimately the development of fruiting bodies (Lindsey *et al.*, 1998). Conversely, over-production of yeast UBP14 also inhibited target degradation by the 26S proteasome (Amerik *et al.*, 1997). These observations strongly suggest that an accurate balance of ubiquitin monomers and multi-ubiquitin chains is critical for cell differentiation and/or reproduction.

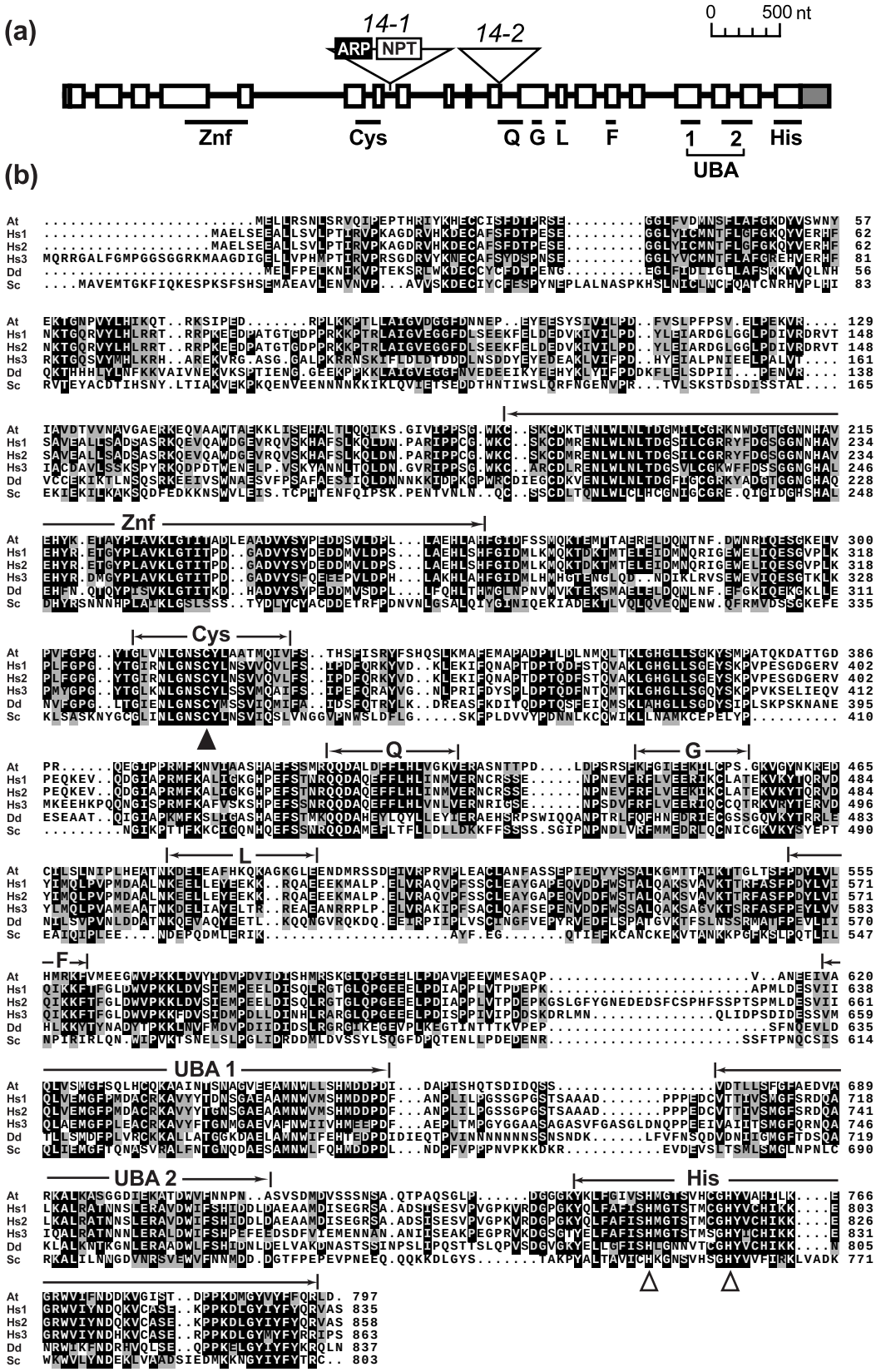
Here we describe *At*UBP14, a member of the *Arabidopsis* UBP family that is an orthologue of *Sc*UBP14, IsoT and UbpA. *At*UBP14 exhibits a similar ability to disassemble free multi-ubiquitin chains and can complement a yeast *ubp14Δ* mutant. T-DNA insertion mutations that disrupt the *AtUBP14* gene cause embryo lethality. The mutant seeds arrest at the globular stage and accumulate abnormally high levels of free multi-ubiquitin chains, indicating that *At*UBP14 activity is essential for early development in higher plants, at least in part by helping to recycle ubiquitin.

Results

Identification of At*UBP14*

Previously, we described a family of 27 *UBP* loci from *Arabidopsis* that could be clustered into 14 subfamilies,

Figure 1. Gene structure of *AtUBP14* and alignment of its derived amino acid sequence with related proteins.
(a) Gene structure of *AtUBP14* (GenBank accession no. AF302664). Introns are indicated by lines. White and shaded boxes identify the coding regions and 5′ and 3′ untranslated regions, respectively. The positions of the Cys, Q, G, L, F and His boxes and the putative zinc finger (Znf) and UBA domains are underlined. The positions of the T-DNA insertion sites for the *Atubp14-1* and *Atubp14-2* mutants are indicated. (b) Amino acid sequence alignment of *Arabidopsis At*UBP14(At, GenBank accession no. AF302664), human isopeptidase T isozymes (IsoT1 (Hs1), accession no. U35116; IsoT2 (Hs2), accession no. P45974I; IsoT3 (Hs3), accession no. U75362), *D. discoideum* UbpA (Dd, accession no. U48271), and *S. cerevisiae Sc*UBP14(Sc, accession no. P38237). Identical and conserved residues are shaded black and grey, respectively. The Cys Q, G, L, F and His boxes and the putative Znf and UBA domains are identified. The catalytic Cys and His residues are indicated by black and white triangles, respectively. The alignments were made using ClustalX (National Center for Biotechnology Information, Bethesda, MD, USA) and displayed using MacBoxshade 2.7 (Institute of Animal Health, Pirbright, Surrey, UK).


(a)
14-1
ARP
NPT
14-2
0
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Znf
Cys
Q G L
F
1
2
His
UBA
(b)
Znf
Cys
Q
G
L
F
UBA 1
UBA 2
His

containing single or multiple members (Yan *et al.*, 2000). Several were predicted to have orthologues in other species, the most obvious being *AtUBP14*, a single gene subfamily with substantial similarity to yeast *UBP14*, human *IsoT* and *Dictyostelium UbpA* (Amerik *et al.*, 1997; Lindsey *et al.*, 1998; Wilkinson *et al.*, 1995). Its closest relatives were human IsoT1 and 2 (61/52% amino acid similarity/identity). The *AtUBP14* gene, deduced by comparing its genomic and cDNA sequences, contains 20 exons and 19 introns (Figure 1a) and encodes a 797 amino acid protein. Amino acid sequence comparisons of *At*UBP14 with its potential orthologues revealed extensive homology throughout the polypeptides (Figure 1b). This pervasive similarity runs counter to comparisons with other UBPs, which showed relatedness only in a few scattered domains (Yan *et al.*, 2000).

