Ubiquitin C-terminal hydrolases 1 and 2 affect shoot architecture in Arabidopsis

Peizhen Yang†, Jan Smalle‡, Sangsook Lee, Ning Yan, Thomas J. Emborg and Richard D. Vierstra *
Department of Genetics, 425-G Henry Mall, University of Wisconsin-Madison, Madison, WI 53706-1574, USA

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*For correspondence (fax +1 608 262 2976; e-mail vierstra@wisc.edu).
†Present address: Department of Biochemistry, 425 Babcock Drive, University of Wisconsin-Madison, Madison, WI 53706, USA.
‡Present address: Department of Plant and Soil Sciences, Cooper and University Drives, University of Kentucky, Lexington, KY 40546-0312, USA.

Summary
Ubiquitin C-terminal hydrolases (UCHs) are a subset of de-ubiquitinating proteases that release covalently linked ubiquitin (Ub), and as such play essential roles in recycling Ub and reversing the action of Ub conjugation. We show here that two related Arabidopsis UCHs, UCH1, and UCH2, are important for shoot development. The UCH1 and 2 genes are ubiquitously expressed, with the corresponding proteins present in both the cytoplasm and nucleus. Unlike their animal and fungal counterparts, we found no evidence that the Arabidopsis UCH1 and 2 proteins stably associate with the 26S proteasome. Altering the levels of UCH1 and 2 has substantial effects on Arabidopsis shoot development, especially with respect to inflorescence architecture, with over-expression and double mutants enhancing and suppressing the outgrowth of cauline branches, respectively. Neither UCH1-over-expressing nor uch1-1 uch2-1 plants have detectably altered sensitivity to cytokinins or auxins individually, but exhibit an altered sensitivity to the ratio of the two hormones. UCH1-over-expressing plants show dramatically enhanced phenotypes when combined with auxin-insensitive mutants axr1-3 and axr2-1, suggesting that one or more aspects of auxin signaling are affected by this enzyme pair. Previous studies revealed that the ubiquitination and degradation of the AUX/IAA family of repressors is a key step in auxin signaling. Here, we show that turnover of a reporter fused to a representative AUX/IAA protein AXR3 is faster in the uch1-1 uch2-1 double mutant but slower in the UCH1 over-expression backgrounds. Taken together, our results indicate that de-ubiquitination helps to modify plant shoot architecture, possibly via its ability to directly or indirectly protect upstream target proteins involved in auxin/cytokinin signaling from Ub-mediated degradation.

Keywords: ubiquitin, de-ubiquitination, shoot development, auxin, cytokinin.

Introduction
Selective attachment by ubiquitin (Ub) is an important post-translational modification for controlling the biologic activity, location and/or half-life of many regulatory and structural proteins in both plants and animals (Smalle and Vierstra, 2004; Weissman, 2001). As the result of an ATP-dependent enzymatic cascade involving the sequential action of Ub-activating enzymes (E1s), Ub-conjugating enzymes (E2s), and Ub–protein ligases (E3s), Ub becomes covalently attached to various targets via an isopeptide bond between Gly76 of Ub and one or more lysines within the target. In some cases, a single Ub is attached. In others, a polymeric chain of Ub monomers is assembled using a lysine within previously bound Ub monomers as the conjugation site. While mono-ubiquitination appears to serve a variety of functions, including roles in transcription, DNA repair and endocytosis, poly-ubiquitination primarily directs proteins to the 26S proteasome for breakdown (Hicke, 2001; Smalle and Vierstra, 2004; Weissman, 2001). Collectively, Ub conjugation affects a number of essential processes in plants, including embryogenesis, morphogenesis, most if not all hormone responses, disease resistance, environmental adaptation, and programmed cell death, to name but a few (Moon et al., 2004; Smalle and Vierstra, 2004). Pertinent to this study are the roles of Ub and the 26S proteasome in leaf morphology (Huang et al., 2006) and shoot branching (Stirnberg et al., 2002), and signaling...
by the hormones auxin (Moon et al., 2004) and cytokinin (Smalle et al., 2002). Auxin perception in particular is initiated by hormone-induced activation of the SCFTIR1 E3 complex, which specifically targets, by ubiquitination, the family of AUX/IAA repressor proteins for breakdown (Dharmasiri et al., 2005; Kepinski and Leyser, 2005).

In addition to Ub attachment, the release of Ub monomers (de-ubiquitination) is now emerging as an important step in regulating the abundance and functions of Ub (Amerik and Hochstrasser, 2004; Chung and Baek, 1999). This reaction is catalyzed by a unique group of processing proteases known as de-ubiquitinating enzymes (DUBs) that specifically release Ub monomers linked through their C-termini by isopeptide/peptide bonds (Wilkinson, 1997). Within the Ub system, DUBs can: (i) generate functional Ub monomers by processing the Ub fusion proteins that are the sole products of the UBO gene families (Callis et al., 1990), (ii) help maintain the pool of active Ub by removing small molecules that become inadvertently attached to the C-terminal Gly and by recycling Ub monomers incorporated into poly-Ub chains, (iii) help rescue Ub by releasing the target-bound form before degradation of the target by the 26S proteasome, and (iv) regulate the stability/function of specific Ub conjugates by selectively removing the Ub (or Ub moieties) (Amerik et al., 2000; Wilkinson, 1997).

Based on their amino acid sequence and enzymatic activities, five major groups of DUBs have been identified, including the Ub C-terminal hydrolases (UCHs), the Ub-specific processing proteases (UBPs), otubain- and ataxin-related families, and the 26S proteasome subunit RPN11 (Amerik and Hochstrasser, 2004; Tran et al., 2003). RPN11 is a metalloprotease that employs a zinc ion for catalysis, whereas the other four groups are cysteine proteases. To date, phylogenetic analyses have identified 30 DUBs in yeast (Saccharomyces cerevisiae), and at least 64 and 110 in Arabidopsis thaliana and humans, respectively (Amerik and Hochstrasser, 2004; Amerik et al., 2000; Yan et al., 2000; P. Yang and R.D. Vierstra, unpublished results). Collectively, these proteases form the second largest group of enzymes within the Ub system, implying important regulatory roles. Despite this prevalence, little is known about DUB function. In animals, individual DUBs have been connected to cell growth and differentiation, neural function, gene regulation, embryogenesis, eye development, stress, and oncogenesis (Amerik and Hochstrasser, 2004; Chung and Baek, 1999). In plants, only a few DUBs have been genetically characterized to date. Plant RPN11, like its animal counterpart, is a core subunit of the 26S proteasome (Yang et al., 2004), and presumably plays a similar role in releasing Ub monomers from poly-ubiquitinated proteins before their breakdown by the protease complex (Verma et al., 2002). Arabidopsis UBP1 and 2 are a related pair that help degrade abnormal proteins (Yan et al., 2000), UBP14 has a central role in disassembling free poly-Ub chains and is essential for embryogenesis (Doelling et al., 2001; Tzafrir et al., 2002), and UBP3 and 4 together are critical for pollen development (Doelling et al., 2007).

UCHs comprise a relatively small family of DUBs that are structurally defined by a signature active site bearing a catalytic triad of positionally conserved cysteine/histidine/aspartic acid residues (Pickart and Rose, 1985). The founding member of this family – yeast UCH1 – was proposed to have an important housekeeping role based on its ability to release small molecules that become inadvertently bound to the C-terminus of Ub as side reactions in the E1–E2–E3 conjugation cascade (Larsen et al., 1998). Recent analyses suggest that UCHs also have specific regulatory roles. In humans, for example, the UCH BAP1 interacts with and enhances the activity of the BRCA1 Ub ligase (Jensen and Rauscher, 1999; Jensen et al., 1998), and the UCH-L1/UCH-L3 pair is highly expressed in brain where it helps to regulate neuronal activity (Kurihara et al., 2001; Lombardino et al., 2005).

