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Functional characterization of the Arabidopsis *ubiquitin-specific protease* gene family reveals specific role and redundancy of individual members in development

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Summary

Ubiquitin-specific proteases (UBPs) are a highly conserved family of proteins in eukaryotes, and play critical roles in protein de-ubiquitination. Here we report a systematic genetic and expression profiling analysis of the *UBP* gene family in the *Arabidopsis thaliana* genome. Mutation analysis of 25 of the 27 member genes representing 13 of the 14 sub-families of the *UBP* gene family revealed that single-gene mutants of three genes in two sub-families exhibit visible phenotypes. Two of these three genes belonging to the *UBP15* sub-family were selected for further characterization. The *ubp15* mutants display narrower, serrated and flat rosette leaves, partially due to a defect in cell proliferation, as well as other phenotypes such as early flowering, weak apical dominance and reduced fertility, while the line over-expressing *UBP15* shows opposite phenotypes. We demonstrated that UPB15 has UBP activity *in vitro*, and that this biochemical activity is essential for its *in vivo* function. A genetic interaction analysis among members of this sub-family revealed that UBP15 and UBP16, but not UBP17, have functional redundancy. Our data thus suggest that distinct UBPs, even within a closely related sub-family, can function in different developmental pathways. Although there are clearly functional redundancies among related sub-family members, those redundancies cannot be inferred simply based on the amino acid identity of the family members.

Keywords: ubiquitin, protein degradation, ubiquitin-specific protease, Arabidopsis, leaf development, gene family.

Introduction

The covalent modification of proteins by ubiquitin plays a central role in diverse cellular pathways such as cell-cycle progression, signal transduction, DNA repair, endocytosis and apoptosis (Hershko and Ciechanover, 1998; Hochstrasser, 1996; Pickart, 2004; Varshavsky, 1997; Weissman, 2001). Protein ubiquitination is catalyzed by a cascade of three enzymes, E1, E2 and E3. The fate of ubiquitinated substrate proteins depends in part on the number of conjugated ubiquitin(s) and the mode of linkage in the ubiquitin chain. The most common form of ubiquitination is a multi-ubiquitin chain linked by Lys48, which acts as a signal for protein degradation by the 26S proteasome.

The cleavage of ubiquitin from proteins by de-ubiquitination enzymes (DUBs) has also been shown to affect the activity and fate of substrates (Amerik and Hochstrasser, 2004; Crosas *et al.*, 2006; Hanna *et al.*, 2006; Wilkinson, 1997). The currently known DUBs carry out two basic biochemical activities, generating mature ubiquitin from ubiquitin precursors and cleaving the isopeptide bonds between covalently linked ubiquitin molecules or between ubiquitin and its attached target protein (Nijman *et al.*, 2005). Cysteine proteases and metalloproteases are the two major types of enzyme in the DUB super-family, with cysteine proteases more numerous in all eukaryotes (Nijman *et al.*, 2005). All the known metalloproteases have a JAMM domain for catalytic activity (Verma *et al.*, 2002). The cysteine protease DUBs can be further divided into four families based on the structure and organization of the catalytic center (Amerik and Hochstrasser, 2004; Nijman *et al.*, 2005; Wilkinson, 1997). Ubiquitin-specific proteases

(UBPs, or USPs in mammals) possess a triad of catalytic residues in highly conserved cysteine and histidine boxes (Hu *et al.*, 2002). Ubiquitin C-terminal hydrolases (UCHs), with a similar triad of catalytic residues in two conserved cysteine and histidine boxes (Johnston *et al.*, 1997, 1999), have smaller overall protein sizes as well as a structural constraints over the catalytic surface, which restricts their hydrolysis ability to small amides and esters at the C-terminus of ubiquitin (Amerik and Hochstrasser, 2004). The other two families, ovarian tumor proteases (OTUs) and Machado–Joseph disease protein domain proteases (MJDs), are less well studied but possess some of the functions of the canonical proteases, at least *in vitro* (Balakirev *et al.*, 2003; Burnett *et al.*, 2003; Nanao *et al.*, 2004; Scheel *et al.*, 2003).

It was estimated in a recent phylogenetic analysis that at least 64 DUBs exist in the model plant Arabidopsis (Yang *et al.*, 2007). Previous reports indicated that these DUBs may perform a variety of essential functions (Chandler *et al.*, 1997; Doelling *et al.*, 2001, 2007; Rao-Naik *et al.*, 2000; Sridhar *et al.*, 2007; Yan *et al.*, 2000; Yang *et al.*, 2004; Yang *et al.*, 2007). However, there has been no systematic analysis on the expression and developmental role of all members of the *UBP* gene family. Further, only few studies have addressed the functional relationship and redundancy among the closely related gene family members.

Here we describe a systematic analysis of the *UBP* gene family in Arabidopsis, and a detailed investigation of one of the 14 sub-families, the *UBP15* sub-family. Comprehensive phenotype characterization of *ubp15* mutants revealed that UBP15 plays a critical role in Arabidopsis leaf development by controlling cell proliferation and reproductive development. Furthermore, the UBP activity of UBP15 was confirmed by *in vitro* assay and shown to be essential for UBP15 functionality *in vivo*. Characterization of single, double and triple mutants among the members of this sub-family revealed specific and redundant function, although the observed patterns bore no direct relationship to the amino acid identity of the family members. This study provides a comprehensive characterization of the role of Arabidopsis UBP de-ubiquitination activity in diverse developmental processes.

Results

A brief summary of the Arabidopsis UBP *gene family*

It has been previously reported that Arabidopsis possesses 27 *UBP* genes (Yan *et al.*, 2000). All of the *UBP* genes encode proteins containing a UBP domain, with two short but well-conserved motifs, known as cysteine (Cys) and histidine (His) boxes, which include a triad of catalytic residues (Cys in the Cys box, His and Asp/Asn in the His box) (Amerik and Hochstrasser, 2004). The triad of catalytic residues is critical for DUB catalytic activity, and specific mutations abolish the activity of UBPs (Baek *et al.*, 2001; Chandler *et al.*, 1997; Doelling *et al.*, 2001; Hanna *et al.*, 2006; Papa and Hochstrasser, 1993; Rao-Naik *et al.*, 2000; Yan *et al.*, 2000). The length of the UBP domains varies from 300 to 900 amino acids; however, except for the two conserved motifs and a few other less-conserved short motifs (Wilkinson, 1999), they show low overall sequence conservation, although they do share a conserved three-dimensional structure. We suggest that this diversity in UBP domains may indicate diversity in the specificity of substrate recognition and function.

Alignment of the 27 UBPs was performed to better visualize conserved domains among sub-families (Figure S1). A phylogenetic analysis of the 27 UBPs was performed (Figure S2), and the 27 proteins were grouped into 14 sub-families. The results were in agreement with those reported by Yan *et al.* (2000). In addition to the shared UBP domain, members within each sub-family also share some other conserved domains. For example, the UBP15 sub-family (comprising five proteins, UBP15–19) contains a signature MYND-type zinc finger domain, which has been reported to be a protein–protein interaction domain in mammalian cells (Gross and McGinnis, 1996; Lutterbach *et al.*, 1998a,b; Masselink and Bernards, 2000). Another type of zinc finger domain (ZnF-UBP) was present in two sub-families, UBP1–2 and UBP14, and is found in other proteins as well as in UBP. The MATH domain in the UBP12–13 sub-family has been shown to be necessary and sufficient for self-association and receptor interaction in related UBP proteins in non-plant organisms (Park *et al.*, 1999; Sunnerhagen *et al.*, 2002; Ye *et al.*, 1999). Furthermore, there is a ubiquitin-associated (UBA) domain in UBP14, a ubiquitin homologue (UBQ) motif in three UBP proteins of two sub-families (UBP6–7, UBP26), and a functionally undefined DUSP domain in UBP26 and four proteins of a five-member sub-family (the UBP5 sub-family).

UBP *genes exhibit non-identical expression profiles*

To obtain expression patterns for the *UBP* genes, we examined our previously described microarray data set covering 18 different Arabidopsis organs (Ma *et al.*, 2005) (Table S1). In that study, 24 of the 27 *UBP* genes were covered. Except for the possible cross-hybridization between *UBP9* and *UBP10*, distinct expression of the other 22 genes was detected in one or more organs. The three *UBP* genes not covered in that microarray study, *UBP13*, *UBP14* and *UBP20*, were reported to be expressed by a prior study (Yan *et al.*, 2000). Thus, all *UBP* genes have some evidence for expression, and most possess largely non-identical expression patterns. As an example, organ-specific expression profiles for the five genes in the *UBP15* sub-family are shown in Figure S3. It is interesting to note that the five genes appear to have distinct expression profiles.

Table 1 Analysis of T-DNA insertion lines for *UBP* genes

Arabidopsis genome ID	Protein	T-DNA line	Insertion site	Phenotype
At2g32780	UBP1	SALK_086190	Promoter	ND
At1g04860	UBP2	SALK_064103	1st exon	ND
		SALK_059858	1st exon	ND
At4g39910	UBP3	SALK_112950	3rd exon	ND
At2g22310	UBP4	SALK_043210	4th exon	ND
At2g40930	UBP5	SALK_088398	5′ UTR	ND
At1g51710	UBP6	SALK_108832	14th intron	ND
At3g21280	UBP7	SALK_014223	ND	ND
At5g22030	UBP8	SALK_034744	5th intron	ND
		SALK_149329	6th intron	ND
		SALK_088692	7th exon	ND
At4g10570	UBP9	SALK_141485	5′ UTR	ND
At4g10590	UBP10	SALK_093503	9th exon	ND
At1g32850	UBP11	SALK_043515	8th intron	ND
At5g06600	UBP12	None		
At3g11910	UBP13	SALK_128312	5th exon	ND
		SALK_024054	6th exon	ND
		SALK_130784	17th exon	ND
		SALK_132368	21th intron	ND
At3g20630	UBP14	SALK_050151	9th exon	Recessive embryo-lethal
		SALK_012863	19th exon	Recessive embryo-lethal
At1g17110	UBP15	SALK_018601	12th exon	Recessive rosette leavesnarrow and serrated
		SALK_015611	8th exon	Recessive rosette leaves narrow and serrated
At4g24560	UBP16	SALK_023552	5th exon	ND
At5g65450	UBP17	SALK_087726	9th exon	ND
		SALK_113300	12th exon	ND
		SALK_009641	8th intron	ND
At4g31670	UBP18	SALK_126252	4th exon	ND
At2g24640	UBP19	SALK_084566	5′ UTR	Recessive embryo-lethal
		SALK_117787	5′ UTR	ND
At4g17890	UBP20	SALK_053104	5th intron	ND
		SALK_046087	5′ UTR	ND
At5g46740	UBP21	SALK_079015	1st exon	ND
At5g10790	UBP22	None		
At5g57990	UBP23	SALK_121772	10th exon	ND
At4g30890	UBP24	SALK_001531	3rd exon	ND
At3g14400	UBP25	SALK_088458	5′ UTR	ND
		SALK_111336	2nd exon	ND
At3g49600	UBP26	SALK_024392	4th intron	ND
At4g39370	UBP27	SALK_067020	5′ UTR	ND
		SALK_027968	1st exon	ND

ND, no difference. Thirty-nine T-DNA knockout lines corresponding to 25 of the 27 *UBP* genes were screened for their phenotype.

Genome-wide isolation and analysis of UBP *gene T-DNA insertion mutants*

As a first step in functional analysis of the *UBP* gene family, a total of 39 T-DNA insertion lines corresponding to 25 *UBP* genes were obtained and verified, as summarized in Table 1. After PCR-based genotyping (primers shown in Table S2), we examined possible phenotypes in those homozygous mutant lines. Only five T-DNA insertion lines in three genes of two sub-families exhibited visible phenotypes (Table 1). Two independent T-DNA alleles for *UBP14* exhibited recessive embryo lethality, similar to a previous report (Doelling *et al.*, 2001). Another three mutant lines were found to belong to the *UBP15* sub-family, with two alleles for *UBP15* showing a leaf morphology phenotype and one allele for *UBP19* showing embryo lethality. As none of the five genes in this sub-family had been characterized previously, we selected this sub-family for in-depth analysis.

Moreover, we searched for homologous genes of At*UBP15* sub-family in rice (*Oryza sativa*) and found four genes with high similarity. Phylogenetic trees for these four proteins, as well as five AtUBP15 sub-family proteins,

indicated that these family members may potentially fall into three branches (Figure S4). Arabidopsis and rice counterparts are grouped into one branch because they are more similar to each other than to sub-family members in another branch but belong to the same species.

The two ubp15 *mutant alleles exhibited similar leaf development defects*

The two T-DNA insertion lines for *UBP15*, designated *ubp15-1* and *ubp15-2*, possess an insertion in the 12th and 8th exons of the *UBP15* gene, respectively (Figure 1a). The full-length mRNA could not be detected by semi-quantitative RT-PCR in either of the two mutants (Figure 1b), suggesting that both are loss-of-function alleles. Both lines segregate a single, recessive Mendelian trait, which co-segregated with the T-DNA and possessed similarity to each other, indicating the phenotype was probably caused by a single insertion in *UBP15*. Compared to wild-type adult plants, both mutants are smaller with narrow and serrated leaves. The leaf phenotype becomes more severe in later rosette leaves (Figure 1c). Under long-day conditions (16 h light/8 h dark), the rosette leaves of the wild-type gradually change their overall pattern, starting from round and flat, then an oval shape and slightly downward-curled, and eventually becoming long and narrow, with a severely downward-curled margin. The *ubp15* mutant leaves are narrow at an earlier stage, with a lower weight of rosette leaf per area, produce fewer rosette leaves before bolting, and subsequently show an earlier flowering phenotype (Figure 1c and Table 2). We plotted the ratio of length and width, defined as the leaf index value (Tsukaya, 2006), of *ubp15-1* and wild-type, which revealed a much stronger reduction in width than in length of the rosette leaf blade in the mutant (Figure 1c and 1g). The mutants also exhibited shorter roots in at the seedling stage (Figure 1d), smaller flowers (Figure 1e), shorter siliques (Figure 1f), and shorter and slimmer stems. These morphological changes are summarized in Table 2.