Like most other UBPs, *At*UBP14 contains the characteristic Cys and His boxes essential for catalysis and the conserved Q, G, L and F domains of unknown functions (Yan *et al.*, 2000). *At*UBP14 also contains a putative zinc finger and two adjacent UBA domains; both of which are also present within the *Sc*UBP14/IsoT/UbpA group (Amerik *et al.*, 1997; Figure 1b). Possible zinc fingers have been found in several other UBPs (Yan *et al.*, 2000), but their role(s) in UBP functions is unknown. UBA domains have been detected within other proteins associated with the ubiquitin/26S proteasome pathway (Bates and Vierstra, 1999; Hofmann and Bucher, 1996). It has been proposed that UBA domains bind ubiquitin non-covalently (Hofmann and Bucher, 1996).

To help reveal the functions of *At*UBP14, its distribution within *Arabidopsis* was examined. Using anti-*At*UBP14 antibodies, we detected *At*UBP14 protein of the expected size (88 kDa) in all organs examined, including developing seeds, etiolated and green seedlings, leaves, stems, roots, flowers and siliques (Figure 2). We expect this species to represent *At*UBP14 because no other *Arabidopsis* UBPs have a related sequence and because the *Arabidopsis* UBPs most similar in size to *At*UBP14 are predicted to be 82 kDa and 95 kDa (Yan *et al.*, 2000). On a protein basis, levels of *At*UBP14 were moderately higher in flowers and siliques containing developing seeds.

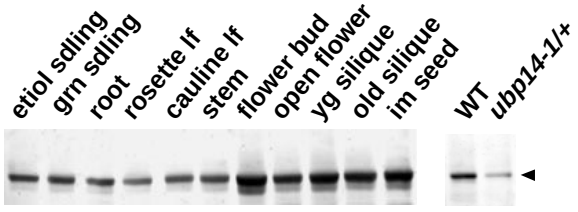

Figure 2. Immunoblot detection of the *At*UBP14 protein in *Arabidopsis*. Left panel: detection of *At*UBP14 in various tissues of wild-type plants. Right panel: comparison of *At*UBP14 levels in 14-day-old wild-type (WT) seedlings and seedlings heterozygous for *Atubp14-1*. Equal amounts of total protein (8 μg) were subjected to SDS–PAGE and immunoblot analysis using anti-*At*UBP14 antibodies. Etiol, etiolated; grn, green; lf, leaf; yg, young; im, immature; sdling, seedling.

At*UBP14 is a functional orthologue of yeast UBP14*

Given the high amino acid sequence similarity of *Arabidopsis* UBP14 with the *Sc*UBP14/IsoT/UbpA group, we considered it likely that *At*UBP14 performs similar function(s). To test this possibility, we attempted to complement a *Scubp14Δ* mutant with cDNAs for either wild-type *AtUBP14* or a mutant in which the active-site Cys317 codon was changed to that for serine (*AtUBP14*$_{C317S}$). Both forms of *AtUBP14* as well as wild-type *ScUBP14* were individually expressed in *Scubp14Δ* cells using the high-copy yeast expression vector pRS426, and tested for growth sensitivity to CAN. As can be seen in Figure 3(a), wild-type yeast but not the *Scubp14Δ* strain grew on 4.4 μM CAN (Amerik *et al.*, 1997). Growth of the *Scubp14Δ* strain was restored by expressing either wild-type yeast or *Arabidopsis UBP14*, but not by expressing the *Arabidopsis UBP14*$_{C317S}$ active-site mutant (Figure 3a).

The expression of *AtUBP14* in the yeast *ubp14Δ* strain also partially restored the normal profile of multi-ubiquitin chains. Whereas wild-type yeast contained mostly free ubiquitin and some multi-ubiquitin chains, the *Scubp14Δ* strain showed dramatically elevated levels of free multi-ubiquitin chains (Amerik *et al.*, 1997; Figure 3b). High levels of multi-ubiquitin chains were still evident in *Scubp14Δ* strains harbouring either an empty pRS426 vector or one expressing the *Arabidopsis UBP14*$_{C317S}$ active-site mutant. However, when the *Scupb14Δ* cells harboured either *ScUBP14* or *AtUBP14*, the levels of multi-ubiquitin chains were substantially reduced (Figure 3B). Yeast *UBP14* was more effective than its *Arabidopsis* counterpart in restoring growth and normal levels of multi-ubiquitin chains. Whether this distinction reflects differences in their catalytic activities or levels of expression is unknown. Nevertheless, the data showed that *At*UBP14 can functionally replace *Sc*UBP14 *in vivo* and that the predicted active-site cysteine (Cys317) is essential for its function.

At*UBP14 displays similar substrate specificities as yeast UBP14*

Yeast UBP14, *Dictyostelium* UbpA and the human IsoTs can cleave ubiquitin attached to a variety of polypeptides, including free multi-ubiquitin chains linked via ε-amino isopeptide bonds through Lys48 (Amerik *et al.*, 1997; Hadari *et al.*, 1992; Lindsey *et al.*, 1998; Wilkinson *et al.*, 1995). To assess the substrate specificity of *Arabidopsis* UBP14, the recombinant protein was assayed *in vitro* with