To help understand how the DUBs regulate growth and development in plants, we characterized two related members of the Arabidopsis UCH family, UCH1 and 2. Enzymatic analysis of UCH2 confirmed their abilities to release Ub linked via peptide or isopeptide bonds both in vivo and in vitro. From analysis of plants over-expressing either UCH1 or UCH2 and of a double mutant disrupting expression of the pair, we discovered that these two DUBs strongly influence shoot architecture. The synergistic effects of 35S:UCH1 when combined with either of two auxin-resistant mutants, axr1-3 or axr2-1, and the ability of UCH1 over-expression and uch1-1 uch2-1 mutants to retard and enhance AUX/IAA protein turnover, respectively, revealed that at least part of this phenotype may be caused by altered auxin signaling.

Results

Characterization of the Arabidopsis UCH family

To help define the UCH family in plants, we used the catalytic domain of a representative member from humans (BAP1) (Jensen et al., 1998) as a query to search the Arabidopsis genome for related sequences. This search uncovered three genes, designated UCH1 (At5g16310), UCH2 (At1g65650), and UCH3 (At4g17510), that encode proteins similar to various UCHs from yeast and animals (Figure 1a). Strong amino acid sequence conservation was particularly evident around the positionally conserved cysteine/histidine/aspartic acid residues that form the signature catalytic triad. Whereas UCH3 encompasses only the N-terminal portion of 230 residues that surrounds the active site, the UCH1 and UCH2 proteins (330 and 334 residues, respectively) also contain related C-terminal extensions of approximately 100 amino acids (Figure 1a).
Phylogenetic analyses using just the active-site domain of approximately 230 amino acids grouped Arabidopsis UCH3 with members of the UCH-L3 subfamily that also contain only this region (Figure 1b). Conversely, UCH1 and 2 clustered most strongly with each other (63/74% amino acid identity/similarity) and with members of the UCH37 family that is found in animals and *Schizosaccharomyces pombe* (approximately 50% similar over the entire length) but is not evident in *Saccharomyces cerevisiae* (Holzl et al., 2000; Li et al., 2001; Stone et al., 2004). In addition to similarity within the N-terminal catalytic domain, all members of this subgroup contain C-terminal extensions related to those in UCH1/2; strong sequence conservation within several stretches of this extension suggests that it is critical for the function(s) of this subgroup (Figure 1a). UCH37 orthologs from mammals, *Drosophila*, and *S. pombe* have been reported to associate with the 26S proteasome and may help ubiquitinated targets enter the protease lumen by trimming their poly-Ub chains (Holzl et al., 2000; Lam et al., 1997; Li et al., 2001; Stone et al., 2004). For human UCH37, this interaction may involve binding to the 26S proteasome subunit RPN12 via the C-terminal extension in UCH37 (Li et al., 2001). A potential nuclear localization signal (NLS) is also present near the C-terminus of Arabidopsis UCH1 and 2 and other members of the UCH37 family (residues 302–304 in UCH1), which may direct these DUBs to the nucleus (Figure 1a).

**UCH2 is active in vivo and in vitro**

Like other DUBs, UCHs are defined by their ability to release Ub attached via peptide (α-amino) and/or isopeptide (ε-amino) bonds to other proteins (Amerik and Hochstrasser, 2004). To confirm this activity for Arabidopsis UCH1/2, we tested recombinant UCH2 protein alongside a Cys82 to should disrupted in the catalytic triad (C82S) and thus should be inactive, and UCH representatives from yeast (ScUbp1p, Tobias and Varshavsky, 1991) and bovine (UCH37, Lam et al., 1997) previously shown to have DUB activity. The Arabidopsis, yeast, and bovine UCHs could be expressed to high levels as soluble proteins in *Escherichia coli*. However, it should be noted that because the amounts of each protein added to the reactions were not normalized, determining their relative activities was not possible.

As can be seen in Figure 2, UCH2 released Ub attached via both peptide and isopeptide linkages. The recombinant

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Figure 1. Amino acid sequence comparison of the Arabidopsis UCH family with orthologs from other species.

(a) Sequence alignment of Arabidopsis (At) UCH1, 2 and 3, human (Hs) UCH37, bovine (Bt) UCH37, *Drosophila melanogaster* (Dm) UCH37, *Schizosaccharomyces pombe* (Sp) Uch2p and *Caenorhabditis elegans* (Ce) 37.7 kDa UCH. Identical and similar amino acids are shown in black and gray boxes, respectively. Asterisks indicate the positions of the cysteine/histidine/aspartic acid residues that comprise the active-site triad. The genomic positions of the T-DNA insertions in the uch1-1 and uch2-1 mutants are shown by arrowheads. The solid and dashed lines identify a potential nuclear localization signal (NLS) and the region in human UCH37 proposed to interact with the 26S proteasome subunit RPN12 (Li et al., 2001), respectively.

(b) Unrooted phylogenetic tree of representative members of the UCH family from Arabidopsis (At), yeast (Sc), *S. pombe* (Sp), rice (Os), *C. elegans* (Ce), *D. melanogaster* (Dm), goldfish (Gg), mice (Mm) and human (Hs). Clades of functionally distinct subtypes are identified by the brackets. The three Arabidopsis UCHs are underlined.

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Arabidopsis UCH2 has DUB activity capable of removing polypeptides linked by z-amino peptide or c-amino isopeptide bonds to the C-terminus of Ub. The substrates were either co-expressed in Escherichia coli with AtUCH2 and its active site mutant C82S, yeast (Sc) Ubp1p or bovine (Bt) UCH37, or mixed with E. coli extracts containing the DUBs. 'Vector' indicates cells or extracts containing an empty expression vector. The substrates and their cleavage products were detected by immunoblot analysis with anti-Ub antibodies. The arrowheads locate free Ub, and either poly-Ub chains containing varying numbers of Ub monomers (a and c), or the initial translation product of AtUBQ1 (b).

(a) Co-expression of the DUBs with AtUBQ10 that contains six Ub repeats linked internally via z-amino peptide linkages.
(b) Co-expression of the DUBs with the Ub extension protein AtUBQ1 that contains a Ub monomer linked via an z-amino peptide linkage to the 52 amino acid ribosomal protein L40.
(c) In vitro incubation of DUBs with poly-Ub chains internally linked via Lys48.

Expression and localization of UCH1 and 2

To help understand the biologic functions of UCH1 and 2, we employed several methods to evaluate their expression patterns. Numerous cDNAs for each were found in the Arabidopsis EST database (11 and 20 for UCH1 and UCH2, respectively; http://www.arabidopsis.org), indicating that both loci are actively transcribed. Mining the cDNA microarray database of Ma et al. (2005) revealed that UCH1 and 2 are expressed throughout the plant, with the relative levels of the UCH2 transcript being approximately two- to ninefold higher than those for UCH1 (Figure 3a). The highest level of UCH2 mRNA was found in cell cultures, implying an important role for the enzyme in actively dividing cells. Scans of the various DNA microarray databases (e.g. Genevestigator; Zimmermann et al., 2004) examining the global gene expression patterns of Arabidopsis exposed to various environments did not detect any substantial changes in UCH1/2 transcript abundance, suggesting that neither gene is strongly regulated by external signals (data not shown).