UBP15 *is expressed ubiquitously but at higher levels in rosette leaves and reproductive organs*

The presence of defects in *ubp15-1* rosette leaves and flowers suggested involvement of UBP15 in the regulation

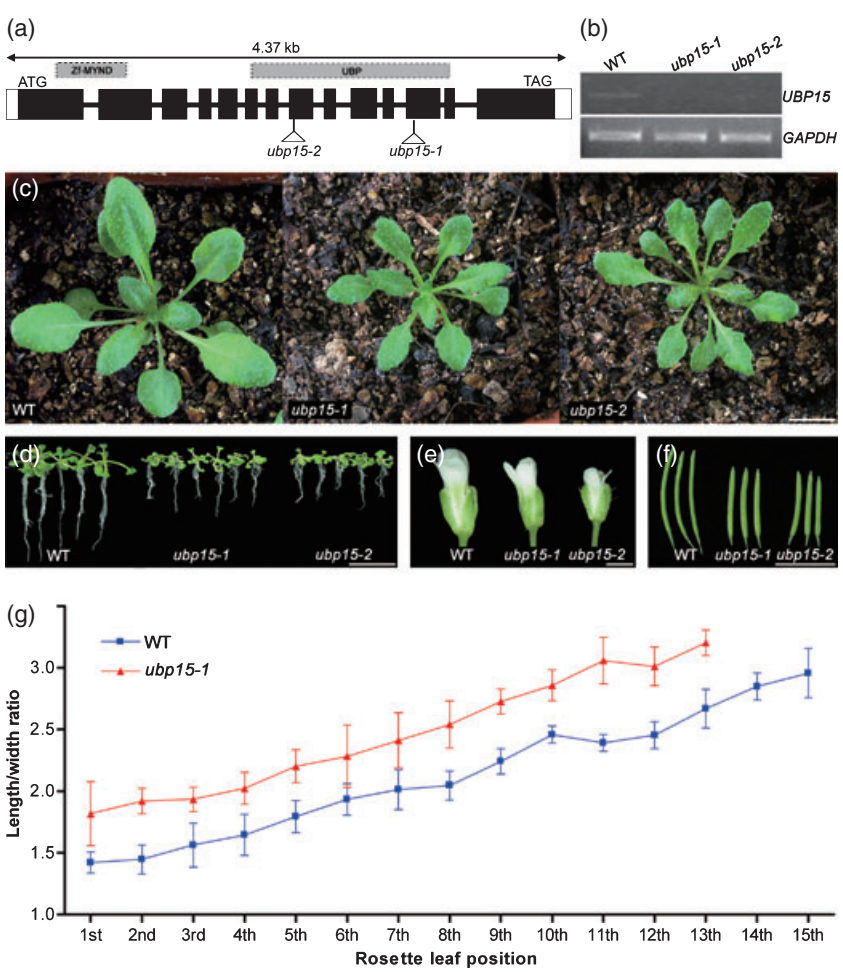

Figure 1. Characterization of the *UBP15* gene.
(a) Gene structure of *UBP15*. Two T-DNA knockout lines showed insertions in the 8th and 12th exons, respectively, each resulting in destruction of the UBP domain.
(b) Expression of *UBP15* in wild-type, *ubp15-1* and *ubp15-2* by RT-PCR using the primers *UBP15FP* and *UBP15RP*. At3g04120/*GAPDH* served as an internal control.
(c, d) One-month-old (c) and 12-day-old (d) plants of wild-type, *ubp15-1* and *ubp15-2*.
(e, f) Flowers (e) and siliques (f) of wild-type, *ubp15-1* and *ubp15-2*.
(g) Length/width ratio of the rosette leaves of wild-type and *ubp15-1* under short-day conditions (8 h light/16 h dark). Error bars represent the standard deviation of the eight repeats.
Scale bars = 1 cm (c,d,f) and 1 mm (e).

Table 2 Morphometric analysis of wild-type, *ubp15-1* mutant and *UBP15* over-expression plants

Parameters	Wild-type	*ubp15-1* mutant	*UBP15* over-expression plants
Number of rosette leaves (LD)[a]	12.58 ± 0.95 (*n* = 26)	10.82 ± 1.36 (*n* = 18)	16.21 ± 1.63 (*n* = 26)
Flowering time (LD) (days)[a]	41.54 ± 3.79 (*n* = 26)	39.13 ± 2.02 (*n* = 46)	46.07 ± 2.37 (*n* = 26)
Number of rosette leaves (SD)[b]	15.92 ± 2.11 (*n* = 7)	13.08 ± 1.77 (*n* = 7)	20.22 ± 2.66 (*n* = 7)
Flowering time (SD) (days)[b]	69.04 ± 1.41 (*n* = 8)	65.69 ± 4.66 (*n* = 21)	75.45 ± 4.87 (*n* = 8)
Fresh weight of the rosette leaves (mg/cm^2)[b]	13.24 ± 0.48 (*n* = 24)	10.74 ± 0.29 (*n* = 40)	15.6 ± 0.46 (*n* = 24)
Silique length (cm)[c]	1.36 ± 0.08 (*n* = 50)	1.09 ± 0.08 (*n* = 50)	1.42 ± 0.07 (*n* = 40)
Root length (cm)[d]	5.87 ± 0.75 (*n* = 22)	4.64 ± 0.74 (*n* = 22)	6.84 ± 0.69 (*n* = 26)
Primary stem length (cm)[c]	26.09 ± 2.68 (*n* = 11)	23.77 ± 2.98 (*n* = 15)	29.25 ± 4.44 (*n* = 6)
Primary stem diameter (mm)[c]	0.80 ± 0.08 (*n* = 14)	0.65 ± 0.09 (*n* = 18)	0.98 ± 0.07 (*n* = 8)

[a]Measurements were taken from bolting plants grown in 16 h light/8 h dark (LD, long days).
[b]Measurements were taken from bolting plants grown in 8 h light/16 h dark (SD, short days).
[c]Measurements were taken from plants 60 days after sowing grown in 16 h light/8 h dark (LD, long days).
[d]Measurements were taken from plants 14 days after sowing grown in 16 h light/8 h dark (LD, long days).

of vegetative and reproductive development. To test this hypothesis and to verify the prior microarray analysis, semi-quantitative RT-PCR was used to examine organ-specific expression of *UBP15*. As shown in Figure 2(a), the mRNA level of *UBP15* was higher in rosette leaves and inflorescences than in other organs. This expression pattern is largely consistent with microarray data (Table S1 and Figure S3) but with anticipated quantitative variations.

We further examined the spatial expression pattern of *UBP15* within a rosette leaf at various different developmental stages. Interestingly, the *UBP15* mRNA level increased from the early to late leaf stages, with higher expression in the leaf margin in the late stage (Figure 2b). These leaf expression patterns during development are consistent with the role of *UBP15* in defining the leaf pattern and shape of the leaf margin, such as serration and curling.

To assess the subcellular distribution of UBP15, constructs containing either *35S:GFP-UBP15* or *35S:UBP15-GFP* were transformed into onion epidermal cells. Transient expression showed that the fusion proteins were ubiquitously present in both the cytosol and nucleus (Figure 2c, and data not shown).

UBP15 possesses de-ubiquitinatination activity in vitro*, and this biochemical activity is essential for function* in vivo

To verify that UBP15 is a bona fide de-ubiquitinating enzyme, GST-fused UBP15 or two mutated forms (with the conserved catalytic Cys447 changed to Ala or Ser) were co-expressed with the model substrates, His-tagged UBQ1 (ubiquitin extension protein) or His-tagged UBQ10 (hexameric polyubiquitin) in *Escherichia coli*. Immunoblot analysis with the anti-ubiquitin antibody showed that UBP15 was capable of cleaving the two substrates, and that Cys447 is essential for this DUB activity (Figure 3a,b, respectively).

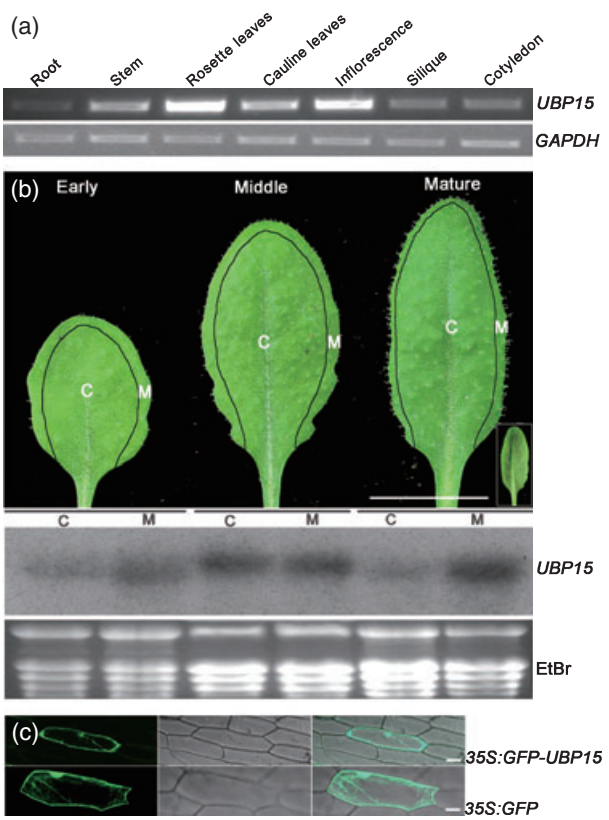

Figure 2. Expression patterns of *UBP15* and subcellular localization of UBP15 in onion epidermal cells.
(a) RT-PCR analysis of the organ expression pattern of *UBP15*. At3g04120/*GAPDH* served as an internal control.
(b) Northern blot analysis of the spatial expression pattern of *UBP15* within a rosette leaf in early, middle and late developmental stages. Leaves were dissected into two parts for RNA preparations: the center (C) and margin (M) as indicated. The RNA gel blot was then hybridized with an α-^{32}P-dCTP-labeled *UBP15* gene-specific probe. The rRNA band pattern was used as a loading control. Scale bar = 1 cm.
(c) Subcellular localization of UBP15 in onion epidermal cells. Scale bar = 100 μm.

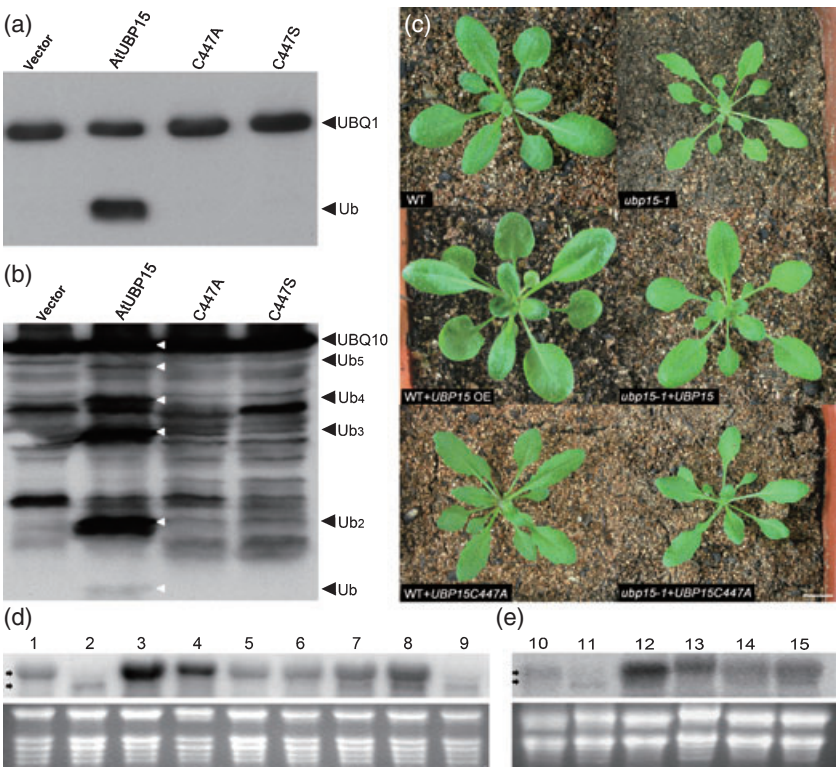

Figure 3. UBP15 is capable of cleaving polypeptides and Cys447 is essential for the function *in vitro* and *in vivo*.
(a, b) UBP15 can cleave substrates UBQ1 (a) and UBQ10 (b). The co-expressed plasmids in *E. coli* were UBQ1 (or UBQ10) with GST vector (lane 1, a and b), GST–UBP15 (lane 2, a and b), GST–UBP15C447A (lane 3, a and b) and GST–UBP15C447S (lane 4, a and b). The cleaved products were detected using anti-ubiquitin antibody. Cleaved products of UBQ10 are indicated by white arrowheads.
(c) Phenotype of *UBP15* transgenic lines. The *UBP15* over-expression line, but not the *UBP15C447A* over-expression lines, exhibits a phenotype opposite to that of the mutants, and expression of *UBP15* in *ubp15-1* restores the wild-type phenotype. One-month-old samples shown from left to right and top to bottom are: wild-type, *ubp15-1*, the *UBP15* over-expression line, the *UBP15*-complemented *ubp15-1* line, the *UBP15C447A* over-expression line, and the *UBP15C447A*-complemented *ubp15-1* line. Scale bar = 1 cm.
(d) RNA gel-blot analysis of *UBP15* expression in transgenic lines. RNA levels from plants of wild-type (lane 1), *ubp15-1* (lane 2), wild-type with the *UBP15* transgene (lanes 3–6) and *ubp15-1* with the *UBP15* transgene (lanes 7–9) were analyzed. Equal amounts of total RNA from plants grown for 4 weeks were used. The gel blot was hybridized with an α-^{32}P-dCTP-labeled *UBP15* gene-specific probe. The rRNA band pattern was used as a loading control. The upper band is the full length of the *UBP15* mRNA, while the lower band is the truncated mRNA. Lanes 3 and 4 were from plants with a *UBP15* over-expression phenotype, while lanes 5 and 6 were from plants similar to wild-type. Lanes 7 and 8 were samples from plants with a restored wild-type phenotype. The lane 9 sample was from a plant showing a *ubp15-1* mutant phenotype.
(e) RNA gel-blot analysis of *UBP15C447A* expression in transgenic lines. RNA levels from plants of wild-type (lane 10), *ubp15-1* (lane 11), wild-type with the *UBP15C447A* transgene (lanes 12 and 13) and *ubp15-1* with the *UBP15C447A* transgene (14 and 15) were analyzed. The sample in lane 12 was from a plant showing the *ubp15-1* phenotype while that in lane 13 was from a plant showing a wild-type phenotypes. The plants from which the samples in lane 14 and 15 were taken still exhibited mutant phenotype despite high expression of exogenous *UBP15C447A*.