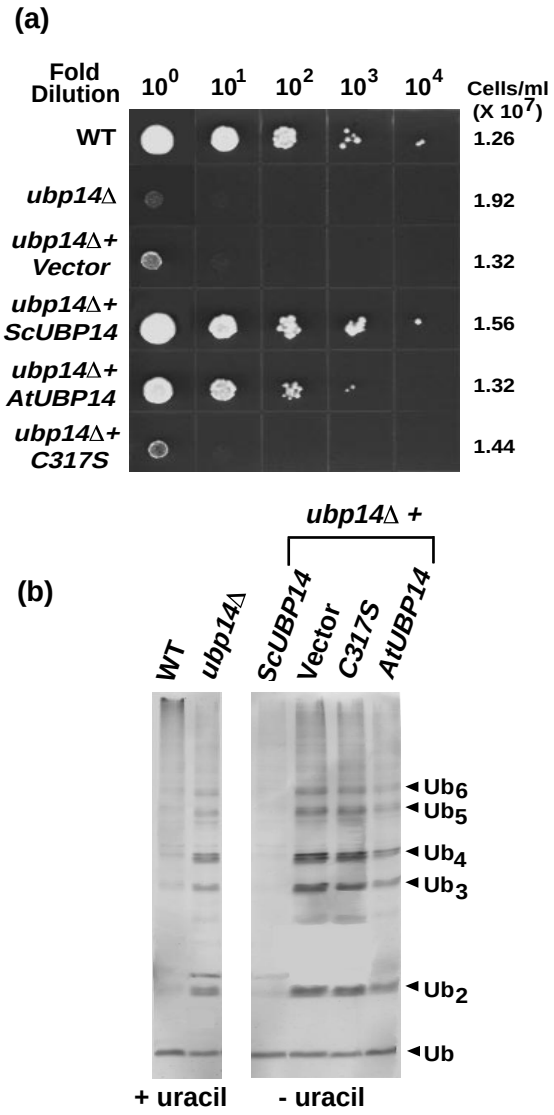

Figure 3. *Arabidopsis* UBP14 is a functional homologue of yeast UBP14.
(a) Growth of various yeast strains on media containing 4.4 μM canavanine (CAN). Cells from wild-type yeast (WT, MHY501) or the *ubp14Δ* mutant (MHY840) expressing *ScUBP14, AtUBP14* or $AtUBP14_{C317S}$ (C317S) were spotted (2 μl) in 10-fold dilutions onto CAN-containing plates. The plates were incubated at 30°C for 4 days. Cell numbers of the cultures prior to plating are indicated on the right. (b) Immunoblot analysis with anti-ubiquitin antibodies of cells from wild-type (WT) yeast or the *Scubp14Δ* strain harbouring vector alone or vectors expressing *ScUBP14, AtUBP14* or the *C317S* mutant. The left and right panels show extracts from cells grown with and without uracil, respectively; uracil-free medium was used to maintain the Ura^+ vectors in the ura^- MHY501 yeast strain. The ubiquitin monomer (Ub) and free ubiquitin chains of various lengths (Ub_n) are indicated on the right.

Lys48-linked multi-ubiquitin chains and *in vivo* with several substrates containing ubiquitin linked via an α-amino peptide bond, including the *Arabidopsis* UBQ10 hexameric polyubiquitin and UBQ1 ubiquitin extension proteins, and ubiquitin-X-β-galactosidase (Ub-X-βgal, where X is either methionine or leucine). As can be seen in Figure 4, yeast and *Arabidopsis* UBP14 appeared to have a similar broad substrate specificity. They both processed multi-ubiquitin chains, polyubiquitin and Ub-X-βgal (Figure 4a,b,d). Consistent with its function as a UBP, *At*UBP14 was inactive when mutated at the active-site Cys317. However, *At*UBP14 and *Sc*UBP14 did not process the UBQ1 ubiquitin extension protein when co-expressed. Under conditions where *Sc*UBP1 completely cleaved UBQ1, releasing both the extension protein and ubiquitin in free forms, no such cleavage was detected for *At*UBP14 or *Sc*UBP14 (Figure 4c).

The data for Ub-X-βgal confirmed that the bond following the C-terminal glycine was cleaved by *Arabidopsis* and yeast UBP14. Previous studies showed that correct cleavage of Ub-M-βgal by UBPs releases M-βgal, which is stable in *E. coli* and thus accumulates (Papa and Hochstrasser, 1993; Varshavsky, 1996). In contrast, correct cleavage of Ub-L-βgal by UBPs releases L-βgal, which fails to accumulate because it is rapidly degraded by the bacterial N-End Rule proteolytic pathway which favors leucine N-termini (Varshavsky, 1996). Using yeast UBP1 as an example, M-βgal but not L-βgal accumulated when the corresponding Ub-X-βgal substrates were co-expressed with *Sc*UBP1 (Figure 4d; Papa and Hochstrasser, 1993). For both *At*UBP14 and *Sc*UBP14, similar results were obtained, i.e. M-βgal but not L-βgal accumulated (Figure 4d).

At*UBP14 mutant analyses*

To help reveal the function(s) of *At*UBP14 in *Arabidopsis*, we studied two independent T-DNA-tagged lines having an insertion within the corresponding gene. The *Atubp14-1* allele was identified in the C24 ecotype by random sequencing of an exon-trap population and encodes a partial *At*UBP14 connected to the ARP-NPTII fusion protein (Babiychuk *et al.*, 1997). The *Atubp14-2* mutant allele was identified by PCR in a population of the WS ecotype (Krysan *et al.*, 1999); its T-DNA insertion bears *NPTII* but is flanked by two LB sequences (Figure 1a). Sequence analysis indicated that the T-DNA of *Atubp14-1* was inserted in the intron just downstream of the Cys box such that the transcribed RNA when spliced encodes ARP-NPTII fused to Arg335 of *At*UBP14. The T-DNA of *Atubp14-2* was inserted after the second nucleotide of the Asp413 codon (Figure 1a). Conceptual translation of *Atubp14-1 and Atubp14-2* revealed that each would encode truncated *At*UBP14 proteins that lacked the Q, G, L, F and His boxes and thus likely represents null alleles. In support, immunoblot analysis of heterozygous *Atubp14-1* or *Atubp14-2* mutant seedlings with anti-*At*UBP14 antisera showed significantly reduced levels of the intact 88-kDa protein compared to wild-type (Figure 2; J. Walker and R. D. Vierstra, unpublished data). We did not detect versions

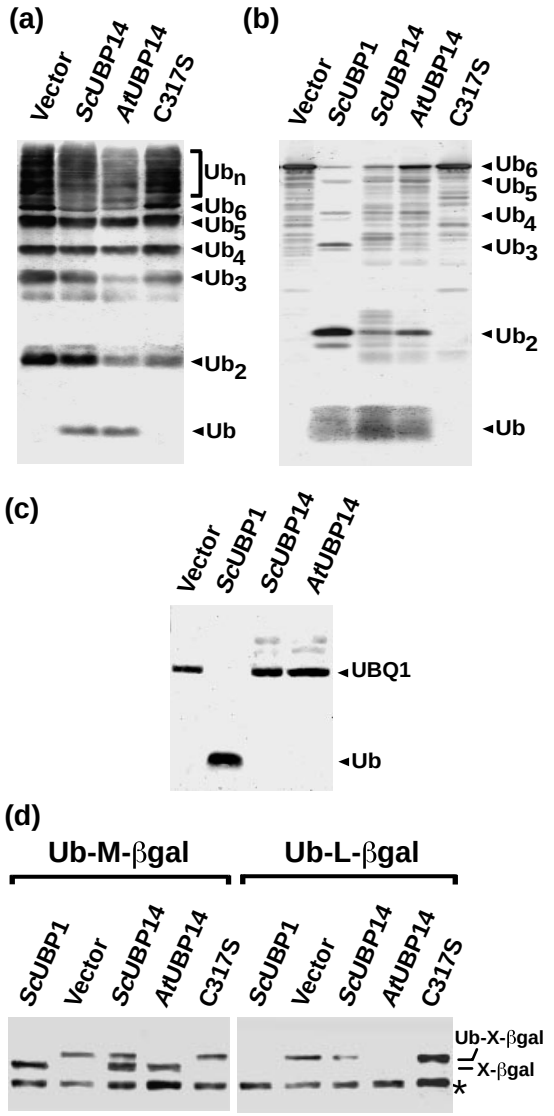

Figure 4. *At*UBP14 de-ubiquitinates substrates linked by (a) ε-amino isopeptide or (b–d) α-amino peptide bonds to the C-terminus of ubiquitin.