To assess the accumulation of the UCH1 and 2 proteins, we generated antisera against His6-tagged recombinant versions of each. As can be seen in Figure 3(b), these antisera could recognize as little as 0.2 ng of UCH1 and <2 ng of UCH2. The anti-UCH1 and anti-UCH2 antibodies recognized less well their respective paralogs UCH2 and UCH1, which proved beneficial for subsequent genetic analyses (see below). Immunoblot analysis of crude Arabidopsis extracts confirmed that the UCH1 and 2 proteins are present in most, if not all, tissues (Figure 3c). The relative levels of UCH1/2 in the various tissues were similar to those of two 26S proteasome subunits, the core protease (CP) 1-subunit PBA1 and the 19S regulatory particle (RP) Lid subunit RPN11.

The subcellular locations of UCH1 and 2 were determined by confocal fluorescence microscopy of Arabidopsis protoplasts transiently expressing UCH1/2-GFP fusions.
Fluorescence from red fluorescent protein (RFP) or GFP fused to the NLS of SV40 large T antigen and chlorophyll autofluorescence were also captured as markers for the nucleus and pockets of cytoplasm, respectively. As can be seen in Figure 3(d), signals from both GFP–UCH1 and GFP–UCH2 were detected in cytoplasm and nucleus, with strong fluorescence emanating from the nucleus. A similar localization pattern was also seen for free GFP, but the nuclear signal for free GFP appeared to be less intense. The patterns for UCH1 and 2 were similar to that of a RFP–RPN10 fusion (Figure 3d) and RPN6 and RPT5 detected immunologically (Kwok et al., 1999), suggesting a potential connection between the two UCHs and the 26S proteasome as has been reported for their animal and S. pombe homologs (Holzl et al., 2000; Lam et al., 1997; Li et al., 2001; Stone et al., 2004).

Human and S. pombe UCH37 were previously reported to interact by yeast two-hybrid analysis and/or co-immunoprecipitation directly with the RPN12 and RPN10 subunits of the 26S proteasome, respectively, suggesting that UCH1/2-type proteins are integral components of the protease complex (Li et al., 2001; Stone et al., 2004). However, when we tested for similar interactions by direct pairing of full-length UCH1 and 2 or their C-terminal domains alone with Arabidopsis RPN12α or RPN10 by the yeast two-hybrid analysis, no interactions were detected using either growth on 3-amino-1',2',4'-triazole or β-galactosidase activity assays (data not shown).

To examine whether UCH1 and 2 directly associate with the 26S proteasome via another mechanism, we employed glycerol gradient ultracentrifugation to enrich for the complex, and tested for the presence of UCH1/2 by immunoblot analysis. Consistent with its large size (Yang et al., 2004), the Arabidopsis 26S proteasome migrated in the glycerol gradient as a single peak of peptidase activity (Figure 4a). The identity of this peak was confirmed by its sensitivity to SDS, which dissociates the complex, and to MG132, which is a reversible inhibitor of its chymotryptic activity (Yang et al., 2004). Known subunits of the 26S proteasome CP (PAC1), and RP Base (RPT1a) and Lid (RPN12α) co-eluted with this peptidase activity, indicating that the CP–RP complex remained intact during this separation (Figure 4b). In contrast, both UCH1 and UCH2 were found at the top of the glycerol gradient where free non-integrated proteins would migrate. Even over-exposures of the immunoblots failed to detect trace amounts of UCH1/2 in the fractions that also

Figure 3. Expression and localization of Arabidopsis UCH1 and UCH2.
(a) Relative abundance of the UCH1/2 transcripts in various tissues as determined by DNA microarray analysis (Ma et al., 2005). Cau, cauline; Cot, cotyledon; Hypo, hypocotyl; Ros, rosette.
(b) Specificity of the anti-UCH1 and 2 antibodies. Increasing concentrations of recombinant UCH1 and 2 were subjected to SDS–PAGE and immunoblot analysis with either anti-UCH1 (upper panel) or anti-UCH2 antibodies (middle panel). Protein (Prot) amounts were verified by silver staining the gel (lower panel).
(c) Immunoblots of various tissues using antibodies recognizing UCH1, UCH2, PBA1 and RPN11.
(d) Localization of UCH1 and 2 in Arabidopsis protoplasts. Protoplasts transfected with plasmids encoding GFP–UCH1, GFP–UCH2, RFP–RPN10 and RFP fused to a NLS were visualized by laser scanning confocal microscopy to detect GFP (green), RFP (yellow) and chloroplast (Chloro) fluorescence (red). Merged pictures of all three images are also shown.
expression of the corresponding genes. The Wassilewskija ecotype (WS) background that disrupt and UCH2, we isolated T-DNA insertion mutants in the To help define the biologic function(s) of Arabidopsis UCH1 Altering UCH1/2 expression modifies shoot architecture and 5a). The 38 bp upstream of the second exon. Genomic PCR using a left border T-DNA primer in combination with gene-specific primers helped to identify homozygous uch1-1 and uch2-1 plants. Kanamycin resistance conferred by the neomycin phosphotransferase gene within the T-DNAs segregated in a 3:1 ratio, suggesting that these mutants contain a single T-DNA insertion.

Gel blot analysis of total RNA hybridized under high stringency failed to detect the UCH1 transcript in the uch1-1 plants, and detected a reduced amount of UCH2 transcript in the uch2-1 plants (Figure 5b). Consistent with the lack of UCH1 mRNA, we found only a trace of cross-reacting protein (which we presume is UCH2) in homozygous uch1-1 seedlings by immunoblot analysis with anti-UCH1 antibodies (Figure 5c). A protein with a slightly lower apparent molecular mass was also seen; this species was present in the uch1-1 uch2-1 double mutant implying that it represents an unrelated protein. Even though we could detect UCH2 mRNA in homozygous uch2-1 seedlings, we could not detect the corresponding protein with anti-UCH2 antibodies, indicating that the uch2-1 mutation strongly dampens expression (Figure 5c). Each single homozygous uch1/2 mutant was phenotypically indistinguishable from wild-type WS plants, implying that the two genes are functionally redundant (Table 1 and data not shown). Consequently, a double uch1-1 uch2-1 mutant line was created by crossing individual uch1-1 with uch2-1 plants, and identifying by PCR the double homozygous mutant in the F2 generation. This line had undetectable levels of both the UCH1 and UCH2 proteins (Figure 5c). Taken together, we conclude that uch1-1 and uch2-1 represent strong alleles for each locus.

We also attempted to increase the levels of UCH1 and 2 by over-expressing each full-length cDNA under the control of the CaMV 35S promoter. Eighty and twenty-four independent transgenic lines containing the 35S:UCH1 and 35S:UCH2 transgenes, respectively, were generated, using hygromycin resistance linked with the transgene for selection. Eleven 35S:UCH1 and five 35S:UCH2 plants displayed similar phenotypes with a range of strengths (see below). A substantial increase in the abundance of the UCH1/2 transcripts and proteins was detected in all phenotypically compromised 35S:UCH1 and 35S:UCH2 seedlings (Figure 5b,c, and data not shown), indicating that the phenotype was indeed caused by over-expression. Furthermore, the UCH2 transcript level was unaffected in the 35S:UCH1 background, suggesting that its expression is not dampened to compensate when UCH1 protein levels are elevated (Figure 5b). Unfortunately, infertility of all the compromised 35S:UCH2 plants precluded a detailed developmental and molecular analysis of these lines (data not shown).