To test the role of the DUB activity of UBP15 in plant development, we introduced wild-type *UBP15* and its Cys447 mutant form under the control of the *UBP15* native promoter into wild-type Arabidopsis and *ubp15-1*. For those lines with the *UBP15* transgene in the wild-type background, we obtained a total of 36 independent T_0 generation plants. Half of the lines exhibited no phenotypic changes, whereas the other half displayed interesting differences from wild-type, opposite to the *ubp15* mutant phenotypes (Figure 3c, middle left). Of those lines with the *UBP15* transgene in the *ubp15-1* background, five (out of 32) showed the wild-type phenotype (Figure 3c, middle right), suggesting that functional rescue of the mutant defect had occurred. RNA gel-blot analysis (Figure 3d) revealed that high-level expression of the *UBP15* transgene in the wild-type background was responsible for the abnormal phenotype that was opposite to that of the *ubp15-1* mutant, while expression of *UBP15* similar to the endogenous full-length gene restores the wild-type phenotype to the *ubp15-1* mutant and has no phenotype effect in the wild-type background. Some plants containing the *UBP15* transgene did not show restoration of the wild-type phenotype, possibly due to insufficient levels of transgene expression.

However, RNA gel-blot analysis showed that the expression of *UBP15C447A* (with the conserved catalytic Cys447 changed to Ala) in *ubp15-1* did not alter the mutant phenotype (Figure 3c, bottom right, and 3e). Interestingly, some lines over-expressing *UBP15C447A* in the wild-type

background exhibited a phenotype similar to *ubp15-1* mutants (Figure 3c, bottom left, and 3e). Those lines with mutant-like phenotype tended to express the mutant transgene at higher levels, and this probably caused dominant-negative interference with endogenous protein function. These results imply that Cys447 is essential for the DUB activity of UBP15 *in vivo*.

Further examination of the phenotypes of *UBP15* over-expression lines revealed larger overall stature of the plants as well as larger rosette leaves. The rosette leaves of the *UBP15* over-expression lines were rounder than those of the wild-type from a very early stage, and were severely downward-curled in the middle to later stages (Figure 3c, middle left). In the later rosette leaves, such as the 9th rosette leaf, they sometimes showed a knot in the leaf tip region (arrow in Figure 4b, compare Figure 4a,b), possibly caused by uneven proliferation of the cells in various positions within the leaf. In addition, the plants over-expressing UBP15 exhibited late flowering, and showed an increase in flower and silique size (Figure 4i,j,l). In the wild-type, only a low frequency of abnormal flowers occurred (in later flowers). The over-expression plants showed abnormal flowers even in the early flowers, with a high rate of abnormality in petal or stamen number (91.3%, 40/46 in one plant) (Figures 4c–e,k). Interestingly, the abnormal flowers are largely fertile, except in extreme cases where damaged sexual organs prevented fertilization (Figure 4h,m). There is also an increased apical dominance in *UBP15* over-expression lines (Table 2 and Figure S5). In some extreme cases, a flower developed in positions where secondary bolts were expected (Figure 4g), in contrast to the wild-type (Figure 4f).

The ubp15 *loss-of-function mutants and* UBP15 *over-expression plants display opposite abnormalities in leaf cell proliferation*

We further characterized the morphogenetic patterns of all leaves, including cotyledons, true leaves and cauline leaves, among mutants and over-expression plants, and compared them with the wild-type. As shown in Figure 5(a), under our growth conditions, wild-type plants had about 11 rosette leaves, while two *ubp15* mutants had about nine rosette leaves. In contrast, *UBP15* over-expression line produced about 14 rosette leaves. To examine the cellular basis for the small plant size and narrow leaf lamina of the *ubp15* mutants, transverse sections of leaves from mutant as well as *UBP15* over-expression lines were compared with the wild-type leaves. The right panel of Figure 5(b) illustrates the position of the representative sections analyzed, and a sample wild-type leaf section is shown in the left panel of Figure 5(b). We selected six of the leaves pictured in Figure 5(a) for measurement – the cotyledon, the 1st, 3rd, 5th and 9th true leaves, and the first grown cauline leaf. The adaxial epidermal cell number and palisade cell number in each line were counted in three or four serial sections under a microscope.

For the adaxial epidermal cells, the number of cells at each position in the three lines is shown in Figure 5(c). At the beginning of leaf development, the adaxial epidermal cell numbers across the lamina were similar in all lines. The differences increased with the development of the leaves, and in the case of the 9th rosette leaf, the numbers of cells across the lamina were approximately 290 in wild-type, approximately 180 in two *ubp15* mutants (a decrease of

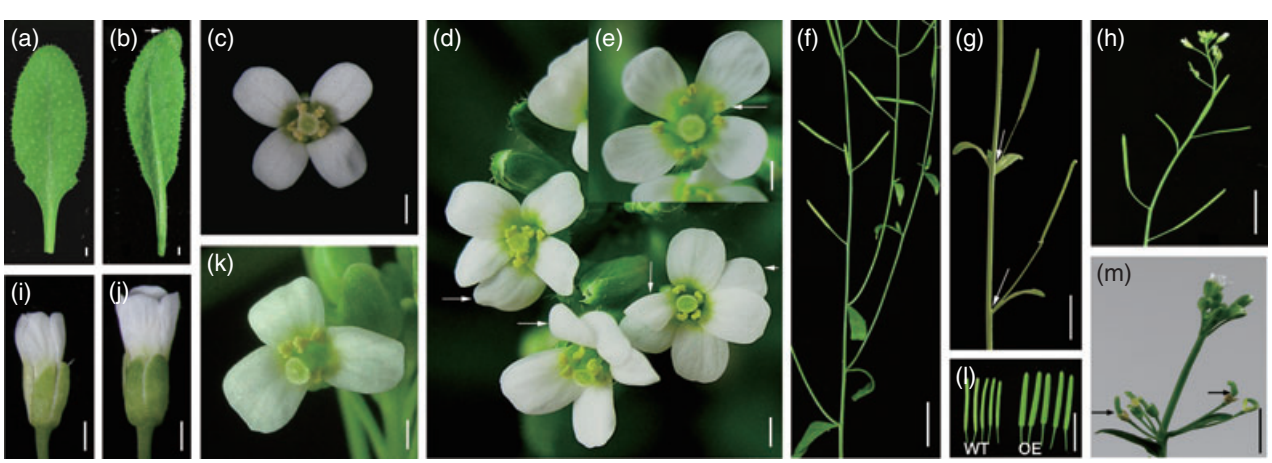

Figure 4. Comparison of wild-type and the *UBP15* over-expression line.
(a,b) Ninth rosette leaf of the wild-type (a) and *UBP15* over-expression line (b).
(c–e, k) Top view of a wild-type flower (c) and *UBP15* over-expression line flowers (d,e,k).
(f, g) Stems of the wild-type (f) and *UBP15* over-expression line (g).
(h, m) Inflorescences and siliques of the wild-type (h) and *UBP15* over-expression line (m).
(i, j) Side view of a wild-type flower (i) and an *UBP15* over-expression line flower (j).
(l) Comparison of the normal siliques of the wild-type (left) and *UBP15* over-expression line (right).
Scale bars = 1 mm (a–e,i–k) and 1 cm (f–h,l,m).

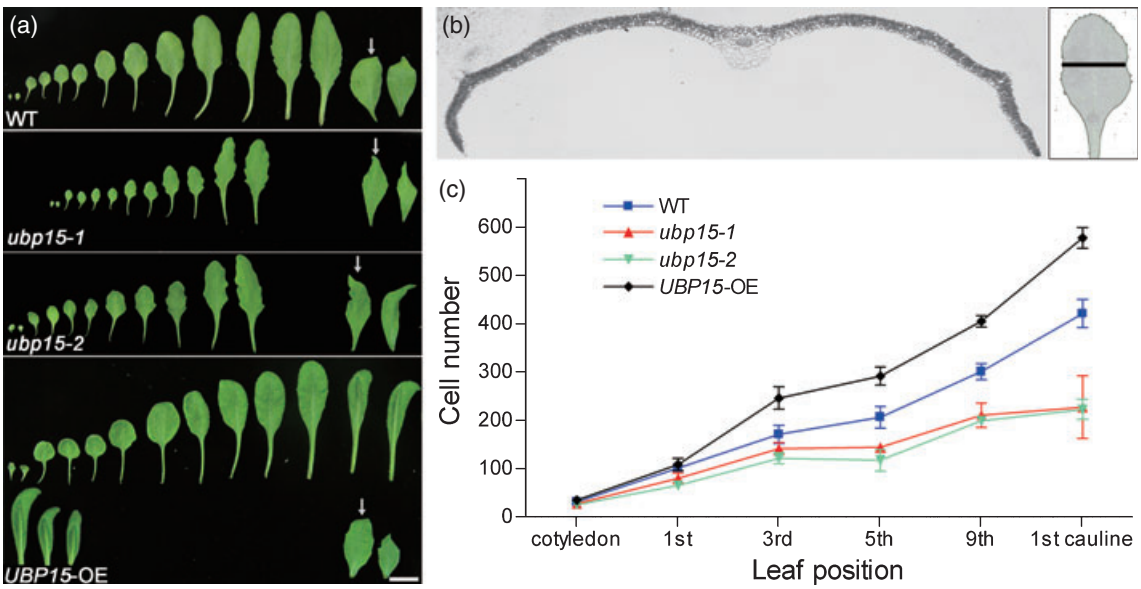

Figure 5. Comparison of the transverse sections of rosette leaves across the lamina in four lines.
(a) Top to bottom: wild-type, *ubp15-1*, *ubp15-2* and the *UBP15* over-expression line. In each line, two cotyledons, rosette leaves from the first to the last, and as the first two cauline leaves are shown from left to right. Arrows indicate the positions of cauline leaves. Scale bar = 1 cm.
(b) A sample wild-type leaf section (left), and the position of the representative section analyzed.
(c) Comparison of adaxial epidermal cell number in transverse sections across the rosette leaves.
Error bars represent standard deviation of three biological repeats.

approximately 40%), and approximately 400 in the *UBP15* over-expression line (an increase of approximately 40%). It is interesting to note that the adaxial epidermal cell number in cauline leaves was higher than that for any rosette leaves. A similar result was observed for the palisade cell numbers (Figure S6). Thus, the mutation in *UBP15* gave rise to a decrease in the adaxial epidermal and palisade cell number in the lateral direction, while over-expression of *UBP15* led to an increase of cell numbers.

We further examined the leaf cellular structure in the transverse sections in the middle region of the leaves. The wild-type leaf had an organized internal anatomy comprising a layer of vertically packed palisade cells beneath the adaxial surface, and layers of spongy parenchyma cells, interspersed with air spaces, loosely arranged below the palisade layer (Figure 6a, first from left). Interestingly, both the *ubp15-1* and *ubp15-2* mutants exhibited reduced a number of spongy cell layers, whereas *UBP15* over-expression lines contained more spongy cell layers (Figure 6a).