Enzymes include yeast *Sc*UBP1, *Sc*UBP14, *Arabidopsis At*UBP14 and the *Arabidopsis* mutant *At*UBP14$_{C317S}$ (C317S). (a) Cleavage of ε-amino Lys48-linked multi-ubiquitin chains *in vitro*. Extracts from *E. coli* containing vector alone or vectors expressing the various UBPs were incubated with multi-ubiquitin chains for 3 h at 37°C. The cleavage products were detected by immunoblot analysis with anti-ubiquitin antibodies. The positions of the multi-ubiquitin chains (Ub$_n$) and ubiquitin monomers (Ub) are indicated on the right. *In vivo* cleavage of (b) hexameric polyubiquitin (UBQ10), (c) ubiquitin extension protein (UBQ1), and (d) Ub-X-βgal (X = methionine (M) or leucine (L)). The UBPs were co-expressed with the various substrates in *E. coli*; the cleavage products were detected by immunoblot analyses with anti-ubiquitin antibodies (b,c) or anti-βgal antibodies (d). The positions of the relevant substrates and products are indicated. The numerous unlabelled species in (b), also present in the vector lane, presumably represent non-specific degradation products of UBQ10. The asterisk in (d) denotes the position of the ω fragment of βgal expressed constitutively in the NovaBlue (DE3) strain.

of *At*UBP14 with altered mass in the *Atubp14-1* or *Atubp14-2* plants, suggesting that the mutant proteins were inefficiently expressed or unstable. However, because the *Atubp14-1* plants were kanamycin-resistant, the *At*UBP14–ARP–NPTII fusion protein must have been expressed, albeit at immunologically undetectable levels. As previously shown by Tang *et al.* (1999) using GFP–NPTII fusions, strong kanamycin resistance can be conferred even by low levels of a NPTII fusion protein.

Both the *Atubp14-1* and *Atubp14-2* mutant alleles were introgressed 2 and 4 times, respectively, into WS to eliminate potential second-site mutations and to create a similar genetic background. We were unable to identify any progeny homozygous for either allele when the heterozygotes were selfed, suggesting that *AtUBP14* is essential. To investigate this possibility in more detail, we looked at the seeds within immature siliques from self-crossed wild-type and heterozygous *Atubp14-1* and *Atubp14-2* mutant plants. Whereas all seeds in wild-type siliques were large and green, approximately 25% of the seeds in the siliques of both mutants developed first as opaque white seeds that then became brown and shrivelled as they matured (Figure 5a). The same lethal phenotype was observed in the progeny of crosses between *Atubp14-1* and *Atubp14-2* heterozygotes, indicating that they are both mutant alleles of the same gene. Siliques of such crosses contained approximately 25% aborted seeds (Figure 5a); of the 16 viable progeny tested, none were identified that contained both mutant alleles (J. H. Doelling and R. D. Vierstra, unpublished data). Genetic crosses between wild-type WS and heterozygous *Atubp14-1* plants demonstrated that the mutant allele could be transmitted to the next generation through either the pollen or the egg (J. H. Doelling and R. D. Vierstra, unpublished data). Collectively, the segregation patterns and transmission efficiency indicated that both *AtUBP14* alleles were recessive, and suggested that a functional copy of *AtUBP14* is required for embryo development after fertilization but not for male and female gametogenesis.

Complementation of At*ubp14 mutants*

The segregation patterns of selfed *Atubp14-1* and *Atubp14-2* plants or crosses between the two mutants strongly suggested that the aborted seeds contained homozygous mutant embryos. In support, approximately two-thirds of the viable seeds collected from selfed plants heterozygous for either allele were kanamycin-resistant due to the co-segregating *NPTII* gene.

To further prove this hypothesis and confirm that the mutant phenotype was directly caused by mutation of the *AtUBP14* gene, we attempted to complement the *Atubp14-1* allele with a genomic fragment encompassing the *AtUBP14* transgene. The transgene and the *Atubp14-1*

allele were tracked by hygromycin B and kanamycin resistance of transgenic plants, conferred by the *hyg*^{r} gene adjacent to the transgene and expression of the *AtUBP14–ARP–NPTII* fusion gene, respectively. Several independent transformants (T1) were identified that contained both the *Atubp14-1* allele and the *AtUBP14* transgene. T2 individuals homozygous for the *Atubp14-1* allele were identified based on the fact that 100% of their progeny tested resistant to kanamycin. These T2 plants were also resistant to hygromycin B, demonstrating indirectly that the *AtUBP14* transgene can substitute for the endogenous *AtUBP14* gene.

To show directly that complementation was successful, plants containing the *AtUBP14* transgene and expected to be homozygous for the *Atubp14-1* allele were crossed to heterozygous *Atubp14-2* mutants, using either mutant line as the pollen donor. Sixteen hygromycin B-resistant plants (thus containing the *AtUBP14* transgene) from the crosses were tested by PCR for the presence of both mutant alleles. Eight of the 16 contained both *Atubp14-1* and *Atubp14-2* (Figure 6) and therefore contained no wild-type copy of the endogenous *AtUBP14* gene. Because these *Atubp14-1*:*Atubp14-2* plants which contained the *AtUBP14* transgene grew normally, we concluded that the transgene can complement the mutant phenotype and that the embryo lethality was caused by the absence of *AtUBP14*.

Loss of At*UBP14 arrests embryo development*

The presence of partially developed seeds suggested that *Atubp14* mutants arrested early in embryogenesis. To determine the stage of arrest, we collected immature seeds from selfed wild-type WS, *Atubp14-1 and Atubp14-2* plants and examined the embryos microscopically. Seeds from wild-type siliques contained embryos at various stages of maturation (globular, heart, torpedo, etc.; Laux and Jurgens, 1997; Meinke, 1994). Similar normal embryo development could be seen for the green seeds collected from the siliques of the *Atubp14-1* and *Atubp14-2* mutants (Figure 5B). However, when embryos from white seeds were analysed, all embryos were either at the globular stage or younger. None showed signs of asymmetric growth characteristic of bilateral differentiation (Laux and Jurgens, 1997) (Figure 5b). From these data, we conclude that loss of *At*UBP14 function induces embryo arrest at the globular stage.