Phenotypic analysis of uch1-1 uch2-1 and 35S:UCH1 plants showed that the corresponding DUBs have an important role in Arabidopsis leaf and shoot development and animal orthologs (Holzl et al., 2000; Lam et al., 2001; Stone et al., 2004).

Altering UCH1/2 expression modifies shoot architecture

To help define the biologic function(s) of Arabidopsis UCH1 and UCH2, we isolated T-DNA insertion mutants in the Wassilewskija ecotype (WS) background that disrupt expression of the corresponding genes. The uch1-1 allele contains a T-DNA within the 4th exon immediately after codon 251, and is followed by a 40 bp deletion (Figures 1a and 5a). The uch2-1 allele contains a T-DNA in the first intron contained the 26S proteasome. While a loose or transient association cannot be discounted, it appears that UCH1/2 are not integral polypeptides of the Arabidopsis 26S proteasome, which is different from the reported location of their S. pombe and animal orthologs (Holzl et al., 1997; Li et al., 2001; Stone et al., 2004).

![Figure 4](image)

Figure 4. UCH1/2 do not associate tightly with the Arabidopsis 26S proteasome.

(b) Detection of UCH1/2 and various 26S proteasome subunits in the glycerol gradient fractions by immunoblot analysis with antibodies against UCH1, UCH2 and subunits CP (PAC1), RP Base (RPT1a), and RP Lid (RPN12a). PEG, PEG precipitate before glycerol gradient ultracentrifugation.
lated more chlorophyll than wild-type, whereas the homozygous *uch1-1* *uch2-1* mutant accumulated less. When grown under a long-day (LD, 16 h light/8 h dark) photoperiod, 14-day-old seedlings of 35S:*UCH1*, wild-type and the *uch1-1 uch2-1* double mutant had 244 ± 10, 201 ± 8 and 176 ± 5 μg chlorophyll g⁻¹ fresh weight, respectively. Both 35S:*UCH1* and *uch1-1 uch2-1* plants had smaller and more compact rosettes than wild-type (0.22 ± 0.08 and 0.16 ± 0.04 versus 0.27 ± 0.05 cm² for the average size of the fifth rosette leaf, respectively), which partially reflected a shortening of the petioles and decreased leaf size (Figure 6a,c).

35S:*UCH1* rosette leaves were also significantly more round than their wild-type and *uch1-1 uch2-1* counterparts (length/width ratios of 1.03 ± 0.11 for 35S:*UCH1* versus 1.59 ± 0.09 for wild-type and 1.64 ± 0.13 for the *uch1-1 uch2-1* mutant). Young 35S:*UCH1* rosettes produced epinastic leaves, while the *uch1-1 uch2-1* rosettes produced hyponastic leaves, compared to the wild-type (Figure 6b, left panel). In addition, the cauline leaves of 35S:*UCH1* lines were more round than those of the wild-type, while the *uch1-1 uch2-1* lines generated more lanceolate cauline leaves (Figure 6b, right panel).

Compared to wild-type WS, fewer numbers of rosette leaves developed on *uch1-1 uch2-1* plants before bolting under LD conditions, whereas more rosette leaves developed on 35S:*UCH1* plants (Figure 6c). However, both mutants flowered and senesced slightly later than wild-type. Flower morphology was also altered. The ratio of pistil and stamen length was decreased in 35S:*UCH1* and increased in *uch1-1 uch2-1* flowers (data not shown). Often the *uch1-1 uch2-1* flowers had mis-shaped petals and abnormally large stigmas (Figure 6f,g). Both the 35S:*UCH1* and *uch1-1 uch2-1* plants developed shorter siliques (Figure 6h). While the fertility of the 35S:*UCH1* plants was unaffected by the over-expression, the *uch1-1 uch2-1* plants appeared less fertile, with aborted seeds often evident upon self-fertilization (Figure 6i).
The most striking phenotype of mature 35S:UCH1 and uch1-1 uch2-1 plants was a substantial change in the emergence and elongation of cauline (or lateral) branches on the primary inflorescence, and in the appearance of rosette (or secondary) inflorescences under both LD and short-day (SD) (8 h light/16 h dark) conditions (Figure 6d,e and Table 1). Under LD conditions, the 35S:UCH1 plants had a shorter primary inflorescence than wild-type (13.6 versus 28.3 cm). While the number of cauline branches was the same (4.0 versus 3.8), they emerged closer to the rosette (6.8 versus 32.1 mm) and their relative length compared to the primary inflorescence was longer in 35S:UCH1 plants (ratio of 0.71 versus 0.50) (Table 1). In most if not all cases, the cauline branches were subtended by cauline leaves for wild-type and all mutant backgrounds under the growth conditions used here. The 35S:UCH1 siliques were also closely spaced on the terminal branches. The combined effects created short bushy plants covered with siliques. The uch1-1 uch2-1 plants under LD conditions more closely resembled wild-type, with a similar length of the primary inflorescence (27.4 versus 28.3 cm). However, the double mutant had slightly fewer cauline branches (3.5 versus 3.8) and a sizeable increase in the number of rosette inflorescences at maturity (10.4 versus 5.0 secondary inflorescences).
Plants include wild-type (WT), 35S:UCH1 transgene. All genotypes, SD conditions, reduced the number of rosette inflorescences. Despite the dramatic changes to the shoot, the growth, architecture and branching of the root appeared to be unaltered for both the uch1-1 uch2-1 and 35S:UCH1 plants, at least when grown for several weeks on vertical agar medium (data not shown).

Reversal of the uch1-1 uch2-1 phenotype by over-expression of UCH1

To confirm that the phenotype of homozygous uch1-1 uch2-1 seedlings was caused by the absence of the UCH1/2 proteins, we attempted to complement the double mutant with the 35S:UCH1 transgene. Homozygous 35S:UCH1 uch1-1 uch2-1 plants were identified in a T3 population of seedlings generated from a cross of a uch1-1 uch2-1 plant with a high-expressing 35S:UCH1 line. As shown in Figure 7(b), these plants now expressed high levels of the UCH1 protein, nearly equivalent to that in the 35S:UCH1 line. By all criteria, the 35S:UCH1 uch1-1 uch2-1 plants were phenotypically indistinguishable from the 35S:UCH1 parent, including a compact stature and altered branching (Figure 7a, and data not shown). Consequently, we concluded that manipulation of the UCH1/2 protein levels was sufficient to elicit the observed range of phenotypes.

Altering UCH1/2 levels does not affect the levels of Ub and RUB1 conjugates or the 26S proteasome

The phenotypic defects associated with altered UCH1/2 levels could be caused by defects in Ub recycling, which would stabilize both Ub conjugates and free poly-Ub chains and potentially reduce the pool of free Ub (Amerik and Hochstrasser, 2004). For example, a dramatic increase in the level of poly-Ub chains was observed in Arabidopsis embryos lacking UBPI4, supporting its role in disassembling Ub polymers (Doelling et al., 2001). In contrast, Figure 5(c) shows that the overall profile of Ub conjugates was not altered in the uch1-1 uch2-1 and the 35S:UCH1 backgrounds, indicating that UCH1/2 do not affect the bulk of Ub conjugates. In addition to recycling Ub, a mammalian relative of UCH1/2 (UCH-L3) can release the Ub-like protein RUB1 (or NEDD8) when attached to its target – the CUL1 subunit of SCF-type E3 ligases (Linghu et al., 2002; Wada et al., 1998). Through this conjugation, RUB1 reversibly regulates SCF activity (Parry and Estelle, 2004). Given the plethora of SCF-type E3s in Arabidopsis (Gagne et al., 2002), the phenotypes of the uch1-1 uch2-1 and 35S:UCH1 plants could be explained by global effects on RUB1–CUL1 abundance. However, we found that both the levels of CUL1 and of the CUL1–RUB1 conjugate were unaffected by altering UCH1/2 protein levels (Figure 5c).