The vascular bundle at the mid-vein of the wild-type comprised several layers of xylem and phloem surrounded by layers of parenchyma cells (Figure 6b, first from left). In

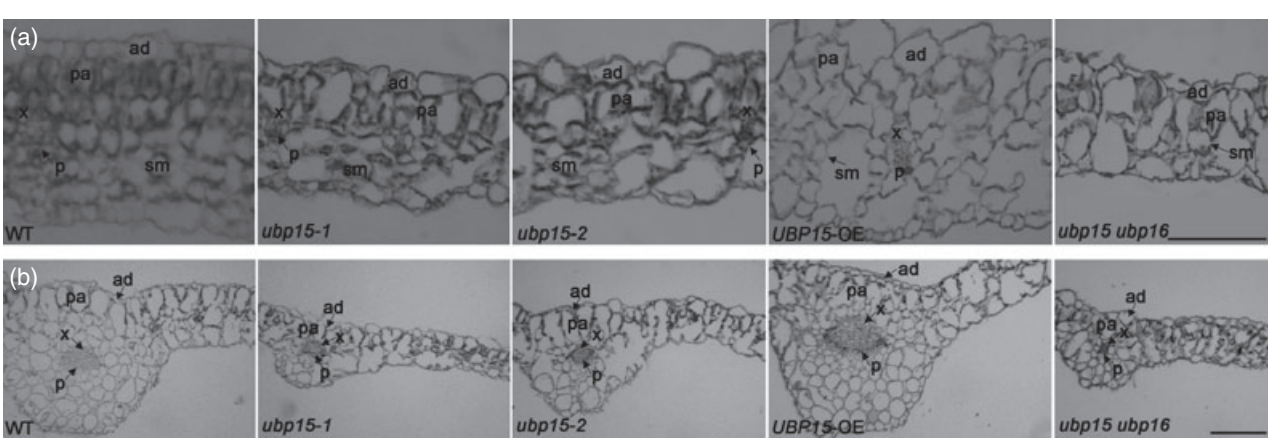

Figure 6. Histological comparison of cell layers in transverse sections of wild-type, *ubp15-1*, *ubp15-2*, the *UBP15* over-expression line and *ubp15 ubp16* mutants.
Mid-vein structure in transverse sections of wild-type, *ubp15-1*, *ubp15-2*, the *UBP15* over-expression line and the *ubp15 ubp16* mutant showing areas without (a) or with (b) a main vascular vein. ad, adaxial; pa, palisade; sm, spongy mesophyll; ab, abaxial; x, xylem; p, phloem. Scale bars = 0.1 mm.

contrast, the vascular bundles of the two mutants were slimmer than in the wild-type due to the lower cell numbers in xylem and phloem. In addition, the numbers of parenchyma cells surrounding the vascular bundles of the two mutants were also reduced, whereas we observed increases in those corresponding cell numbers in the *UBP15* over-expression lines (Figure 6b). Interestingly, a small but significant increase in the cell size was detected in the *ubp15* mutants, implying partial compensation for the loss of cell number.

The UBP15 *mutation altered expression of a large number of genes*

To explore the possible molecular processes underlying the cell proliferation defects observed in *ubp15* mutants, we performed a genome-wide expression analysis on the 9th rosette leaf as soon as it had emerged using an Arabidopsis microarray (Ma *et al.*, 2005). This analysis identified a total of 804 differentially expressed genes (4% of the expressed genes) between *ubp15-1* and wild-type at an adjusted *P*-value < 0.15, with 406 (50.5%) up-regulated and 398 (49.5%) down-regulated (Table S3). To validate the microarray results, real-time RT-PCR was performed two or three times for nine selected genes exhibiting expression changes in the *ubp15-1* mutant (Figure S7). The real-time RT-PCR results for all the analyzed genes correlated with our microarray results.

To characterize the biological processes affected, the gene ontology (GO) categories (Maere *et al.*, 2005) for those genes with altered expression in the *ubp15-1* mutant (Table S4). It is interesting to note that two of the genes that were differentially expressed in *ubp15-1* were found to be related to the cell cycle (Table S4). ICK1 was up-regulated and the Cyclin-like F-box domain-containing protein was down-regulated, with changes in expression of 1.72- and 0.3-fold, respectively. Both changes are consistent with the observed cell proliferation phenotype. Furthermore, expression of two flowering genes *CAL* and *MAF5* was also consistent with the delayed flowering phenotypes. Expression of the *CAL* gene, which positively regulates flower development, was slightly increased (by 1.98-fold), while the expression of *MAF5*, a negative regulator, decreased by 0.31-fold (see Table S4).

The *ubp15-1* mutant was found to be defective in numerous plant metabolic pathways at the transcriptional level, suggesting that secondary effects had caused the observed growth defects. This observation suggested that the compromised genes in the *ubp15-1* mutant also affected other pathways or groups of pathways involved in biosynthetic metabolism, chlorophyll biosynthesis, photosynthesis and signal transduction. These genes also include a large number of transcription factors (Table S4), which could affect many developmental processes.

UBP15 and UBP16 are partially redundant, but not with UBP17

UBP16 and UBP17 are two closely related proteins (Figure S2), and their overall sequences show 32% identity. Recessive mutants for each of the two genes (*ubp16-1*, *ubp17-1* and *ubp17-2*) (Figure 7a) did not exhibit an observable phenotype. To examine the functional redundancy of these two genes, we constructed the double mutants *ubp16-1 ubp17-1* and *ubp16-1 ubp17-2* and did not observe a noticeable phenotype. To further test for additional functional redundancy among this sub-family, we constructed a series of double mutants *ubp15 ubp16*, *ubp15 ubp17* and *ubp16 ubp17*, as well as the triple mutant *ubp15 ubp16 ubp17* (Figure 7b–d, *ubp16 ubp17* not shown). The phenotype of the *ubp15 ubp16* mutant is similar to but more severe than that of the *ubp15* mutant, with more extreme narrow rosette leaves, dwarf plants, smaller flowers and aborted siliques (Figure 7b–d). A detailed comparison of *ubp15-1* and *ubp15-1 ubp16-1* revealed additional defects in the double mutant. The alteration in cell number was conspicuous, and was evident in a strikingly degenerated vascular bundle (Figure 6a, first from right, and Figure 6b, first from right). In the case of the 9th rosette leaf, the adaxial epidermal cell number across the leaf lamina decreased by 60% compared with the wild-type (Figure S8), as did palisade cell number (data not shown). From these experiments, we conclude that *UBP15* and *UBP16* redundantly influence plant size and leaf development, and that *UBP15* is the major player. On the other hand, *ubp15 ubp17* did not show more severe defects compared to *ubp15* mutants, implying that UBP17 has minimal functional contribution. The phenotype of the triple mutant *ubp15 ubp16 ubp17* was similar to that of the double mutant *ubp15 ubp16*, confirming that UBP17 plays a lesser role.

Discussion

Characterization of the phenotypes of *ubp15* mutants and the *UBP15* over-expression line provides a basis for further analysis of this gene in plant leaf development. Both *ubp15* mutants produce narrow rosette leaves that are serrated and flat, and exhibit a decrease in the cell number in a transverse section across the lamina, whereas the *UBP15* over-expression line shows an opposite phenotype. This indicates that UBP15 can affect the leaf shape by controlling cell proliferation, possibly by regulating cell-cycle proteins. More recently, *hub1* was found to exhibit narrower rosette leaves. This phenotype was caused by a decrease in cell numbers. A microarray assay revealed that the number of expressed genes related to cell cycle and cytokinesis had changed (Fleury *et al.*, 2007). In the current microarray study, we found that two proteins (ICK1 and the Cyclin-like F-box domain-containing protein) related to the cell cycle had an

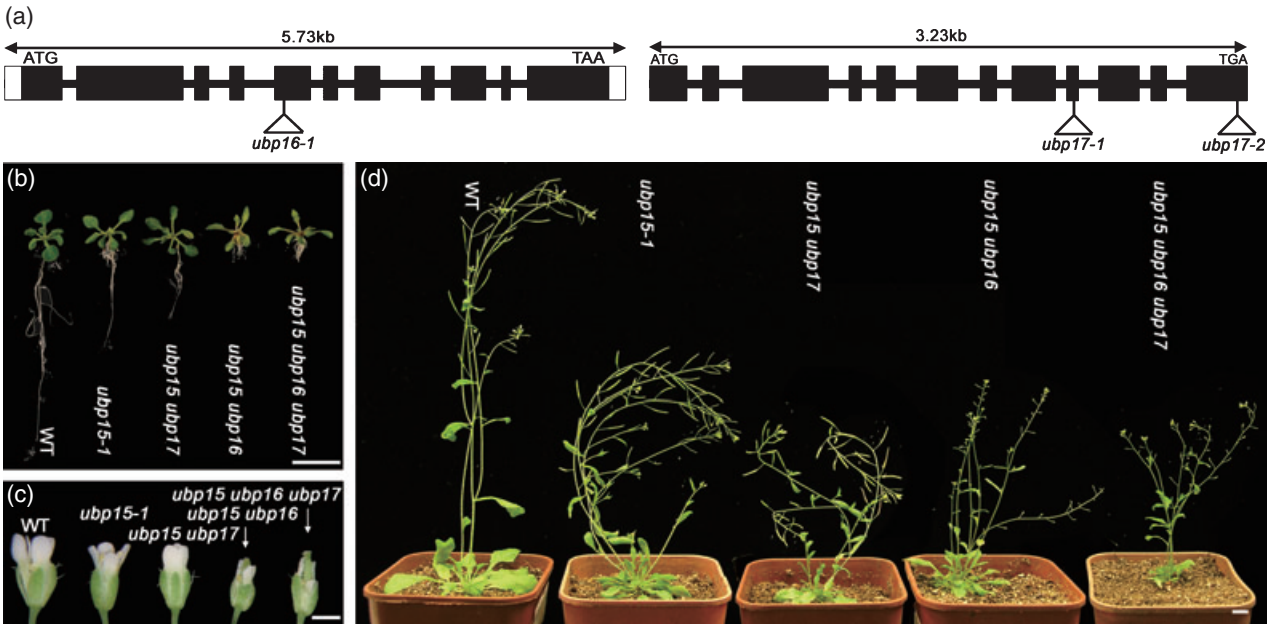

Figure 7. Characterization of the *UBP16* and *UBP17* genes.
(a) Gene structure of *UBP16* (left) and *UBP17* (right).
(b, d) Twelve-day-old (b) and two-month-old (d) plants of wild-type, *ubp15-1*, *ubp15 ubp17*, *ubp15 ubp16* and *ubp15 ubp16 ubp17*. Scale bars = 1 cm.
(c) Flowers of wild-type, *ubp15-1*, *ubp15 ubp17*, *ubp15 ubp16* and *ubp15 ubp16 ubp17*.
Scale bar = 1 mm.

altered gene expression level in the mutant, and the patterns of both were consistent with the observed decrease in cell number. However, we do not know whether the expression of many other genes distantly related to the cell cycle that were altered in *ubp15-1* contributed to the phenotype.

The rosette leaves in the *ubp15* mutants were flat, while downward-curled leaves characterized the *UBP15* over-expression line. The expression level of *UBP15* in rosette leaves increased in the margin of the leaves as plant development progressed, suggesting that UBP15 is specifically involved in the margin shape of the rosette leaves. A similar result was found for *iamt1-D*, which displays dramatic hyponastic leaf phenotypes caused by increased expression of *IAMT1* at the margin of the rosette leaves (Qin *et al.*, 2005). These similarities suggest that auxin could be a common mediator for the observed phenotype.

The leaf index increased in the *ubp15-1* mutant because of narrowing of the mutant rosette leaves. Polarized growth of leaf blades in the leaf-width direction is governed by polar elongation of leaf cells or polar cell proliferation (Tsukaya, 2006). Genes that are known to alter cell proliferation in the leaf-width direction include *GIF1* (also named *AN3*) (Kim and Kende, 2004), *GRF5* (Horiguchi *et al.*, 2005) and *HUB1* (Fleury *et al.*, 2007; Horiguchi *et al.*, 2005; Kim and Kende, 2004). They all are important in the positive control of cell number across the width of the leaf. HUB1 is an E3 ligase that mono-ubiquitinates histone H2B, and its mutant exhibits a similar phenotype to that of *ubp15* mutants, thus suggesting that it may share the same pathway as UBP15. However, our microarray data did not show a change in the expression level of *HUB1*. Therefore, further genetic and biochemical data will be required to elucidate these possible relationships.

In the UBP15 sub-family, UBP16 and UBP17 have 32% amino acid identity over their entire length, while UBP18 and UBP19 exhibit 64% identity over their entire length. However, UBP15 is less similar to the other four, showing 24, 25, 26 and 27% identity, respectively, with UBP16, UBP17, UBP18 and UBP19 over their entire length, and this weak sequence identity could explain why the *ubp15* mutants exhibit the observed phenotype. The *ubp16 ubp17* double mutants did not show any apparent phenotype. On the other hand, the phenotypes of the *ubp15* mutants were further enhanced in *ubp15 ubp16* double mutant plants. This phenotype pattern indicates that UBP15 and UBP16 may have partial functional redundancy, with UBP15 as the major player, but UBP17 makes a minor contribution, if any.

Therefore, although UBP15 is more related to UBP18/UBP19 than to UBP16 in terms of protein sequence, the phenotype of *ubp15 ubp16* suggests that UBP15 is closer to UBP16 than to UBP18/UBP19 with regard to its function. It is also interesting to note that, although UBP18 and UBP19 are extremely similar to each other, a *ubp19* mutant showed a recessive embryo phenotype while a *ubp18* mutant did not show any detectable defect (Figures S9 and S10). In other words, it appears that proteins closely related in amino acid sequence do not necessarily share functional similarity. This

detailed analysis of the UBP15 sub-family members reveals their distinct roles in different or overlapping cellular and developmental pathways to regulate protein de-ubiquitination.

Experimental procedures

Plant materials and growth conditions

The wild-type *Arabidopsis thaliana* plants used in this study were of the Col-0 ecotype. The 39 T-DNA insertion lines listed in Table 1 were obtained from the SALK collection. Double and triple mutants were obtained by crossing, and homozygous progeny were confirmed by PCR analysis (see Appendix S1).

Surface-sterilized seeds were sown on 1× MS medium/1% sucrose/0.3% Phytagel (Sigma, http://www.sigmaaldrich.com) plate, kept for 48 h at 4°C in the dark, and then placed in chamber with 16 h illumination per day (or 8 h under short-day conditions) at 22°C. After 7 days, seedlings were transferred to soil and were grown in standard long-day or short-day growth rooms.