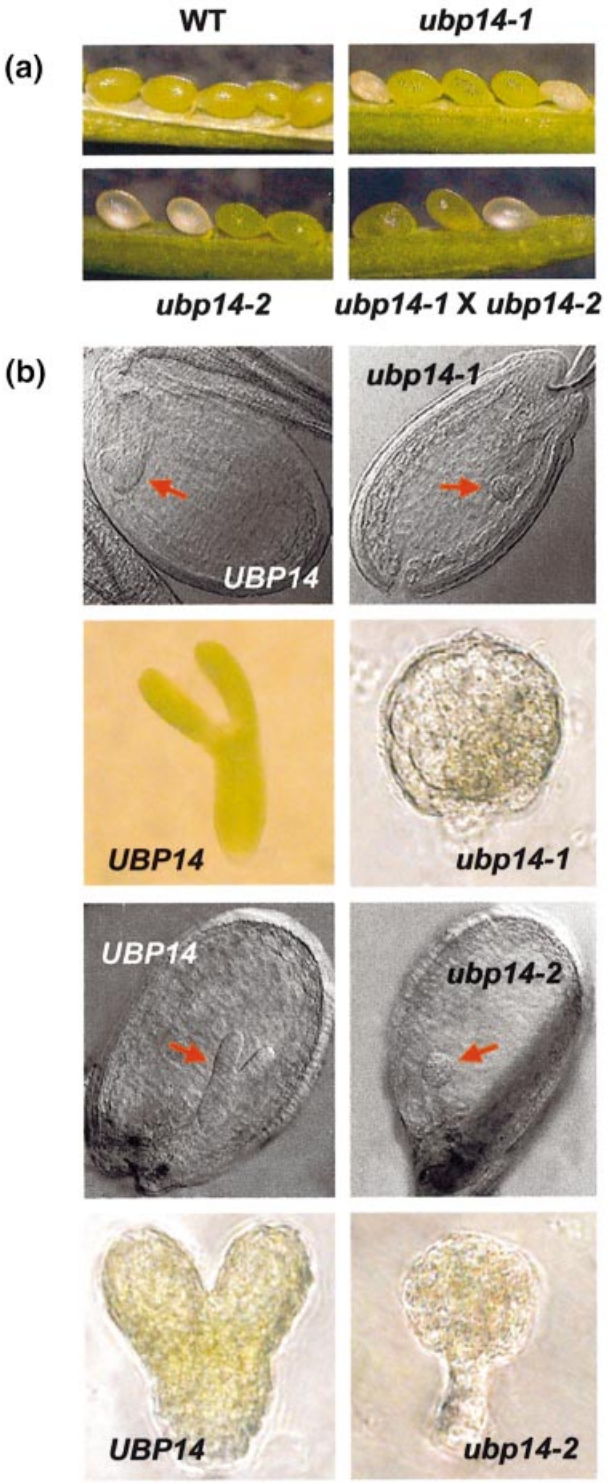

Figure 5. *Atubp14* mutants are embryo lethal.
(a) Immature siliques containing seeds from selfed wild-type (WT) plants, selfed heterozygous *Atubp14-1* and *Atubp14-2* plants, or a cross between heterozygous *Atubp14-1* and *Atubp14-2* plants. Whereas the green seeds are viable, the white seeds eventually shrivel and turn brown at maturity and are non-viable. (b) Microscopic examination of embryos from green (containing at least one wild-type copy of *AtUBP14*) and white seeds (homozygous for the *Atubp14* mutations) from the same *Atubp14-1* and *Atubp14-2* siliques. Upper panels: seeds cleared for inspection of the embryos (arrowheads). Lower panels: embryos dissected from the siliques. The torpedo-stage embryo is shown at one-quarter of the magnification of the other three embryos.

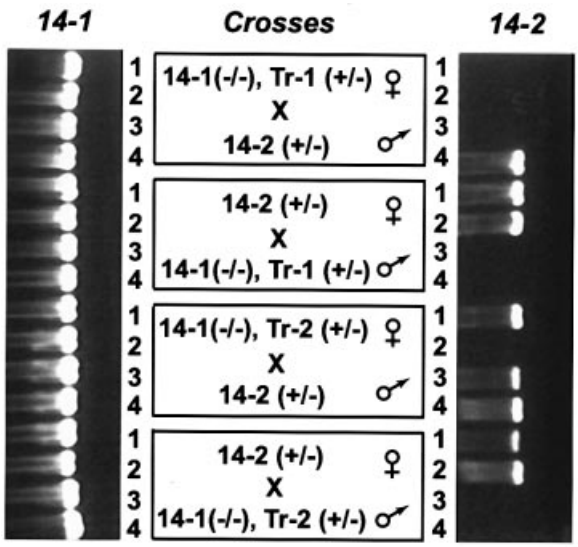

Figure 6. Complementation of the *Atubp14* mutants by an *AtUBP14* transgene.
Two independently transformed lines homozygous for the *Atubp14-1* allele and containing an *AtUBP14* transgene [14-1(–/–), Tr(+/–)] were crossed to heterozygous *Atubp14-2* plants [14-2(+/–)]. The genotypes of viable hygromycin-resistant plants (containing the *AtUBP14* transgene) were determined by PCR using *Atubp14-1*- or *Atubp14-2*-specific primers. The four sets of PCR reactions represent individual progeny of these two crosses using either mutant as the pollen donor. Half of the plants contained both *Atubp14-1* and *Atubp14-2* and thus no wild-type copy at the endogenous locus, indicating rescue by the *AtUBP14* transgene.

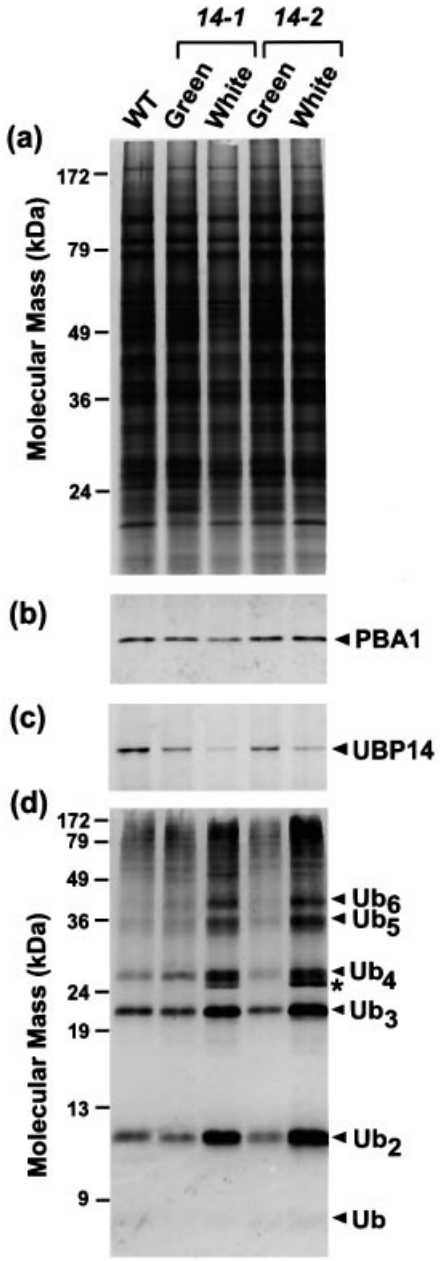

Figure 7. Loss of *AtUBP14* increases the levels of multi-ubiquitin chains and ubiquitin conjugates in homozygous mutant seeds.
Crude protein extracts prepared from green seeds of wild-type (WT) plants and green or white seeds of selfed *Atubp14-1* and *Atubp14-2* plants were subjected to SDS–PAGE. (a) Total protein detected by silver staining. (b) Immunoblot analysis with anti-*At*PBA1 antibodies. (c) Immunoblot analysis with anti-*At*UBP14 antibodies. (d) Immunoblot analysis with anti-ubiquitin antibodies. The ubiquitin monomer (Ub) and free multi-ubiquitin chains (Ub_n) of various lengths are indicated. The asterisk identifies a new ubiquitin species of 25 kDa present in the *Atubp14-1* and *Atubp14-2* mutants. Equal amounts of total protein (6, 8, 8 and 4 μg for (a)–(d), respectively) were analysed in each lane.