Although our data did not establish an interaction between UCH1/2 and the 26S proteasome, it remains formally possible that UCH1/2 bind to and promote degradation by the 26S proteasome, with the UCH mutants reflecting a defect in 26S proteasome capacity. For bona fide subunits of the 26S proteasome in Arabidopsis, yeast and animals, diminished activity or expression often upregulates the expression of other 26S proteasome subunits in an attempt to compensate for the defect (Lundgren et al., 2005; Xie and Varshavsky, 2001; Yang et al., 2004). To determine whether a similar upregulation occurs in the uch1-1 uch2-1 and 35S:UCH1 backgrounds, we compared the accumulation of various 26S proteasome subunits to that of wild-type. However, immunoblot analysis with antibodies that recognize the subunits of the 26S proteasome CP (PBA1 and PAC1) and RP Base (RPT1 and RPN1) and Lid (RPN5) failed to detect any increases in subunit accumulation (Figures 5c and 7d, inset). Consequently, the

Figure 7. Complementation of the uch1-1 uch2-1 double mutant with the 35S:UCH1 transgene.
Plants include wild-type (WT), uch1-1 uch2-1, and either WT or uch1-1 uch2-1 homozygous plants expressing the 35S:UCH1 transgene. (a) Plants were grown for 37 days under LD conditions.
(b) Immunoblot analysis of extracts from 10-day-old seedlings with antibodies against UCH1 and UCH2 and subunits of the 26S proteasome RP Base (RPT1a) and RP Lid (RPN1a).

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UCH1/2 mutants do not appear to affect 26S proteasome abundance or capacity.

Changes in UCH1/2 levels potentially affect auxin and cytokinin signaling

In the absence of marked changes in the overall pattern of Ub conjugates and free Ub chains, it is likely that UCH1/2 target specific ubiquitinated proteins. One influential set of targets could be those involved in auxin and cytokinin perception, based on the collective phenotypes for the uch1–1 uch2–1 double mutant and UCH1 over-expression lines. Particularly informative were the effects on shoot architecture and stem elongation, which are, at least in part, under the combined control of these two hormones (Berleth et al., 2004; Cline, 1994; Leyser, 2003). Additional features diagnostic of auxin and/or cytokinin defects include: (i) leaf epinasty, which is a characteristic of many auxin-resistant mutants (e.g. axr2-1, Timpte et al., 1994), and (ii) changes in the polarity of pistil, stamen and cauline leaf expansion (Grebe, 2004) and in the number of leaves generated before bolting (Reinhardt et al., 2000).

As a first test for a change in auxin and cytokinin responsiveness, we examined the inhibitory effects on root growth of various compounds with auxin [indoleacetic acid (IAA), 1-naphtylacetic acid (1-NAA), picloram and 2,4-dichlorophenoxyacetic acid (2,4-D)] or cytokinin activity (6-benzylaminopurine and kinetin). Wild-type Arabidopsis is sensitive to sub-micromolar concentrations of these compounds. When tested in parallel, the uch1–1 and uch2–1 single mutants, the uch1–1 uch2–1 double mutant and the 35S:UCH1 seedlings showed no significant hyper-or hyposensitivity compared to wild-type at all concentrations tested (Figure 8a, and data not shown).

Because shoot growth and architecture can be controlled by the combined action of auxins and cytokinins (Cline, 1994; Leyser, 2003), we tested the response of the 35S:UCH1 and uch1–1 uch2–1 seedlings to various auxin/cytokinin ratios, using the combined ability of these two hormones to promote callus induction and shoot regeneration in hypocotyls. In Figure 8(b), hypocotyls were incubated with a fixed concentration of the natural auxin IAA (1 µM) and a concentration range (0–32 µM) of the cytokinin 2-isopentyladenine (2iP). Wild-type hypocotyls developed callus tissue and eventually formed green shoots at 2iP concentrations above 1 µM when mixed with 1 µM IAA. The response was inhibitory above 16 µM 2iP. In contrast, the 35S:UCH1

Figure 8. Response of plants with altered UCH1/2 levels to auxins and cytokinins.
Plants include wild-type (WT), double homozygous uch1–1 uch2–1 seedlings, and seedlings homozygous for the 35S:UCH1 transgene.
(a) Effects of 1-naphthylacetic acid (NAA), picloram, benzyladenine (BA) and kinetin on root growth. Seedlings were grown for 4 days on hormone-free medium and then transferred to medium containing various concentrations of each hormone. After 6 days, root growth of each seedling was measured and expressed as a percentage (±SD) of that obtained in hormone-free medium.
(b and c) Effect of IAA and the cytokinin 2-isopentyladenine (2iP) on the formation of callus and shoots from hypocotyl segments. The segments were incubated for 2 weeks on solid medium containing (b) 1 µM IAA and a range of 2iP concentrations, or (c) 4 µM 2iP and a range of IAA concentrations.
hypocotyls responded poorly with only a narrow range of 2iP (around 4 μM) able to stimulate callus and shoot formation in the presence of IAA (Figure 8b). Conversely, the uch1-1 uch2-1 mutant responded well to a wide range of cytokinin concentrations plus IAA, with levels of 2iP as low as 0.5 μM and as high as 32 μM still able to promote callus and shoot regeneration. We also performed a parallel experiment in which the concentration of 2iP was fixed at 4 μM and the concentration of IAA was varied from 0 to 16 μM. In this case, comparable changes in IAA/2iP sensitivities were observed; uch1-1 uch2-1 hypocotyls responded to a wider range of IAA concentrations, whereas the 35S:UCH1 hypocotyls responded to a narrower range (Figure 8c). These two sets of analysis together suggest that the shoots of the UCH mutants are altered in their response to the combination of auxin and cytokinins.

35S:UCH1 synergistically enhances auxin-resistant mutants

To further dissect the effects of the UCH mutants on auxin perception, we constructed double mutants between 35S:UCH1 and the auxin-resistant mutants axr1-3 and axr2-1. The axr1-3 mutation attenuates the activity of the AXR1 protein, the E1 that promotes the attachment of RUB1 to CUL1 and thus is required to activate E3 ligases such as SCFTIR1 (Leyser et al., 1993; Parry and Estelle, 2004). By blocking SCFTIR1 activation, the axr1-3 mutation stabilizes members of the AUX/IAA protein family that repress auxin signaling. The axr2-1 mutant bears a dominant mutation in domain II of the AUX/IAA protein IAA7, which inhibits recognition of IAA7 by SCFTIR1 (Gray et al., 2001). Consequently, the axr2-1 protein is less prone to ubiquitination by this E3, thus allowing it to constitutively repress auxin signaling even in the presence of the hormone (Nagpal et al., 2000; Timpte et al., 1994).