Sequence alignment and phylogenetic analysis

For phylogentic analysis of the 27 UBP proteins, their variable regions were eliminated and the conserved domains within each protein were used. The conserved domains were defined by submitting the protein sequences to both the Pfam (http://pfam.sanger.ac.uk/search) and National Center for Biotechnology Information (NCBI) CDART (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd=rps) databases. Alignment of the assembled conserved domains of each 27 proteins was performed by Clustal W (Thompson *et al.*, 1994). The phylogenetic tree was constructed using MEGA version 3.1 (Kumar *et al.*, 2004) by identifying conserved positions of the alignment, using the neighbor-joining algorithm with 1001 bootstrap replicates.

Measurement of the length of seedling roots and siliques, and the width and length of rosette leave

See Appendix S1.

Measurement of flowering time and rosette leaf number

See Appendix S1.

RNA isolation and RT-PCR

RNA was isolated using Trizol reagents [Invitrogen (http://www.invitrogen.com/)]. RNA samples (3 μg) were subjected to reverse transcription using a Superscript™ II RNase H^- reverse transcriptase kit (Invitrogen) and random primers. The reaction mixture was diluted 10-fold, and 1 μl was subjected to the PCR using rTaq DNA polymerase (TaKaRa, http://www.takara-bio.com). At3g04120/*GAPDH* was used as an internal control. The linearity of the PCR reaction was monitored by comparing relative amounts of PCR products after 22, 30 and 35 cycles. Forward and reverse primer sequences used for detection of gene transcripts were as follows At3g04120/*GAPDH*, 5′-CACTTGAAGGGTGGTGCCAAG-3′ and 5′-CCTGTTGTCGCCAACGAAGTC-3′; *UBP15*, 5′-TCGAGAGGCAACAGTTATGCTG-3′ and 5′-CTCAGGCCTCCAGTAACTGTAAGTTCTATCCTG-3′.

RNA gel-blot analysis

RNA blotting was performed as described previously (Yang *et al.*, 2005). The total RNA from various regions of the leaves was extracted, and 20 μg of total RNA for each lane were separated on an agarose gel, transferred to Hybond N^+ membrane (Amersham Biosciences, http://www5.amershambiosciences.com/), and hybridized with a *UBP15*-specific probe. rRNA served as the loading control. The primers used for *UBP15* were 5′-TCGAGAGGCAACAGTTATGCTG-3′ and 5′-CTCAGGCCTCCAGTAACTGTAAGTTCTATCCTG-3′.

Transient expression in onion epidermal cells

The procedure for transient expression in living onion (*Allium cepa*) epidermal cells using particle bombardment was as described previously (Ang *et al.*, 1998). After bombardment, onion cell layers were incubated for 24 h at 22°C in the light. The cell layers were then examined by confocal microscopy.

Creation of construct and transgenic lines

To obtain *UBP15*-over-expressing and *UBP15*-complemented plants, a *Hin*dIII/*Xba*I-digested *UBP15* promoter fragment (1.8 kb upstream of ATG) and an *Xba*I/*Stu*I *UBP15* coding sequence (CDS) fragment (2.8 kb) were inserted into the pCAMBIA1300 binary vector. The resulting construct was then transformed into wild-type and *ubp15-1* by *Agrobacterium*-mediated transformation. A construct with a mutation in the Cys position (*pUBP15:UBP15C447A*) was also produced in the same way and transformed into wild-type and *ubp15-1*. Transgenic plants were selected with hygromycin (20 μg/ml; Roche, http://www.roche.com) on MS plates.

To produce the *35S:GFP-UBP15* (or *35S:UBP15-GFP*) constructs, the CaMV 35S promoter together with GFP fused to full-length *UBP15* CDS (or full-length *UBP15* CDS fused to GFP) was subcloned into the *Xba*I/*Sph*I site of pCAMBIA1300.

In vitro *DUB activity assay*

The ability of UBP15 to cleave ubiquitin linked via α-amino linkages was determined in *E. coli* using the His-tagged substrates poly-ubiquitin UBQ10 and ubiquitin extension protein UBQ1. Each of the two α-amino substrates (UBQ1 in pET28a and UBQ10 in pACYCDuet-1) was co-expressed with either GST, GST–*UBP15*, GST–*UBP15C447A* or GST–*UBP15C447S* in pGEX4T-3 in the *E. coli* strain NovaBlue (DE3) under standard conditions (induction at 22°C) (Novagen, http://www.merckbiosciences.com). Lysates were analyzed by Western blot with anti-ubiquitin antibody (Sigma, http://www.sigmaaldrich.com/) or anti-His antibody (Sigma). An enhanced chemiluminescence system (Amersham) was used for detection.

Tissue fixation and embedding for histological sections

For observation of the transverse structure of leaves, we fixed samples for more than 24 h in FAA (50% ethanol, 5% acetic acid and 5% formaldehyde) at room temperature, dehydrated them in a graded series of mixtures of H_2O/95% alcohol/tert-butyl alcohol (4:5:1, 3:5:2, 1.5:5:3.5, 0:5:5, 0:2.5:7.5, then 100% tert-butyl alcohol twice) with a 2 h incubation in each solution, and then infiltrated the samples by adding paraffin slices gradually (tert-butyl alcohol/paraffin in the ratios 3:1, 2:2 and 1:3. The samples were transferred to the melted pure paraffin twice, following the embedding.

Embedded tissues were cut into 8 μm sections using a microtome (Leica RM2255, http://www.leica.com). Sections were placed onto poly-L-lysine-coated slides, de-paraffinized and rehydrated using an ethanol series (ethanol/H_2O in the ratios 1:0, 1:0, 9.5:0.5, 9:1, 8:2, 7:3 and 1:1) with a 5 min incubation in each solution. Then sections were stained with 1% Safranin O (Sigma) and 0.02% Fast Green FCF (Sigma). The cover glasses were affixed to the slides using resin. The number of adaxial epidermal and palisade cells was counted under a microscope.

Microarray analysis and quantitative RT-PCR

The procedures used in the microarray experiments and data analysis were as described previously (Ma *et al.*, 2002). We use early-emerged 9th rosette leaves of wild-type and *ubp15-1* as samples. Appendix S1 provides a detailed description of the protocol.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Figure S1. Alignment of 27 UBP proteins.

Figure S2. Phylogenetic analysis of the *UBP* gene family of Arabidopsis.

Figure S3. Gene expression pattern of the *UBP15* sub-family.

Figure S4. Phylogenetic analysis of the AtUBP15 sub-family and its homologues in rice (*Oryza sativa*).

Figure S5. Two-month-old plants of wild-type, two *ubp15* mutants and the *UBP15* over-expression line.

Figure S6. Comparison of palisade cell number in transverse sections across the lamina of rosette leaves.

Figure S7. Real-time PCR confirming the microarray results.

Figure S8. Comparison of the 9th rosette leaf of wild-type and mutants as well as transgenic lines.

Figure S9. Characterization of the *UBP18* and *UBP19* genes.

Figure S10. Tissue expression pattern of *UBP19* determined using *pUBP19:GUS* transgenic lines.

Table S1. Microarray data for the expression of 27 UBP genes in various organs.

Table S2. Primers designed to identify the genotype of T-DNA insertion lines.

Table S3. Trascriptome analysis of *ubp15-1.*

Table S4. Microarray data for selected genes with various functions.

Appendix S1. Supplementary experimental procedure.

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                  1                                                  50
 UBP9    (1) NLKQGNLYFVISKRWYTSWQEYVEN----------SANECSTGESSEAP
UBP10    (1) NLKEGNLYFVISKRWYTSWEKYVEQ----------STKEYISGESSEAS
UBP11    (1) -LKEGNLYFVISNRWYTRWQRFVGL----------LTEEFRSGEPSEVT
 UBP5    (1) NSKEGDTFYLITQRWWQEWIEYVNQDQPCNTNDGSSLSEHCDSPGSSTLK
 UBP8    (1) --------------------------------------------------
UBP24    (1) --------------------------------------------------
 UBP3    (1) --------------------------------------------------
 UBP4    (1) --------------------------------------------------
UBP12    (1) --------------------------------------------------
UBP13    (1) --------------------------------------------------
UBP26    (1) --------------------------------------------------
UBP14    (1) --------------------------------------------------
 UBP6    (1) --------------------------------------------------
 UBP7    (1) --------------------------------------------------
 UBP1    (1) --------------------------------------------------
 UBP2    (1) --------------------------------------------------
UBP27    (1) --------------------------------------------------
UBP22    (1) --------------------------------------------------
UBP20    (1) --------------------------------------------------
UBP21    (1) --------------------------------------------------
UBP23    (1) --------------------------------------------------
UBP25    (1) --------------------------------------------------
UBP15    (1) --------------------------------------------------
UBP16    (1) --------------------------------------------------
UBP17    (1) --------------------------------------------------
UBP18    (1) --------------------------------------------------
UBP19    (1) --------------------------------------------------

                 51                                         100
 UBP9   (40) RPGPIDNHDIIES---DSDINDPQLRRLLVEGEDYVLVPKQVWKRLVEWY
UBP10   (40) RPGPIDNHDIIES---ESDVNDPQLRRLLMERVDYVLVPQEVWKRLVEWY
UBP11   (39) RPGPIDNHDIIDS---ESDASDPQLRMMLEEGVDYTLVQQEVWRKLVKWY
 UBP5   (51) KPSRIDNSDLIYDSSLEDPSNTSEIIETLQEGRDYVLLPQEVWNQLRSWY
 UBP8    (1) --------------------------------------------------
UBP24    (1) --------------------------------------------------
 UBP3    (1) --------------------------------------------------
 UBP4    (1) --------------------------------------------------
UBP12    (1) ----------------------------------LKFTWTIPNFSRQ
UBP13    (1) ----------------------------------LKFTWTIPMFTRL
UBP26    (1) --------------------------------------------------
UBP14    (1) --------------------------------------------------
 UBP6    (1) --------------------------------------------------
 UBP7    (1) --------------------------------------------------
 UBP1    (1) --------------------------------------------------
 UBP2    (1) --------------------------------------------------
UBP27    (1) --------------------------------------------------
UBP22    (1) --------------------------------------------------
UBP20    (1) --------------------------------------------------
UBP21    (1) --------------------------------------------------
UBP23    (1) --------------------------------------------------
UBP25    (1) --------------------------------------------------
UBP15    (1) --------------------------------------------------
UBP16    (1) --------------------------------------------------
UBP17    (1) --------------------------------------------------
UBP18    (1) --------------------------------------------------
UBP19    (1) --------------------------------------------------
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                  101                                                 150
 UBP9    (87) SGGPPIERKLICQGFYTRSYSVEVYPLCLMLTDGRDESRTVIRLGKQASI
UBP10    (87) SGGPPIERKLICQGFYTRSYSVEVYPLCLMLTDGRDESRTVIRLGKQASI
UBP11    (86) KGGPPVPRKLISQGFYTKSFSVEVYLLCLTLTDSRDESTTIIRLSKQASI
 UBP5   (101) GGGPTLARRVISSGLSQTELAVEVYPLRLQLLLMPKSDHSAIRISKKETI
 UBP8     (1) --------------------------------------------------
UBP24     (1) --------------------------------------------------
 UBP3     (1) --------------------------------------------------
 UBP4     (1) --------------------------------------------------
UBP12    (14) NTRKHYSDVFVVGGYKWRILIFPKGNNVDHLSMYLDVSDAASLPYGWSRY
UBP13    (14) NTRKHYSDVFVVGGYKWRILIFPKGNNVDHLSMYLDVADAANLPYGWSRY
UBP26     (1) --------------------------------------------------
UBP14     (1) ----------------------------------------CSKCDKTENLWLN
 UBP6     (1) -------------------------------------------TVSVKWQKK
 UBP7     (1) ------------------------------------------LTVSVKWQKK
 UBP1     (1) ----------------------------------------RNAIWLCLECGYY
 UBP2     (1) -----------------------------------------AIWLCLECGCY
UBP27     (1) --------------------------------------------------
UBP22     (1) --------------------------------------------------
UBP20     (1) --------------------------------------------------
UBP21     (1) --------------------------------------------------
UBP23     (1) --------------------------------------------------
UBP25     (1) --------------------------------------------------
UBP15     (1) --------------------------------------------------
UBP16     (1) --------------------------------------------------
UBP17     (1) --------------------------------------------------
UBP18     (1) --------------------------------------------------
UBP19     (1) --------------------------------------------------