Both yeast and *Dictyostelium* null mutants of *UBP14/UbpA* accumulate high levels of multi-ubiquitin chains, presumably because of a defect in disassembly (Amerik *et al.*, 1997; Lindsey *et al.*, 1998). To test for a similar defect in *Arabidopsis*, we collected immature white seeds (representing homozygous mutant progeny) and green seeds (representing heterozygous mutant and wild-type progeny) from young siliques of selfed *Atubp14-1 and Atubp14-2* plants, and compared their ubiquitination patterns to that of immature wild-type seeds of a similar age. As can be seen in Figure 7(a), SDS–PAGE profiles of total protein from the white and green seeds of the two mutants were similar to those of wild-type seeds, indicating that the *Atubp14* mutations did not alter appreciably the protein content of these young, developing seeds despite an obvious difference in seed colour. Immunoblot analysis with antibodies against a β-subunit (PBA1) of the 20S core particle (Fu *et al.*, 1998), also indicated that the abundance of the 26S proteasome was unaffected by the *Atubp14* mutations (Figure 7b).

As expected, the level of the *At*UBP14 protein was significantly reduced in the white homozygous *Atubp14* mutant seeds as compared to wild-type seeds or green seeds harvested from the selfed *Atubp14-1* and *Atubp14-2* siliques (Figure 7c). We presume that most of the *At*UBP14 protein in the white seeds was from the seed coat that surrounds each developing embryo; because it is maternal tissue, it should be heterozygous for either of the *Atubp14* mutations. When the same protein extracts were examined with anti-ubiquitin antibodies, a substantial increase

in free multi-ubiquitin chains (Ub_{2-6}) was evident in the white seeds as compared to green seeds from the mutant or wild-type plants (Figure 7d). Although not as dramatic, the levels of multi-ubiquitinated proteins with an even higher molecular mass (> 50 kDa) were elevated as well. We also detected a new ubiquitinated species of 25 kDa in the *Atubp14-1* and *Atubp14-2* mutants (Figure 7d). This could represent an unprocessed tetrameric polyubiquitin (Callis *et al.*, 1995), a partially degraded tetrameric free chain, or a ubiquitin–protein conjugate of unknown identity. Surprisingly, little free ubiquitin was evident in either the wild-type or mutant extracts, suggesting that most of the ubiquitin in developing seeds is conjugated to itself or other proteins.

Discussion

UBPs play an essential role in ubiquitin metabolism by generating free ubiquitin monomers from their initial translation products, by removing ubiquitins bound to various targets, and by recycling free multi-ubiquitin chains (D'Andrea and Pellman, 1998; Wilkinson, 1997; Yan *et al.*, 2000). Here, we describe *Arabidopsis* UBP14 that is essential for early embryo development, possibly by helping recycle multi-ubiquitin chains back to ubiquitin monomers. Sequence comparisons helped assign *At*UBP14 as a member of the yeast *Sc*UBP14, human IsoT and *Dictyostelium* UbpA subfamily. This similarity extends beyond the motifs necessary for catalysis and includes several additional domains (potential zinc finger and two UBA domains) that may be important for substrate specificity. Like other members of this group (Amerik *et al.*, 1997; Lindsey *et al.*, 1998; Wilkinson *et al.*, 1995), *Arabidopsis* UBP14 will digest multi-ubiquitin chains (in addition to other substrates) and complement a yeast *ubp14Δ* mutant, indicating functional homology as well. Whereas loss of *Sc*UBP14 has only subtle effects in yeast, loss of the single gene that encodes *At*UBP14 has a profound developmental effect in *Arabidopsis*, inducing embryo lethality. Consequently, it is likely that ubiquitin recycling plays an influential role in early plant development.

Enzymatic analysis of *Arabidopsis* UBP14 showed that it, like *Sc*UBP14, IsoT and UbpA (Amerik *et al.*, 1997; Lindsey *et al.*, 1998; Wilkinson *et al.*, 1995), will process ubiquitins linked together at Lys48 through ε-amino isopeptide bonds. Kinetic studies of *Sc*UBP14 and IsoT suggest that these enzymes prefer free multi-ubiquitin chains, releasing the ubiquitin monomers by a sequential exo mechanism that begins with the proximal end of the chain (i.e. the ubiquitin moiety at the C-terminal end) (Amerik *et al.*, 1997; Lindsey *et al.*, 1998; Wilkinson *et al.*, 1995). Based on observations that chains bearing a proximal des-GlyGly ubiquitin (UbΔGG) are poor substrates, Wilkinson *et al.* (1995) proposed that these enzymes have a specificity pocket for the free C-terminus of the chain and one or more pockets that bind the globular body of the ubiquitins. Given the proposed role of UBA domains in ubiquitin recognition (Hofmann and Bucher, 1996), it is possible that the two UBA domains preceding the His box could form these body-binding pockets. We also found that *Arabidopsis* and yeast UBP14 will cleave ubiquitins linked via α-amino peptide bonds. However, neither yeast nor *Arabidopsis* UBP14 works with all peptide-linked ubiquitin fusions, as both enzymes failed to digest the UBQ1 ribosomal extension protein. Although we did not compare the reaction kinetics for each substrate, these data suggest that *At*UBP14 and *Sc*UBP14 (and probably others in their subfamily) can digest a much broader repertoire of substrates than just multi-ubiquitin chains. Whether this relaxed specificity translates into a wider range of *in vivo* substrates/functions is unknown.