As shown in Figure 9(a), both the axr1-3 and axr2-1 mutant phenotypes were strongly enhanced in the 35S:UCH1 background. axr1-3 seedlings have irregular and epinastic rosette leaves, slightly reduced shoot height and decreased auxin sensitivity (Lincoln et al., 1990). The double 35S:UCH1 axr1-3 mutant was compromised even further, with a dramatic decrease in rosette growth and shortened petioles. Plants containing the axr2-1 mutation also grow as dwarfs, with defects in auxin perception, including resistance to 2,4-D and 1-NAA (Timpte et al., 1994). The 35S:UCH1

Figure 9. UCH1/2 selectively modify the stability of the AUX/IAA protein.
(a) Mutants in auxin signaling act synergistically with over-expression of UCH1. Lines tested include wild-type (WT), homozygous axr1-3 and axr2-1 seedlings, and axr1-3 and axr2-1 seedlings homozygous for the 35S:UCH1 transgene.
(b) UCH1 and 2 selectively modify the stability of the AUX/IAA reporter protein AXR3NT–GUS. Seven-day-old wild-type and UCH mutant seedlings that are expressing the AXRNT–GUS fusion protein were heat-shocked at 37°C for 2 h, cooled to 24°C for 20 min, and then incubated with or without 10 μM 1-NAA. Tissue was collected at the indicated times after 1-NAA addition, frozen, homogenized, and GUS activity was measured fluorometrically in the crude extracts. GUS activity was reported as fluorescent units, and converted to a percentage using the fluorescent activity at the 20 min time point as 100%.
(c) Turnover of PhyA. Wild-type, 35S:UCH1 and uch1-1 uch2-1 seedlings were grown for 6 days in the dark and then exposed to continuous red light (R). Immunoblots were probed with an anti-phyA monoclonal antibody.
(d) Turnover of HY5. Six-day-old dark-grown seedlings of wild-type and UCH mutants were subjected to a 15 h exposure to white light. Immunoblots were probed with an anti-HY5 antibody. The UBC1 E2 antibody was included to verify equal loading.
axr2-1 double mutant was even more compromised, with dramatically reduced leaf initiation and expansion, stem elongation and seed production (Figure 9a, and data not shown). Whereas the 35S:UCH1 or axr2-1 single mutants had normal and reduced growth of lateral roots, respectively, lateral root formation in the double mutant was nearly abolished (data not shown).

**UCH1 and 2 regulate the stability of AUX/IAA proteins directly or indirectly**

The synergistic effects between 35S:UCH1 and axr1-3/axr2-1 implied a possible connection between UCH1 abundance and AUX/IAA protein turnover. To test this possibility more directly, we exploited an HS-AXR3NT–GUS (β-glucuronidase) transgene, which expresses domains I and II of AXR3/IAA17 fused to GUS, under the control of the soybean heat shock promoter HS6871 (Gray et al., 2001). The NLS in domain I ensures that the fusion is nuclear-localized like the parent AXR3 protein, whereas domain II provides the recognition site for SCFTIR1 that directs its Ub-mediated turnover (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). In contrast to the natural AUX/IAA proteins, which are difficult to detect in vivo, this HS-AXR3NT–GUS transgene provides a sensitive reporter to track AUX/IAA protein synthesis and turnover via GUS activity assays (Gray et al., 2001).

uch1-1 uch2-1, 35S:UCH1 and wild-type WS plants containing this reporter were heat-shocked at 37°C for 2 h, returned to room temperature for 20 min, exposed to 0 or 10 μM concentrations of 1-NAA, and then assayed for GUS activity at 20-min intervals thereafter. In the absence of 1-NAA, AXR3NT–GUS levels decreased slowly in wild-type seedlings after the heat shock, a breakdown presumably initiated by SCFTIR1-mediated ubiquitination (Figure 9b, upper panel). Conversely, GUS activity remained high in 35S:UCH1 plants but dropped dramatically in the uch1-1 uch2-1 mutants, implying that the turnovers of AXR3NT–GUS were faster and slower, respectively. In the presence of 1-NAA, GUS activity decreased more rapidly in all backgrounds, consistent with enhanced turnover of AXR3NT–GUS following auxin-induced activation of SCFTIR1 (Dharmasiri et al., 2005; Gray et al., 2001; Kepinski and Leyser, 2005). The breakdown of AXR3NT–GUS in wild-type seedlings was now similar to that of uch1-1 uch2-1 seedlings, but still faster than that of 35S:UCH1 seedlings (Figure 9b, lower panel).

To examine whether changes in UCH levels selectively affect the turnover of AUX/IAA proteins rather than all Ub substrates, we tested two other known targets of the Ub/26S proteasome proteolysis pathway, phytochrome A (phyA) and long hypocotyl 5 (HY5), which are degraded by different mechanisms. While HY5 is degraded rapidly in dark-grown plants but stabilized in the light (Osterlund et al., 2000), phyA is stabilized in the dark but degraded rapidly in the light (Clough et al., 1999). As shown in Figure 9(c,d), breakdown of both phyA and HY5 was not significantly altered in the 35S:UCH1 and uch1-1 uch2-1 backgrounds.

**Discussion**

The Ub/26S proteasome system plays a key role in many aspects of plant biology (Moon et al., 2004; Smalle and Vierstra, 2004). Although most research on the plant system has focused on the enzymes involved in ubiquitination, both the number and diversity of de-ubiquitinating enzymes imply that they are also important to Ub dynamics (Smalle and Vierstra, 2004; Yan et al., 2000). Previous studies have confirmed a global role for some plant DUBs (Doelling et al., 2001, 2007). Here, we show that other DUBs can have more subtle and selective roles, based on the genetic analysis of UCH1 and 2, a pair of related Arabidopsis UCHs that specifically modifies shoot architecture.

Through both in vivo and in vitro assays, we confirmed that Arabidopsis UCH1/2 have de-ubiquitinating activities. While UCH2 will release Ub monomers linked by either α- and ε-amino peptide linkages, it remains unclear which type of substrate(s) UCH2 prefers. Phylogenetically, UCH1/2 are similar to the UCH37 group in yeast and animals, which can be easily distinguished from other UCH types by a conserved C-terminal domain of approximately 120 amino acids that extends beyond the catalytic core. Members of the UCH37 group from other eukaryotes have been shown to associate with the 26S proteasome where they possibly help to release Ub moieties from various ubiquitinated targets before breakdown of the targets by the 26S proteasome (Holzl et al., 2000; Lam et al., 1997; Li et al., 2001). The C-terminal extension of bovine UCH37 has been reported to interact with the RPN12 subunit of the 26S proteasome (Li et al., 2001), while the C-terminal extension of S. pombe Uch2p has been reported to interact with the RPN10 subunit (Stone et al., 2004). Even though Arabidopsis UCH1/2 have a related extension and show tissue and intracellular distributions similar to those of the 26S proteasome, we failed to detect a direct association of UCH1/2 with the protease complex, either by analysis of the 26S proteasome isolated under gentle conditions or by directed yeast two-hybrid assays of UCH1 with RPN10 or RPN12. Consequently, Arabidopsis UCH1/2 may serve a function(s) within the Ub system independent of 26S proteasome binding.

Arabidopsis plants missing both UCH1 and 2 are viable and complete their life cycle from seed germination to flowering and embryogenesis normally. Compared to wild-type, uch1-1 uch2-1 seedlings and those over-expressing UCH1 (35S:UCH1) displayed no changes in the abundance and patterns of Ub–protein conjugates or free Ub chains, indicating that the overall dynamics of Ub cycling is not substantially altered. The abundance of free monomeric Ub was also unaffected, suggesting that UCH1/2 do not help to
supply free Ub by processing the initial translation products of UBO genes (Amerik and Hochstrasser, 2004).

Despite unaltered Ub profiles, the uchi-1/2 mutant and over-expression lines showed a striking change in shoot development and architecture. 35S:UCH1 seedlings have a shorter primary inflorescence and longer cauline branches, but a similar number of rosette branches, whereas the uchi-1/2 seedlings are normal in height but have shorter cauline branches. Our demonstration that the 35S:UCH1 transgene can reverse the phenotype of uchi-1/2 seedlings indicates that of 35S:UCH1 seedlings indicates that the absolute levels of UCH1/2 are important for this effect.