                  151                                                 200
 UBP9   (137) RELYEKVCAMTGVPQEKAHIWDYFDKRKNGLLDPLSYKSLEESSLHMDQD
UBP10   (137) RELYEKVCALTGVPQEKAHIWDYFDKRKNGLLDSLSYKSLEESSLHMDQD
UBP11   (136) GQLYEMVCAGKGVAKEKARIWDYFEKKKSVLLDPSSEQSVEEAGLQFNQD
 UBP5   (151) RELHRRACEIFDLDSEHVRIWDYYGHQKYSLMNDLD-KTLDDANLQMDQD
 UBP8     (1) --------------------------------------------------
UBP24     (1) --------------------------------------------------
 UBP3     (1) --------------------------------------------------
 UBP4     (1) --------------------------------------------------
UBP12    (64) AQFSLAVVNQIHTRYTVRKETQHQFNARESDWGFTSFMPLSELYDPSRGY
UBP13    (64) SQFSLAVVNQVNNRYSIRKETQHQFNARESDWGFTSFMPLSELYEPTRGY
UBP26     (1) --------------------------------------------------
UBP14    (14) LTDGMILCGRKNWDGTGGNNHAVEHYKETAYPLAVKLGTITADLEAADVY
 UBP6    (10) VLDGIEIDVSLPPYVFKAQLYDLTGVPPERQKIMVKGGLLKDD---GDWA
 UBP7    (11) VFESIEIDTSQPPFVFKAQLYDLSGVPPERQKIMVKGGLLKDD---ADWS
 UBP1    (14) VCGDVGLPTEAQSHVMGHNRLTRHRLVIQCKNPQLRWCFSCQSLLPFDNE
 UBP2    (12) VCGGVGLPNGPQSHVLRHSRVTRHRLVIQWENPQLRWCFPCQLLLPVEKE
UBP27     (1) --------------------------------------------------
UBP22     (1) --------------------------------------------------
UBP20     (1) --------------------------------------------------
UBP21     (1) --------------------------------------------------
UBP23     (1) --------------------------------------------------
UBP25     (1) --------------------------------------------------
UBP15     (1) -----------------CARCFGPAKTRCSRCKSVRYCSGKCQIIHWRVA
UBP16     (1) -----------------CPVCYCLATTRCSRCKAVRYCSGKCQIIHWRQG
UBP17     (1) -----------------CAVCLYPTTTRCSQCKSVRYCSSKCQILHWRRG
UBP18     (1) -----------------CSVCGNFSTKKCSRCKSVRYCSAECQRSDWSSG
UBP19     (1) -----------------CSVCGKATTKKCSRCKSVRYCSAACQTSDWKSG
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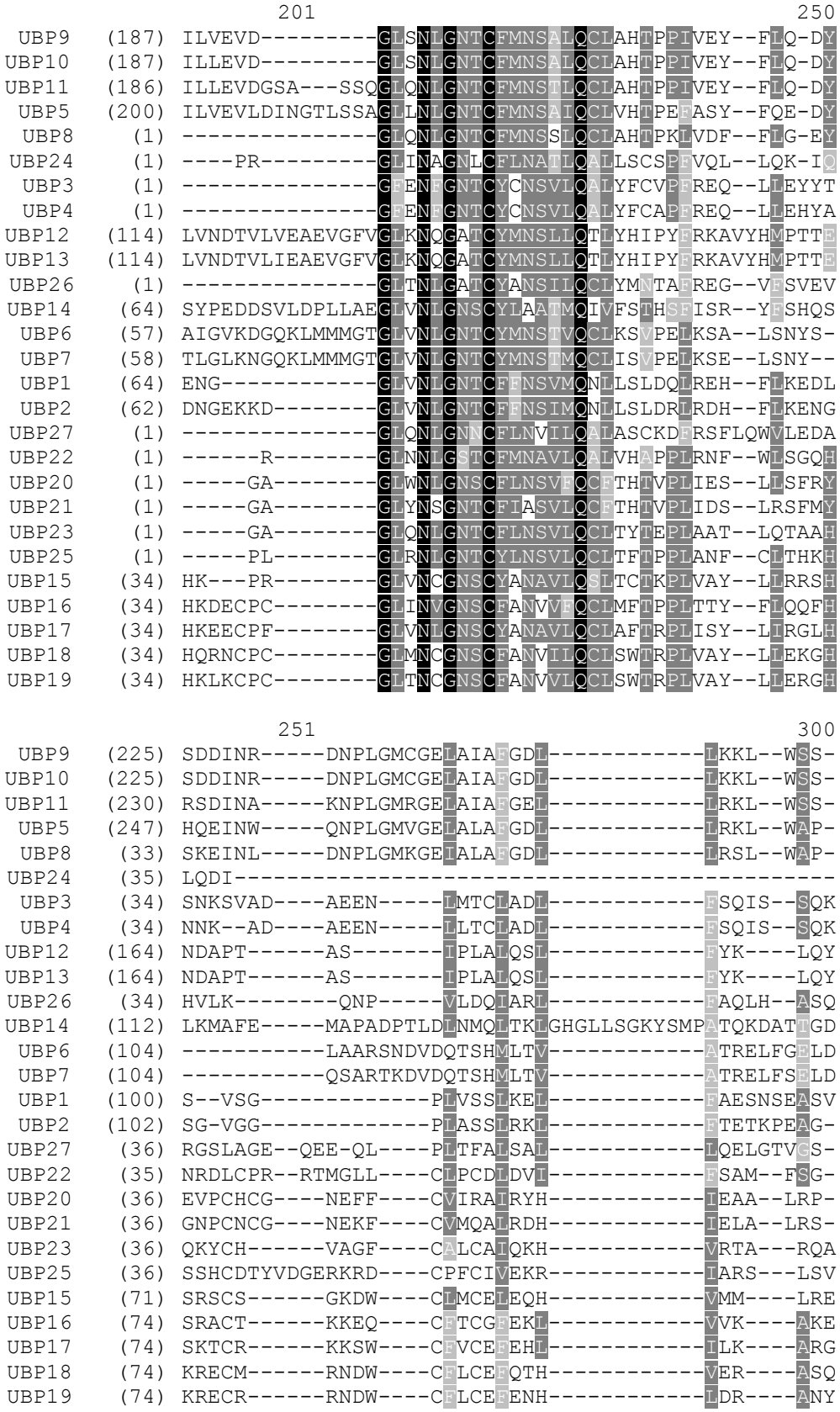
201
250
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UBP10 (187) ILLEVD---------GLSNLGNTCFMNSALQCLAHTPPIVEY--FLQ-DY
UBP11 (186) ILLEVDGSA---SSQGLQNLGNTCFMNSTLQCLAHTPPIVEY--FLQ-DY
UBP5 (200) ILVEVLDINGTLSSAGLLNLGNTCFMNSAIQCLVHTPEFASY--FQE-DY
UBP8 (1) ---------------GLQNLGNTCFMNSSLQCLAHTPKLVDF--FLG-EY
UBP24 (1) ----PR---------GLINAGNLCFLNATLQALLSCSPFVQL--LQK-IQ
UBP3 (1) ---------------GFENFGNTCYCNSVLQALYFCVPFREQ--LLEYYT
UBP4 (1) ---------------GFENFGNTCYCNSVLQALYFCAPFREQ--LLEHYA
UBP12 (114) LVNDTVLVEAEVGFVGLKNQGATCYMNSLLQTLYHIPYFRKAVYHMPTTE
UBP13 (114) LVNDTVLIEAEVGFVGLKNQGATCYMNSLLQTLYHIPYFRKAVYHMPTTE
UBP26 (1) ---------------GLTNLGATCYANSILQCLYMNTAFREG--VFSVEV
UBP14 (64) SYPEDDSVLDPLLAEGLVNLGNSCYLAAIMQIVFSTHSFISR--YFSHQS
UBP6 (57) AIGVKDGQKLMMMGTGLVNLGNTCYMNSTVQCLKSVPELKSA--LSNYS-
UBP7 (58) TLGLKNGQKLMMMGTGLVNLGNTCYMNSTMQCLISVPELKSE--LSNY--
UBP1 (64) ENG------------GLVNLGNTCFFNSVMQNLLSLDQLREH--FLKEDL
UBP2 (62) DNGEKKD--------GLVNLGNTCFFNSIMQNLLSLDRLRDH--FLKENG
UBP27 (1) ---------------GLQNLGNNCFLNVIIQALASCKDFRSFLQWVLEDA
UBP22 (1) ------R--------GLNNLGSTCFMNAVLQALVHAPPLRNF--WLSGQH
UBP20 (1) -----GA--------GLWNLGNSCFLNSVFQCFTHTVPLIES--LLSFRY
UBP21 (1) -----GA--------GLYNSGNTCFIASVLQCFTHTVPLIDS--LRSFMY
UBP23 (1) -----GA--------GLQNLGNTCFLNSVLQCLTYTEPLAAT--LQTAAH
UBP25 (1) -----PL--------GLRNLGNTCYLNSVLQCLTFTPPLANF--CLTHKH
UBP15 (34) HK---PR--------GLVNCGNSCYANAVLQSLTCTKPLVAY--LLRRSH
UBP16 (34) HKDECPC--------GLINVGNSCFANVVFQCLMFTPPLTTY--FLQQFH
UBP17 (34) HKEECPF--------GLVNLGNSCYANAVLQCLAFTRPLISY--LIRGLH
UBP18 (34) HQRNCPC--------GLMNCGNSCFANVIIQCLSWTRPLVAY--LLEKGH
UBP19 (34) HKLKCPC--------GLTNCGNSCFANVVLQCLSWTRPLVAY--LLERGH
251
300
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UBP11 (230) RSDINA-----KNPLGMRGELAIAFGEL----------LRKL--WSS-
UBP5 (247) HQEINW-----QNPLGMVGELALAFGDL----------LRKL--WAP-
UBP8 (33) SKEINL-----DNPLGMKGELALAFGDL----------LRSL--WAP-
UBP24 (35) LQDI----------
UBP3 (34) SNKSVAD----AEEN-----LMTCLADL----------FSQIS--SQK
UBP4 (34) NNK--AD----AEEN-----LLTCLADL----------FSQIS--SQK
UBP12 (164) NDAPT------AS-------IPLALQSL----------FYK----LQY
UBP13 (164) NDAPT------AS-------IPLALQSL----------FYK----LQY
UBP26 (34) HVLK--------QNP-----VLDQIARL----------FAQLH--ASQ
UBP14 (112) LKMAFE-----MAPADPTLDLNMQLTKLGHGLLSGKYSMPATQKDATLGD
UBP6 (104) -----------LAARSNDVDQTSHMLTV----------ATRELFGFLD
UBP7 (104) -----------QSARTKDVDQTSHMLTV----------ATRELFSFLD
UBP1 (100) S--VSG----------PLVSSLKEL----------FAESNSEASV
UBP2 (102) SG-VGG----------PLASSLRKL----------FTETKPEAG-
UBP27 (36) RGSLAGE--QEE-QL----PLTFALSAL----------LQELGTVGS-
UBP22 (35) NRDLCPR--RTMGLL----CLPCDLDVL----------FSAM--FSG-
UBP20 (36) EVPCHCG----NEFF----CVIRAIRYH----------IEAA--LRP-
UBP21 (36) GNPCNCG----NEKF----CVMQALRDH----------IELA--LRS-
UBP23 (36) QKYCH------VAGF----CALCAIQKH----------VRTA---RQA
UBP25 (36) SSHCDTYVDGERKRD----CPFCIVEKR----------IARS---LSV
UBP15 (71) SRSCS------GKDW----CLMCELEQH----------VMM----LRE
UBP16 (74) SRACT------KKEQ----CFTCGFEKL----------VVK----AKE
UBP17 (74) SKTCR------KKSW----CFVCEFEHL----------ILK----ARG
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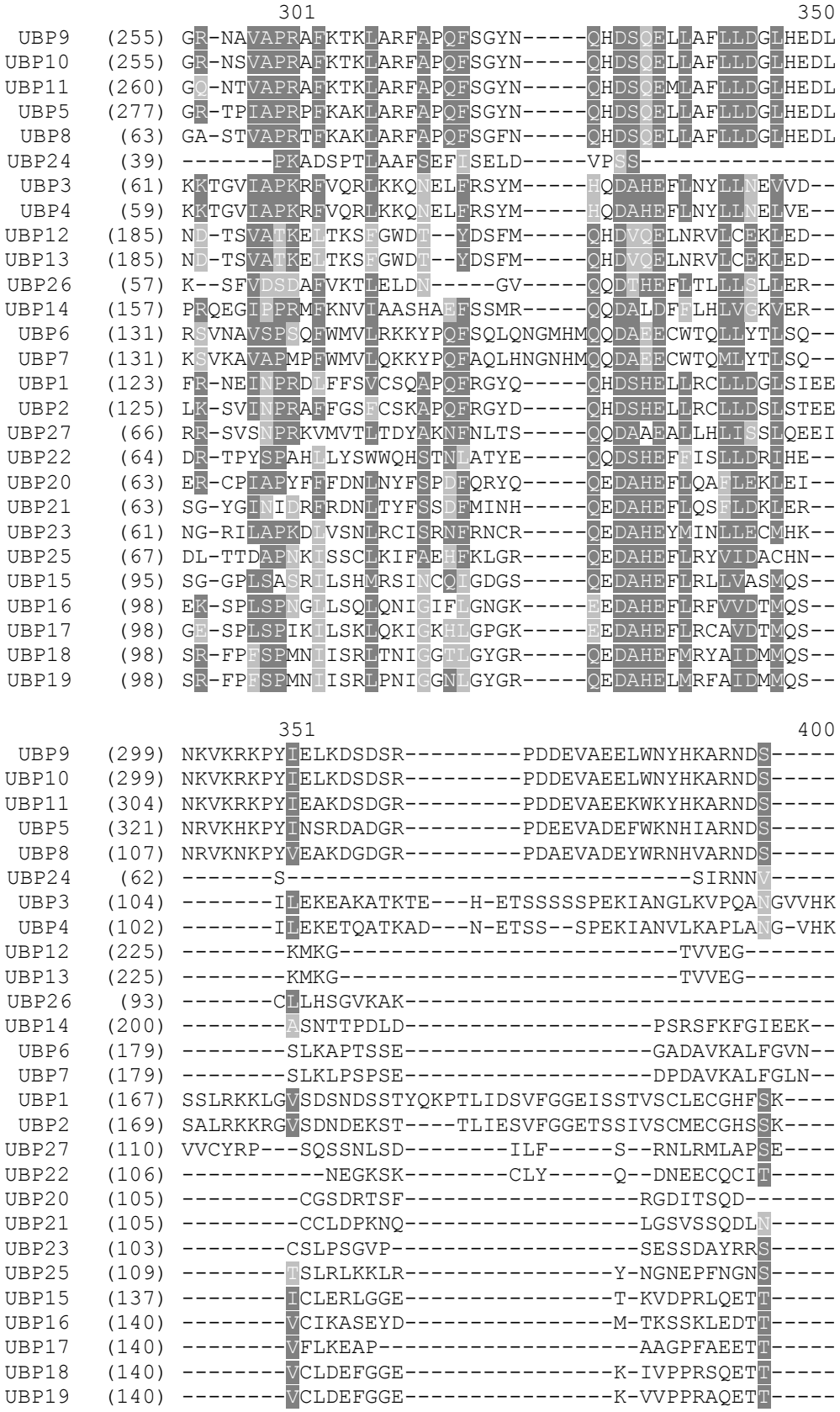
UBP19 (74) KRECR------RNDW----CFLCEFENH----------LDR----ANY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UBP5 (277) GR-TPIAPRPFKAKLARFAPQFSGYN-----QHDSQELLAFLLDGLHEDL
UBP8 (63) GA-STVAPRTFKAKLARFAPQFSGFN-----QHDSQELLAFLLDGLHEDL
UBP24 (39) -------PKADSPTLAAFSEFISELD-----VPSS---------------
UBP3 (61) KKTGVIAPKRFVQRLKKQNELFRSYM-----HQDAHEFLNYLLNEVVD--
UBP4 (59) KKTGVIAPKRFVQRLKKQNELFRSYM-----HQDAHEFLNYLLNELVE--
UBP12 (185) ND-TSVATKEITKSFGWDT--YDSFM-----QHDVQELNRVLCEKLED--
UBP13 (185) ND-TSVATKEITKSFGWDT--YDSFM-----QHDVQELNRVLCEKLED--
UBP26 (57) K--SFVDSDAFVKTLELDN-----GV-----QQDTHEFLTLLLSLLER--
UBP14 (157) PRQEGIPPRMFKNVIAASHAFFSSMR-----QQDALDFFLHLVGKVER--
UBP6 (131) RSVNAVSPSQFWMVLRKKYPQFSQLQNGMHMQQDAEECWTQLLYTLSQ--
UBP7 (131) KSVKAVAPMPFWMVLQKKYPQFAQLHNGNHMQQDAEECWTQMLYTLSQ--
UBP1 (123) FR-NEINPRDIFFSVCSQAPQFRGYQ-----QHDSHELLRCLLDGLSIEE
UBP2 (125) LK-SVINPRAFFGSFCSKAPQFRGYD-----QHDSHELLRCLLDSLSTEE
UBP27 (66) RR-SVSNPRKVMVTLTDYAKNFNLTS-----QQDAAEALLHLISSLQEEI
UBP22 (64) DR-TPYSPAHILYSWWQHSTNLATYE-----QQDSHEFFISLLDRIHE--
UBP20 (63) ER-CPIAPYFFFDNLNYFSPDFQRYQ-----QEDAHEFLQAFLEKLEI--
UBP21 (63) SG-YGINIDRFRDNLTYFSSDFMINH-----QEDAHEFLQSFLDKLER--
UBP23 (61) NG-RILAPKDIVSNLRCISRNFRNCR-----QEDAHEYMINLLECMHK--
UBP25 (67) DL-TTDAPNKISSCLKIFAEHFKLGR-----QEDAHEFLRYVIDACHN--
UBP15 (95) SG-GPLSASRILSHMRSINCQIGDGS-----QEDAHEFLRLLVASMQS--
UBP16 (98) EK-SPLSPNGILSQLQNIGIFLGNGK-----FEDAHEFLRFVVDTMQS--
UBP17 (98) GF-SPLSPIKILSKLQKIGKHLGPGK-----FEDAHEFLRCAVDTMQS--