Phenotypic analysis of deletion strains for yeast *UBP14* and *Dictyostelium UbpA* showed that each is not essential (Amerik *et al.*, 1997; Lindsey *et al.*, 1998). As expected from their presumed roles in multi-ubiquitin recycling, loss of either protein increases the steady-state levels of free multi-ubiquitin chains and ubiquitinated proteins. Under standard culture conditions, haploid *Scubp14Δ* strains grow normally. However, like other mutants in the yeast ubiquitin pathway, the *Scubp14Δ* mutants exhibit a strong sporulation defect, stabilize several short-lived proteins, and are hypersensitive to the arginine analogue CAN, the latter phenotype indicating that the breakdown of abnormal proteins is compromised (Amerik *et al.*, 1997). Likewise, *Dictyostelium ubpA* amoebae grow normally and respond like wild-type to starvation. However, a number of defects become evident in the *ubpA* lines following the switch to the sporulation phase, including a reduction in aggregation, chemotaxis, cAMP relay and cell adhesion, caused in part by the attenuated expression of specific genes (Lindsey *et al.*, 1998).

In contrast, our data indicate that *AtUBP14* is absolutely essential in *Arabidopsis*. This was proven by the ability of two mutant *Atubp14* alleles to generate the same embryo-lethal phenotype and by rescue of the phenotype by complementation with an *AtUBP14* transgene. Embryos presumably homozygous for *Atubp14* mutations reached the globular stage, but did not differentiate further to produce viable seeds. Because both mutant alleles could be efficiently transferred through either the pollen or egg, meoisis as well as male and female gametogenesis must proceed normally without a functional *AtUBP14* gene. And because the pollen tube must grow several millimeters before reaching the egg, it is likely that the general physiology and growth of this metabolically active haploid tissue are not significantly attenuated.

However, once the zygote is created, only a limited number of further cell divisions occurs before embryo arrest. Why do *Atubp14* mutants specifically arrest at the globular stage? Analysis of immature seeds of presumed *Atubp14* mutation indicate that they, like those of the yeast *Scubp14Δ* and *Dictyostelium ubpA* strains, have substantially increased levels of both free multi-ubiquitin chains and ubiquitinated proteins. One possibility is that the accumulated free chains globally block ubiquitin-mediated protein breakdown. Both *in vitro* and *in vivo* studies indicate that free multi-ubiquitin chains can be effective inhibitors of the 26S proteasome by competing with ubiquitinated substrates (Amerik *et al.*, 1997; Arnason and Ellison, 1994; Beal *et al.*, 1996; Piotrowski *et al.*, 1997). Consistent with this notion is the fact that homozygous *Arabidopsis* mutants for several genes essential for basic housekeeping processes also arrest at the globular stage, including the genes for ribosomal protein S16 (Tsugeki *et al.*, 1996), glycine acyl tRNA synthetase (Uwer *et al.*, 1998), and one required for biotin synthesis (Patton *et al.*, 1998). The fact that the vegetative phase of both yeast and *Dictyostelium* can survive with similarly elevated levels of chains suggests that *Arabidopsis* is more sensitive to excess multi-ubiquitin chains. Whether this is true for other multicellular organisms remains to be demonstrated.

A second possibility is that developmental progression beyond the globular stage specifically requires AtUBP14 directly, presumably by controlling the level of a key developmental regulator. Interestingly, it is during the early globular stage that cell differentiation of the embryo first becomes visible, and then, upon entering the heart stage, the apical–basal pattern emerges (Meinke, 1994). This regulatory factor could be necessary to transform the relatively non-differentiated cells within the globular stage to cells that rapidly develop to produce a heart-stage embryo containing both the root and shoot primordia. Consistent with this idea is the effect of *ubpA* mutations on *Dictyostelium* development. The *ubpA* mutants display an aberrant phenotype only when they transition to the reproductive phase that requires the coordinated effort of the individual cells to produce the pseudopod (Lindsey *et al.*, 1998). However, it should be emphasized that because *Atubp14* mutants arrest early in embryogenesis, this does not preclude role(s) for this enzyme later on in *Arabidopsis* development. Arrest at the globular stage probably demarcates the first developmental stage where possession of a functional *AtUBP14* gene becomes essential. In fact, the detection of *At*UBP14 protein in all tissue types probably reflects a general role for this enzyme in ubiquitin metabolism.

Our data showing that levels of free multi-ubiquitin chains and ubiquitinated proteins increase upon loss of *At*UBP14 are consistent with the proposal that the UBP14 subfamily is required for a homeostatic mechanism that balances the pools of free ubiquitin and multi-ubiquitin chains to promote optimal function of the ubiquitin/26S proteasome pathway (Amerik *et al.*, 1997; Lindsey *et al.*, 1998; Wilkinson *et al.*, 1995). If levels of UBP14 drop too low, free multi-ubiquitin chains would accumulate, which in turn would block protein turnover by competing with ubiquitinated substrates for recognition by the 26S proteasome. Conversely, if the levels of UBP14 became too high (e.g. by over-expression), inappropriate disassembly of multi-ubiquitin chains could occur. Such chain shortening could impair binding of the substrate to the 26S proteasome, especially if chain length fell below that required for recognition (four ubiquitin monomers; Beal *et al.*, 1996; van Nocker *et al.*, 1996). Moreover, if free chains were disassembled, their use as preformed multi-ubiquitin chains would be impaired (van Nocker and Vierstra, 1993). By modulating chain assembly/disassembly, it is possible that the UBP14 subfamily provides a general strategy for regulating protein turnover under changing environmental and developmental conditions. Such a mechanism could control the turnover of all proteins or selectivity affect the half-lives of selected substrates.

Experimental procedures

Identification of the At*UBP14 gene and plasmid construction*

AtUBP14 was identified by analysis of the T-DNA insertion line L1-85 (see below) and by BLAST searches of the *A. thaliana* (ecotype Columbia) DNA sequence database using the consensus Cys and His box sequences from the *At*UBP family as queries (Yan *et al.*, 2000). Searches of *At*UBP14 protein sequence for functional domains used the National Center for Biotechnology Information conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Genomic clones for *AtUBP14* were isolated from the BACs T8D5 and T15I10 (*Arabidopsis* Biological Resource Center (ABRC), Columbus, Ohio, USA), using as the probe a PCR product of *AtUBP14* amplified from L1-85 genomic DNA. A full-length *AtUBP14* cDNA was PCR-amplified from a 3–6 kb size-selected cDNA library (Kieber *et al.*, 1993) using Pfu polymerase (Stratagene) and the 5′ primer GGATCCATGGAGCTCCTCCGATCCAACTTGTC and 3′ primer GTCGACATCAAGCCGCTGAAAGAAGTAGACATAACC, designed to introduce a *Bam*HI and a *Sal*I restriction site (underlined) at the predicted start and stop codons, respectively. The Cys → Ser mutant p*AtUBP14*$_{C317S}$ was generated by QuickChange site-directed mutagenesis (Stratagene) using the primers GTCAATCTTGGGAACAGTTCCTACTTGGCAGCTACG and CGTAGCTGCCAAGTAGGAACTGTTCCCAAGATTGAC (the altered nucleotides are underlined). The amplified product was cloned into the *Eco*RV site of pBluescript. For expression in *Escherichia coli*, the cDNAs of *AtUBP14* and *AtUBP14*$_{C317S}$ were both subcloned into the *Bam*HI and *Sal*I sites of pET23a and pET32a (Novagen, Madison, Wisconsin, USA). For expression in yeast (*S. cerevisae*), the cDNAs were re-amplified, using the same 5′ primer in

conjunction with the 3′ primer GTCGAC*TCA*ATCAAGCCGCTGAAAGAAGTAGAC which reintroduced the natural stop codon (italic) in addition to a *Sal*I site (underlined), and ligated into the yeast vector pRS426 (Stratagene).