Recently, several other shoot morphology mutants have been described that affect components of the Ub26S proteasome system (e.g. Huang et al., 2006; Stirnberg et al., 2002). In particular, the bushy phenotypes associated with UCH1/2 over-expression as compared to those of wild-type are superficially similar but not identical to those of max2 mutants. The MAX2 locus encodes an F-box subunit of an E3 Ub–protein ligase that may be involved in perception by a presently unidentified growth regulator (Booker et al., 2005; Stirnberg et al., 2002). In contrast to the 35S:UCH1 plants, the max2 plants have an increased number of rosette branches, fasciated stems and elongated petioles, but no change in node distance. These collective differences suggest that UCH1/2 and MAX2 do not share the same target(s).

It is also known that fertility can affect Arabidopsis inflorescence architecture, with male-sterile mutants displaying similar reductions in cauline branch length, increased number of rosette branches, and longer flowering times as observed here for uchi-1/2 plants (e.g. Hensel et al., 1994). Given that the uchi-1/2 double mutants show partial infertility, their altered branch morphology could be accentuated by reduced seed production.

Prior to this study, UCHs had not been associated with specific targets in plants. The collective phenotypes observed here for uchi-1/2 and 35S:UCH1 seedlings suggest a role for Arabidopsis UCH1 and/or UCH2 in auxin and cytokinin signaling. These phenotypes included the effects on shoot growth, leaf epinasty, floral development and leaf number before bolting, which are under the control of cytokinins and/or auxins (Berleth et al., 2004; Grebe, 2004; Reinhardt et al., 2000; Timpte et al., 1994). We also found that the mutant and/or over-expressing lines were altered in the ability of auxins and cytokinins to stimulate callus and shoot growth from hypocotyl sections, and in the stability of an AUX/IAA protein reporter (AXR3NT–GUS). The 35S:UCH1 developmental phenotype was also strongly synergistic with those of the auxin-insensitive mutants arx1-3 and arx2-1.

While we cannot yet conclude that UCH1/2 interact directly with components of auxin signaling, the simplest explanation is that UCH1/2, as part of its functions, selec-
nuclear targets are possible. Clearly, identifying proteins that directly interact with UCH1/2 will be important in demonstrating their action. Whatever the roles, the specific phenotypes elicited by altering UCH1/2 levels clearly indicate that some members of the DUB superfamily can be highly selective in the targets they de-ubiquitinate. They also potentially add another layer to the regulation of auxin perception by the Ub system.

Experimental procedures

Identification of the Arabidopsis UCH1 and 2 genes

Arabidopsis thaliana UCH genes were identified by BLAST of the Columbia (Col-0) ecotype DNA database (http://www.arabidopsis.org) using the amino acid sequence of human BAP1 as the query (Jensen and Rauscher, 1999). Full-length UCH1 and 2 cDNAs (1D4T7P and 21D4D177P, respectively) were obtained from the EST collection at the Arabidopsis Biological Resource Center (ABRC, Ohio State University). The correct sequence of each was confirmed by DNA sequence analysis. Intron/exon boundaries and coding regions were determined by comparing sequences from genomic loci, full-length and partial cDNAs, and expressed sequence tags (ESTs) (http://www.arabidopsis.org/). Amino acid sequences were aligned using Clustal X version 1.83 (European Molecular Biology Laboratory) Heidelberg, Germany (Thompson et al., 1997) and displayed using Macboxshade version 2.11 (Institute of Animal Health, Pirbright, Surrey, UK). The unrooted phylogenetic tree was generated in MEGA 2.1 by the neighbor-joining, Poisson distance method, using a 2000 replicate (Kumar et al., 2001).

UCH1 and 2 cloning and antibody production

The UCH and 2 coding regions were PCR-amplified from the full-length cDNA clones and cloned into the pET28 and pET23 vectors, respectively, for expression in E. coli strain BL21 (DE3) (Novagen; http://www.emdbiosciences.com). Following a 4 h induction with 1 mm isopropyl- β-D-thiogalactopyranoside, the cells were harvested and disrupted in Bugbuster buffer (Novagen), and the insoluble fraction was collected from the crude extract by centrifugation at 16 000 g. The UCH1 and 2 proteins were purified by nickel-chelate affinity chromatography following the insoluble protein purification procedure (Qiagen; http://www.qiagen.com/) and injected directly into rabbits (Polyclonal Antibody Service; http://www.polysciences.com). Yeast two-hybrid analyses of full-length UCH1 and 2 or their C-terminal domains alone paired with full-length Arabidopsis RPN12a or RPN10 were accomplished using the pGBK-T7 and 2 or their C-terminal domains alone paired with full-length Arabidopsis RPN12a or RPN10 were accomplished using the pGBK-T7 and pGBKT7-Rec system (Clontech; http://www.clontech.com/). Yeast two-hybrid analyses of full-length UCH1 and 2 or their C-terminal domains alone paired with full-length Arabidopsis RPN12a or RPN10 were accomplished using the pGBK-T7 and pGBKT7-Rec system (Clontech; http://www.clontech.com/).

De-ubiquitinating enzyme activity assays

The ability to release Ub linked via α-amino linkages was assayed in vivo using the substrates ubiquitin extension protein AtUBQ1 (p8185) (Chandler et al., 1997) and the hexameric polyubiquitin AtUBQ10 (Callis, 1995); the latter had the transcription start site modified to attenuate expression (pAtUBQ10-LE as described by Yan et al., 2000). The coding regions for each substrate was introduced into a pACYC184-based plasmid co-expressed in the E. coli strain NovaBlue (DE3) (Novagen) with wild-type UCH2 or its C82S mutant version in pET32a. Yeast (S. cerevisiae), Ubp1p (Tobias et al., 1991), and bovine UCH37 (Lam et al., 1997) expressed from plasmid RB293 were used as positive controls.

The cleavage of Ub monomers connected via α-amino isopeptide bonds using Lys48-linked poly-Ub chains synthesized in vitro by the wheat E2 UBC7 as described previously (van Nocker and Vierstra, 1993). Extracts containing the recombinant enzymes were prepared by concentrating cells expressing the corresponding proteins in 1/20 of the original culture volume and sonicating the cells in 50 mM Tris-HCl (pH 8.0), 5% v/v glycerol, 1 mM dithiothreitol and 1 mM Na4EDTA. Lysates (37.5 μl) clarified at 10 000 g for 2 h at 37°C with 2.5 μl (50 ng) of poly-Ub chains. The reactions were quenched by heating after the addition of 50 μl SDS–PAGE sample buffer. The processing of the various substrates for UCHs was monitored by SDS–PAGE and immunoblot analysis using anti-Ub antibodies (Yan et al., 2000).