UBP18 (98) SR-FPFSPMNIISRLTNIGGTLGYGR-----QEDAHEFMRYAIDMMQS--
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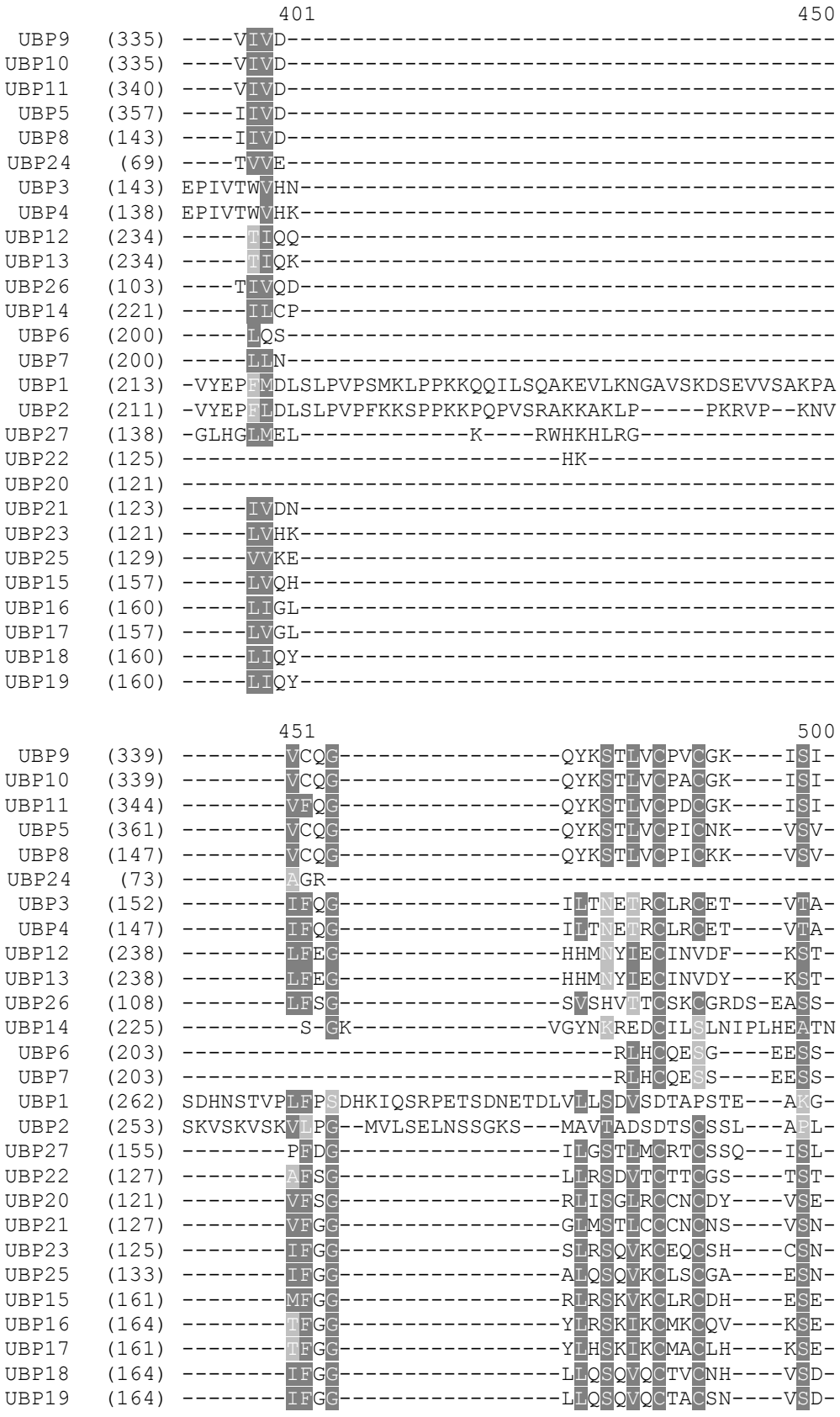
351
400
UBP9 (299) NKVKRKPYIELKDSDSR---------PDDEVAEELWNYHKARNDS-----
UBP10 (299) NKVKRKPYIELKDSDSR---------PDDEVAEELWNYHKARNDS-----
UBP11 (304) NKVKRKPYIEAKDSDGR---------PDDEVAEEKWKYHKARNDS-----
UBP5 (321) NRVKHKPYINSRDADGR---------PDEEVADEFWKNHIARNDS-----
UBP8 (107) NRVKNKPYVEAKDGDGR---------PDAEVADEYWRNHVARNDS-----
UBP24 (62) -------S-----------------------------SIRNNV-----
UBP3 (104) -------ILEKEAKATKTE---H-ETSSSSSPEKIANGLKVPQANGVVHK
UBP4 (102) -------ILEKETQATKAD---N-ETSS--SPEKIANVLKAPLANG-VHK
UBP12 (225) --------KMKG--------------------------TVVEG-------
UBP13 (225) --------KMKG--------------------------TVVEG-------
UBP26 (93) -------CLLHSGVKAK---------------------------------
UBP14 (200) --------ASNTTPDLD-------------------PSRSFKFGIEEK--
UBP6 (179) --------SLKAPTSSE-------------------GADAVKALFGVN--
UBP7 (179) --------SLKLPSPSE-------------------DPDAVKALFGLN--
UBP1 (167) SSLRKKLGVSDSNDSSTYQKPTLIDSVFGGEISSTVSCLECGHFSK----
UBP2 (169) SALRKKRGVSDNDEKST----TLIESVFGGETSSIVSCMECGHSSK----
UBP27 (110) VVCYRP---SQSSNLSD--------ILF-----S--RNLRMLAPSE----
UBP22 (106) -----------NEGKSK--------CLY-----Q--DNEECQCIT-----
UBP20 (105) ---------CGSDRTSF-------------------RGDITSQD-------
UBP21 (105) ---------CCLDPKNQ-------------------LGSVSSQDLN-----
UBP23 (103) --------CSLPSGVP-------------------SESSDAYRRS-----
UBP25 (109) --------TSLRLKKLR-----------------Y-NGNEPFNGNS-----
UBP15 (137) --------ICLERLGGE-----------------T-KVDPRLQETT-----
UBP16 (140) --------VCIKASEYD-----------------M-TKSSKLEDTT-----
UBP17 (140) --------VFLKEAP--------------------AAGPFAEETT-----
UBP18 (140) --------VCLDEFGGE-----------------K-IVPPRSQETT-----
UBP19 (140) --------VCLDEFGGE-----------------K-VVPPRAQETT-----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UBP10 (335) ----VIVD
UBP11 (340) ----VIVD
UBP5 (357) ----IIVD
UBP8 (143) ----IIVD
UBP24 (69) ----TVVE
UBP3 (143) EPIVTWVHN
UBP4 (138) EPIVTWVHK
UBP12 (234) -----IIQQ
UBP13 (234) -----IIQK
UBP26 (103) ----TIVQD
UBP14 (221) -----ILCP
UBP6 (200) -----LQS
UBP7 (200) -----LLN
UBP1 (213) -VYEPFMDLSLPVPSMKLPPKKQQILSQAKEVLKNGAVSKDSEVVSAKPA
UBP2 (211) -VYEPFLDLSLPVPFKKSPPKKPQPVSRAKKAKLP-----PKRVP--KNV
UBP27 (138) -GLHGLMEL-------------K----RWHKHLRG----------
UBP22 (125) ----------HK----------
UBP20 (121) ----------
UBP21 (123) -----IVDN----------
UBP23 (121) -----LVHK----------
UBP25 (129) -----VVKE----------
UBP15 (157) -----LVQH----------
UBP16 (160) -----LIGL----------
UBP17 (157) -----LVGL----------
UBP18 (160) -----LIQY----------
UBP19 (160) -----LIQY----------
451
500
UBP9 (339) --------VCQC----------QYKSTLVCPVCGK----ISI-
UBP10 (339) --------VCQC----------QYKSTLVCPACGK----ISI-
UBP11 (344) --------VFQC----------QYKSTLVCPDCGK----ISI-
UBP5 (361) --------VCQC----------QYKSTLVCPICNK----VSV-
UBP8 (147) --------VCQC----------QYKSTLVCPICKK----VSV-
UBP24 (73) --------AGR----------
UBP3 (152) --------IFQC----------ILTNETRCLRCET----VTA-
UBP4 (147) --------IFQC----------ILTNETRCLRCET----VTA-
UBP12 (238) --------LFEC----------HHMNYIECINVDF----KST-
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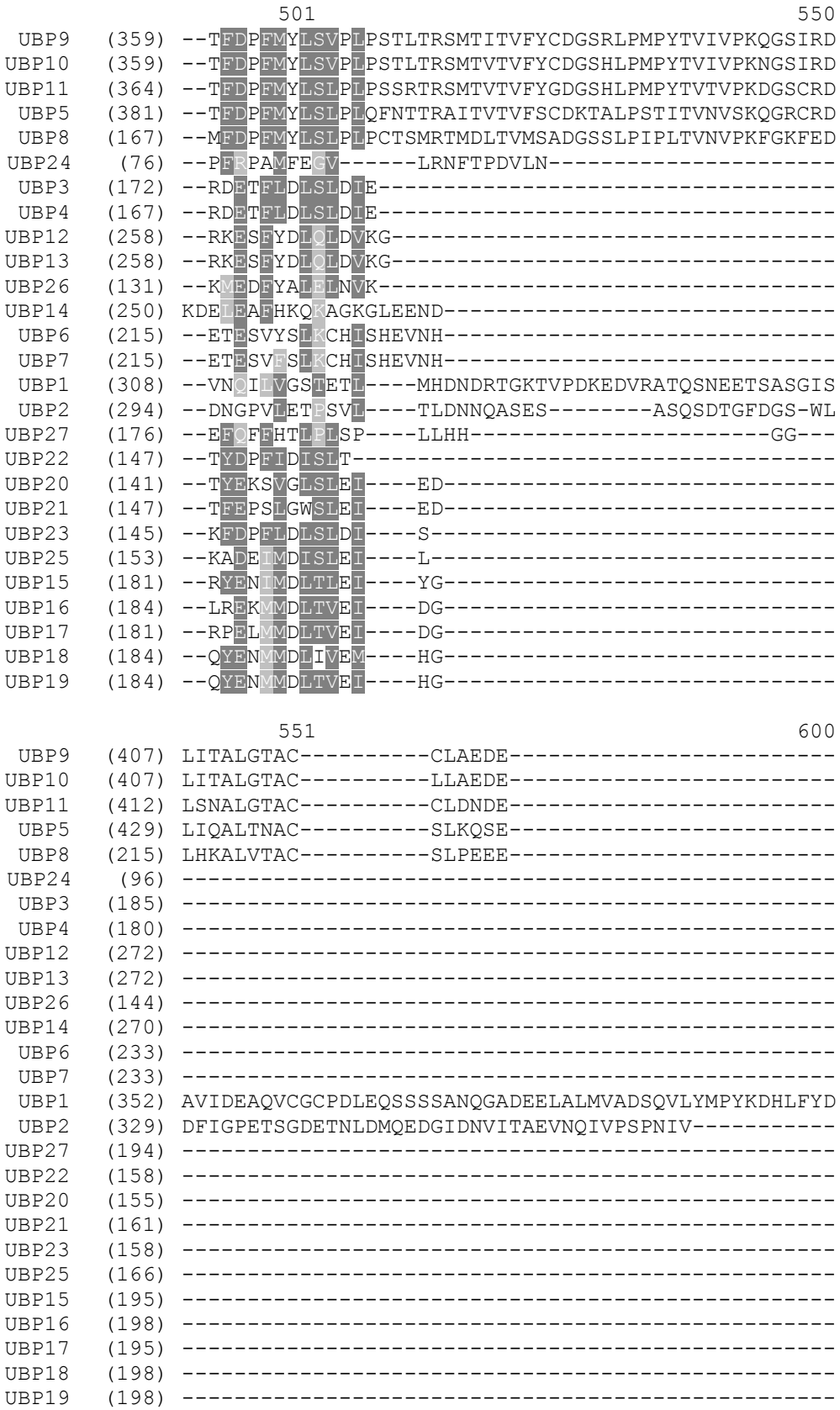
UBP26 (108) --------LFSC----------SVSHVTTCSKCGRDS-EASS-
UBP14 (225) ---------S-CK----------VGYNKREDCILSLNIPLHEATN
UBP6 (203) ----------RLHCQESG----EESS-
UBP7 (203) ----------RLHCQESS----EESS-
UBP1 (262) SDHNSTVPLFPSDHKIQSRPETSDNETDLVLLSDVSDTAPSTE---AKG-
UBP2 (253) SKVSKVSKVLPG--MVLSELNSSGKS---MAVTADSDTSCSSL---APL-
UBP27 (155) --------PFDC----------ILGSTLMCRTCSSQ---ISL-
UBP22 (127) --------AFSC----------LLRSDVTCTTCGS----TST-
UBP20 (121) --------VFSC----------RLISGLRCCNCDY----VSE-
UBP21 (127) --------VFGC----------GLMSTLCCCNCNS----VSN-
UBP23 (125) --------IFGC----------SLRSQVKCEQCSH----CSN-
UBP25 (133) --------IFGC----------ALQSQVKCLSCGA----ESN-
UBP15 (161) --------MFGC----------RLRSKVKCLRCDH----ESE-
UBP16 (164) --------TFGC----------YLRSKIKCMKCQV----KSE-
UBP17 (161) --------TFGC----------YLHSKIKCMACLH----KSE-
UBP18 (164) --------IFGC----------LLQSQVQCTVCNH----VSD-
UBP19 (164) --------IFGC----------LLQSQVQCTACSN----VSD-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550
UBP9 (359) --TFDPFMYLSVPLPSTLTRSMTITVFYCDGSRLPMPYTVIVPKQGSIRD
UBP10 (359) --TFDPFMYLSVPLPSTLTRSMTVTVFYCDGSHLPMPYTVIVPKNGSIRD
UBP11 (364) --TFDPFMYLSLPLPSSRTRSMTVTVFYGDGSHLPMPYTVTVPKDGSCRD
UBP5 (381) --TFDPFMYLSLPLQFNTTRAITVTVFSCDKTALPSTITVNVSKQGRCRD
UBP8 (167) --MFDPFMYLSLPLPCTSMRTMDLTVMSADGSSLPIPLTVNVPKFGKFED
UBP24 (76) --PFRPAMFEGV------LRNFTPDVLN----------
UBP3 (172) --RDETFLDLSLDIE----------
UBP4 (167) --RDETFLDLSLDIE----------
UBP12 (258) --RKESFYDLQLDVKG----------
UBP13 (258) --RKESFYDLQLDVKG----------
UBP26 (131) --KMEDFYALELNVK----------
UBP14 (250) KDELEAFHKQKAGKGLEEND----------
UBP6 (215) --ETESVYSLKCHISHEVNH----------
UBP7 (215) --ETESVFSLKCHISHEVNH----------
UBP1 (308) --VNQILVGSTETL----MHDNDRTGKTVPDKEDVRATQSNEETSASGIS
UBP2 (294) --DNGPVLETESVL----TLDNNQASES--------ASQSDTGFDGS-WL
UBP27 (176) --EFQFFHTLPLSP----LLHH----------GG---
UBP22 (147) --TYDPFIDISLT----------
UBP20 (141) --TYEKSVGLSLEI----ED----------
UBP21 (147) --TFEPSLGWSLEI----ED----------
UBP23 (145) --KFDPFLDLSLDI----S----------
UBP25 (153) --KADEIMDISLEI----L----------
UBP15 (181) --RYENIMDLTLEI----YG----------
UBP16 (184) --LREKMMDLTVEI----DG----------
UBP17 (181) --RPELMMDLTVEI----DG----------
UBP18 (184) --QYENMMDLIVEM----HG----------
UBP19 (184) --QYENMMDLTVEI----HG----------
551
600
UBP9 (407) LITALGTAC----------CLAEDE----------
UBP10 (407) LITALGTAC----------LLAEDE----------
UBP11 (412) LSNALGTAC----------CLDNDE----------
UBP5 (429) LIQALTNAC----------SLKQSE----------
UBP8 (215) LHKALVTAC----------SLPEEE----------
UBP24 (96) ----------
UBP3 (185) ----------
UBP4 (180) ----------
UBP12 (272) ----------
UBP13 (272) ----------
UBP26 (144) ----------
UBP14 (270) ----------
UBP6 (233) ----------
UBP7 (233) ----------
UBP1 (352) AVIDEAQVCGCPDLEQSSSSANQGADEELALMVADSQVLYMPYKDHLFYD
UBP2 (329) DFIGPETSGDETNLDMQEDGIDNVITAEVNQIVPSPNIV----------
UBP27 (194) ----------
UBP22 (158) ----------
UBP20 (155) ----------
UBP21 (161) ----------
UBP23 (158) ----------
UBP25 (166) ----------
UBP15 (195) ----------
UBP16 (198) ----------
UBP17 (195) ----------
UBP18 (198) ----------
UBP19 (198) ----------

```
                    601                                       650
 UBP9   (422) -----------S----------------------------LLL-AEVYD
UBP10   (422) -----------S----------------------------LLL-AEVYD