Complementation of yeast ubp14Δ

The yeast strains used were MHY501 (*MATα his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1*) and MHY840 (*MATα his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 ubp14-Δ1::HIS3*) (Amerik *et al*., 1997). For complementation assays, MHY840 cells were transformed by the LiAc/SS-DNA/PEG method (Gietz *et al*., 1995) with the vector pRS426 harbouring *ScUBP14*, *ATUBP14* or $AtUBP14_{C317S}$. Transformed cells were grown in liquid minimal synthetic medium (SD medium) supplemented with 3% galactose instead of dextrose and without uracil. MHY501 and MHY840 cells were grown with uracil and 3% galactose. At mid-log phase, cells were harvested and resuspended in SD medium without arginine, and diluted 10-, 100-, 1000- and 10 000-fold. Aliquots (2 μl) of each dilution were spotted onto solid SD medium containing 3% galactose and 4.4 μM CAN but without dextrose and arginine, and incubated for 4 days at 30°C. For immunoblot analysis, cells grown to the mid-log phase were lysed directly in hot 1 × SDS–PAGE sample buffer. Lysates from an equal number of cells (determined by optical density) of each genotype were subjected to SDS–PAGE and immunoblot analysis using anti-ubiquitin antibodies.

UBP activity assays

In vitro cleavage of ubiquitin ε-amino isopeptide linkages was measured using Lys48-linked multi-ubiquitin chains as the substrate (Yan *et al*., 2000) and *AtUBP14* expressed in pET32a. *In vivo* cleavage of ubiquitin attached by α-amino linkages was determined using the substrates polyubiquitin *At*UBQ10-LE (Yan *et al*., 2000), ubiquitin extension protein *At*UBQ1 (p8185; Chandler *et al*., 1997) and ubiquitin-X-β-galactosidase (Ub-X-βgal, where X = methionine or leucine; Papa and Hochstrasser, 1993). Each of the three substrates (encoded on a pACYC184-based plasmid) was co-expressed with the wild-type or a mutant form of *AtUBP14* (in pET23a), wild-type *ScUBP1* (in pRB293; Tobias and Varshavsky, 1991; R.T. Baker, unpublished) or *ScUBP14* (in pGEX-KG; Amerik *et al*., 1997) in the *E. coli* strain NovaBlue DE3 (Novagen) using standard conditions except that induction was at 22°C. Detection of the cleavage products was performed according to the method described by Yan *et al*. (2000).

T-DNA insertion mutants of At*UBP14*

The *Arabidopsis* T-DNA insertion line L1-85 (renamed as *Atubp14-1*) was identified by Drs Elena Babiychuk and Sergei Kushnir (Universiteit of Gent, Gent, Belgium) from an exon-trap population of *A. thaliana* (ecotype C24) transformed with the *apurinic/apyrimidinic endonuclease* (*ARP*)*–neomycin phosphotransferase II* (*NPTII*) coding region (Babiychuk *et al*., 1997). The DNA sequence at the 5′ *AtUBP14* T-DNA junction was obtained following PCR amplification of genomic DNA using the gene-specific primer CGGAGAATCTCTGGCTTATTCTTACCGATG and the T-DNA-specific primer CCCCTGCGCTGACAGCCGGAACAC.

The *Atubp14-2* T-DNA mutant was identified by a PCR-based screen (Krysan *et al*., 1999) of a T-DNA-transformed population of *A. thaliana* (ecotype WS) generated by Ken Feldman (E.I. duPont de Nemours & Company, Wilmington, Delaware, USA) using the 5′ GTCTTGGGTCTATTGGGTAAATACAAACTC or 3′ GCTTGTGAAAGGCTTCTAATTCATCTTGA gene-specific primers in combination with either a left border (LB) or a right border (RB) T-DNA-specific primer (Krysan *et al*., 1996). The T-DNA insertion within *Atubp14-2* conferred kanamycin resistance and was bracketed on both ends by LB sequence. The *Atubp14-1 and Atubp14-2* mutants were backcrossed four and two times, respectively, to wild-type *Arabidopsis* (ecotype WS).

For complementation of *Atubp14-1*, a 10.6 kb genomic DNA fragment (*Hin*dIII–*Xba*I) from BAC T15I10 encompassing the entire *AtUBP14* gene (also includes part of the open reading frame for a hypothetical protein) was cloned into pBluescript. The plasmid was digested with *Kpn*I and *Xba*I, and the resulting fragment was then subcloned into the corresponding sites of pCAMBIA1301 (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK). pCAMBIA-*AtUBP14* was introduced into *Agrobacterium tumefaciens* strain GV3101, which then was used to transform heterozygous *Atubp14-1* plants by the floral dip method (Clough and Bent, 1998). The transformed T1 seedlings were selected by hygromycin B resistance. T2 seedlings and their progeny were genotyped by PCR and antibiotic resistance. Several T3 seedlings were crossed to heterozygous *Atubp14-2* plants; progeny seedlings were tested by PCR for the presence of the *Atubp14-1* and *Atubp14-2* alleles.

Siliques at different stages of maturity were harvested, opened, and cleared for 12–48 h at room temperature with a chloral hydrate:water (8:3 v/v) solution (Meinke, 1994). Cleared or dissected wild-type and mutant embryos were examined using an Olympus CH30 microscope.

Immunoblot analysis for At*UBP14 and ubiquitin*

Anti-*At*UBP14 antibodies were generated against recombinant protein expressed in *E. coli* using pET23a (see above). The protein was purified by washing the inclusion body fraction. Plant tissues were harvested from adult *Arabidopsis* plants grown under a long-day photoperiod (16 h light/8 h dark) at 21°C. Etiolated and green seedlings were grown for 5 days in the dark and under continuous white light, respectively. For analysis of the *Atubp14* mutants, white and green seeds were collected separately from young, green siliques of selfed heterozygous mutant plants. Care was taken to harvest only young seeds with embryos at or prior to the torpedo stage. As a control, green seeds of the same developmental age were collected from wild-type siliques. All tissues were homogenized in extraction buffer (3 ml g^{-1} fresh weight) containing 50 mM Tris (pH 8.0), 1 mM Na_4EDTA, 1% Triton X-100, 100 mM KCl and 30 mM $Na_2S_2O_5$, and clarified by low-speed centrifugation. Equal amounts of total protein (determined by BioRad Bradford assay; Hercules, California, USA) were subjected to SDS–PAGE and silver staining or to immunoblot analysis using either anti-*At*UBP14, anti-*At*PBA1 or anti-ubiquitin antibodies.

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