Plant materials and growth conditions

The uch1-1 and uch2-1 T-DNA insertion lines were identified by PCR in the University of Wisconsin-Madison A. thaliana (ecotype WS) T-DNA-transformed population (http://www.biotech.wisc.edu/ Arabidopsis) using T-DNA-specific primers and 5′- and 3′-gene-specific primers (Krysan et al., 1999). Both alleles were back-crossed three times to wild-type WS to potentially eliminate second site mutations. The homozygous single and double mutants were identified by PCR in progeny from heterozygous parents. To generate UCH1/2-over-expressing plants, the UCH1 and 2 coding regions were PCR-amplified from their corresponding cDNAs using primer pairs constructed to add XbaI sites before and after the translational start and stop codons. The fragments were digested with XbaI and ligated into pGSEV9 vector designed to express the gene under the control of the 35S promoter (E. Babychuk and S. Kushnir Laboratory for Genetics, Universiteit Gent, Gent, Belgium, unpublished data). These transgenes were introduced into WS Arabidopsis by the Agrobacterium-mediated floral-dip method (Clough and Bent, 1998). Transformed plants were selected by hygromycin resistance, allowed to self, and T1 plants containing the 35S: UCH1 and UCH2 transgenes were screened for the homozygous lines. Homozygous 35S: UCH1 plants were crossed into other backgrounds using its pollen as the donor. The Arabidopsis mutants axr1-1 and axr2-1 in the Col-0 ecotype (Lincoln et al., 1990; Timpte et al., 1994) were obtained from ABRC. Plants expressing HS-AXR38NT–GUS were as described previously (Gray et al., 2001). For complementation of uch1-1 uch2-1, the 35S: UCH1 transgenic line was crossed into the double mutant WS background, and the progeny were bearing hygromycin B and kanamycin resistance. T2 and T3 seedlings bearing all three loci were tracked by PCR.

For various phenotypic assays, wild-type and mutant seeds were surface-sterilized in 50% bleach, stratified at 4°C for 4 days in the dark, and then allowed to germinate on solid Gamborg’s B5 growth medium (GM; Gibco BRL; http://www.invitrogen.com) at 21°C under LD (16 h light/8 h dark) or SD (8 h light/16 h dark) photoperiods. Root length and leaf morphology were measured using IMAGE J version 1.33 (National Institutes of Health; http://www.rsb.info.nih.gov/). For the shoot induction experiments, hypocotyl segments were dissected from 5-day-old light-grown seedlings, transferred to GM containing 1 μM IAA without 2-IP or with a range of 2-IP concentrations, or containing 4 μM 2IP without IAA or with a range of IAA concentrations, and incubated for another 4 weeks under LD conditions. Leaf chlorophyll was extracted overnight at 4°C into 1 ml of 96% ethanol and quantified spectrophotometrically (Wintermans and de Mots, 1965). Morphometric analysis of shoot
architecture was conducted on plants grown to maturity under LD or SD conditions.

Intracellular localization of Arabidopsis UCH1 and 2

The intracellular localization of UCH1 and 2 was determined by transient expression of GFP fusions in Arabidopsis thaliana ecotype Col-0 protoplasts. To generate the 35S::GFP fusions for UCH1 and 2, the full-length UCH1/2 cDNAs were first cloned into pENTR vector using BP reaction clonase (Invitrogen; http://www.invitrogen.com/), then swapped into vector pMDC43 (Curtis and Grossniklaus, 2003) to generate N-terminal GFP fusions using the Gateway LR reaction kit (Invitrogen). The 35S::RFP fusion vector for RPN10 was generated by PCR amplifying the full-length coding sequence from the cDNA template using primers designed to add HindIII and EcoRI restriction sites to the 5′- and 3′-ends, digesting the product with HindIII and EcoRI, and then inserting the products into similarly digested 326-RFP plasmid (Lee et al., 2001). The RFP-NLS and GFP-NLS reporters were as described by Lee et al. (2001). Purified plasmids were introduced into leaf tissue protoplasts by polyethylene glycol-mediated transfection (Lee et al., 2001). GFP::RFP expression was monitored 24 h after transformation using a Zeiss 510-Meta scanning laser confocal microscope (http://www.zeiss.com/) in combination with the BP505–530 (excitation 488 nm, emission 505–530 nm), Chs 560–615 (excitation 543 nm, emission 560–615 nm), and Chs 650–670 (excitation 633 nm, emission 650–670 nm) filter sets to detect GFP, RFP, and chlorophyll autofluorescence, respectively.

RNA gel blot analyses

Total RNA was isolated from young A. thaliana ecotype WS seedlings using Trizol reagent (Invitrogen), separated by agarose gel electrophoresis (10 µg per lane), and bound to Zeta-Probe membranes (Bio-Rad; http://www.bio-rad.com/). [32P]-labeled probes were either generated by RT-PCR using 2 µl of total RNA and SuperScript reverse transcriptase (Stratagene; http://www.stratagene.com/) and labeled by random priming, or synthesized as riboprobes using the appropriate linearized plasmids and the Riboprobe Gemini II core system (Promega; http://www.promega.com/). The UCH1 and UCH2 templates used the respective full-length clones that were digested with PstI. The β-TUB4 template was as described previously (Smalle et al., 2003).

Glycerol gradient enrichment of the 26S proteasome

Crude extracts were prepared from 10-day-old Arabidopsis seedlings grown in liquid culture following the method described by Yang et al. (2004). After the 10% polyethylene glycol precipitation step, the pellet was resuspended in buffer A (20 mM Tris-HCl, pH 7.5, 2 mM ATP, 5 mM MgCl2, 1 mM dithiothreitol, 10 mM creatine phosphate, 10 µg ml−1 creatine kinase) plus 40% glycerol. The crude extracts (0.5 ml of approximately 3 mg protein ml−1) were applied on top of a 10 ml 10–40% v/v glycerol gradient in buffer A, and centrifuged at 180 000 g for 18 h at 4°C. Fractions (0.5 ml) were assayed for peptidase activity of the 26S proteasome using N-succinyl Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin as the substrate (Yang et al., 2004), or subjected to SDS–PAGE and immunoblot analysis with various 26S proteasome antibodies. The refractive indices for gradient fractions were used to cross-reference peak assignments among the different gradients.

De-ubiquitination modifies shoot architecture

SDS–PAGE and immunoblot analysis

Immunoblot analysis was performed according to the method described by Yang et al. (2004). Antibodies against HY5, PAC1, RPT1a, RPN11a, RPN12a, and UBC1 were as described previously (Osterlund et al., 2000; Smalle et al., 2002; Yang et al., 2004). Anti-CUL1 antibodies were as described by Gray et al. (1998). Arabidopsis phytochrome A was detected using the monoclonal antibody O73D generated against oat phytochrome A (Clough et al., 1999).

Heat induction and GUS assays

Analysis of the protein expressed by the HS-AXR3NT–GUS transgene in various Arabidopsis backgrounds was performed as previously described (Gray et al., 1999). Six-day-old seedlings grown at 25°C were submerged in liquid GM, subjected to a 37°C heat shock for 2 h, and then returned to 25°C. 1-NAA was added 20 min after the heat shock. Plants were collected at 20-min intervals thereafter. For quantification of GUS activity, 10 seedlings from each time point were rapidly frozen, homogenized in 50 ml Na2HPO4, pH 7.0, 0.1% sodium n-lauryl sarcosinate, 0.1% Triton X-100, 100 mM β-mercaptoethanol, 10 mM Na2EDTA, and 1 mM phenylmethylsulfonyl fluoride. The clarified extracts were mixed with 0.33 ml 4-methylumbelliferyl-β-D-glucuronide (MUG) dissolved in the extraction buffer, incubated for 1 h at 37°C, and then assayed fluorimetrically for GUS activity using a Wallac Victor microtitre plate fluorimeter (Perkin-Elmer; http://www.perkinelmer.com). GUS activity was calculated by the rate of MUG hydrolysis and normalized relative to total protein level.

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Accession numbers: The EMBL/GenBank accession numbers for UCH1 and UCH2 cDNAs are AF412059 and AB005242, respectively.