UBP11   (427) -----------S----------------------------LLL-AEVYD
 UBP5   (444) -----------E----------------------------LKL-AEIRN
 UBP8   (230) -----------T----------------------------LLV-TEVYN
UBP24    (96) ---------------------------------------------NMSG
 UBP3   (185) --------------------------------------------------
 UBP4   (180) --------------------------------------------------
UBP12   (272) --------------------------------------------------
UBP13   (272) --------------------------------------------------
UBP26   (144) --------------------------------------------------
UBP14   (270) --------------------------------------------------
 UBP6   (233) --------------------------------------------------
 UBP7   (233) --------------------------------------------------
 UBP1   (402) DYMVAEASSSFVSGDHEPKKDYFDFSSFLDEPEIREGPVFRPLSKSEVYE
 UBP2   (368) ------ANSSVSSGDQ-----------------TLEGNTERLMQD---YE
UBP27   (194) --------------------------------------------------
UBP22   (158) --------------------------------------------------
UBP20   (155) --------------------------------------------------
UBP21   (161) --------------------------------------------------
UBP23   (158) --------------------------------------------------
UBP25   (166) --------------------------------------------------
UBP15   (195) --------------------------------------------------
UBP16   (198) --------------------------------------------------
UBP17   (195) --------------------------------------------------
UBP18   (198) --------------------------------------------------
UBP19   (198) --------------------------------------------------

                    651                                       700
 UBP9   (431) HKIFRYFEIPLDSLSAIKDDEHIVAYRLNQIPKGSRKAKLEILHGGQERA
UBP10   (431) HKIFKYFENPLDSLSSIKDDEHIVAYRLNQMPKGSGKAKLEILHGGQKRP
UBP11   (436) HKVFKYYENPRELLNGIKDNEHIVAYRFKQMHKGPGKVKLEILHGEQEK-
 UBP5   (453) NFIHRLFEDPLIPLSSIKDDDHLAAYKLSKSSENTTLLRLVLRRRDQKAG
 UBP8   (239) NRIIRFLEEPTDSLTLIRDGDKLVVYRLKKDANNS--PLIVYMHQKLEEQ
UBP24   (100) RPR---QEDAQEFLSFIMDQMHDELLKLKE--------------------
 UBP3   (185) --------------------------------------------------
 UBP4   (180) --------------------------------------------------
UBP12   (272) --------------------------------------------------
UBP13   (272) --------------------------------------------------
UBP26   (144) --------------------------------------------------
UBP14   (270) --------------------------------------------------
 UBP6   (233) --------------------------------------------------
 UBP7   (233) --------------------------------------------------
 UBP1   (452) AGFKADCS-DDKTVSAGKGEASSSFISSDHEQNIDYVDFSSFFDEPEISE
 UBP2   (392) EIAKAEANLDEKDVQAMQSDEC---------------------------P
UBP27   (194) -------------YNIMSG--C----------------------------
UBP22   (158) -------------LDSMNG--F----------------------------
UBP20   (155) --------------------------------------------------
UBP21   (161) --------------------------------------------------
UBP23   (158) --------------------------------------------------
UBP25   (166) --------------------------------------------------
UBP15   (195) --------------------------------------------------
UBP16   (198) --------------------------------------------------
UBP17   (195) --------------------------------------------------
UBP18   (198) --------------------------------------------------
UBP19   (198) --------------------------------------------------
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                   701                                                750
 UBP9   (481) VLDSVRGSDVKLFGTPFVTYVNT-EPLSGTDIDAVISGFLSPLHK-----
UBP10   (481) ILESVRGRDVKLFGTPFVTYVNT-EPLSGADIDAVLSRFLSPLHK-----
UBP11   (485) --SSDRGP--KCFGTPLVTYINK-EPLSGTDIATSISGLLSPLRR-----
 UBP5   (503) --ERESTVQLKPCGTPLLSSASCGDALTKGKIHCLVQNMLSPFRREE---
 UBP8   (287) FISGKSSPTWKAFGIPLVSRLC--DVENGSDVENLYLKLLSSFKMP----
UBP24   (127) --QS-----------PKVTASK----------------------------
 UBP3   (185) ------------------------------------------------
 UBP4   (180) ------------------------------------------------
UBP12   (272) ------------------------------------------------
UBP13   (272) ------------------------------------------------
UBP26   (144) ------------------------------------------------
UBP14   (270) ------------------------------------------------
 UBP6   (233) ------------------------------------------------
 UBP7   (233) ------------------------------------------------
 UBP1   (501) RPFFRPLSKSEVSEAGFKADCSDDKTVSAGKGEASSSFVSSDHEQNIDYV
 UBP2   (415) -------ATSGISAEFSQASCIG---CDPGIGESSS---SVNP-------
UBP27   (201) ----------------TLEHCL----------------------------
UBP22   (165) ----------------SPADCR----------------------------
UBP20   (155) ------------------------------------------------
UBP21   (161) ------------------------------------------------
UBP23   (158) ------------------------------------------------
UBP25   (166) ------------------------------------------------
UBP15   (195) ------------------------------------------------
UBP16   (198) ------------------------------------------------
UBP17   (195) ------------------------------------------------
UBP18   (198) ------------------------------------------------
UBP19   (198) ------------------------------------------------

                   751                                                800
 UBP9   (525) -----VHAPSKIHNGSDNGHLADAT-VDQASGILSSPDTEIDNASD---R
UBP10   (525) -----VHAPSKIHNGSENGHLPDAT-VDEASEILSSPDTEIDDASD---R
UBP11   (525) -----VHMSCVVNSGNENGHVP-----DESSRSILSRDTETEDN-D---R
 UBP5   (548) ----SVGKKGNSDSSIPERRSARFNNTEEEDKVGGLKKAKKSNSSDLGAS
 UBP8   (331) ----TEFFTENLENPTEEEATDKTD-TDGTTSVEDTNSTDVKETTES-LP
UBP24   (136) --------SSVISSANDDG--------DEWETVGPKNKSAVT--------
 UBP3   (185) ------------------------------------------------
 UBP4   (180) ------------------------------------------------
UBP12   (272) ------------------------------------------------
UBP13   (272) ------------------------------------------------
UBP26   (144) ------------------------------------------------
UBP14   (270) ------------------------------------------------
 UBP6   (233) ------------------------------------------------
 UBP7   (233) ------------------------------------------------
 UBP1   (551) DFSSFFDEPEISERPFFRPLSKSEVSEAGFMAVSGNDKTVRAGKGET---
 UBP2   (445) -----WDEEELP-----LVVADSQILYMPYKEISCNDKSVEG-ECEA---
UBP27   (207) ------------------------------------K--KF--------
UBP22   (171) ------------------------------------K--NR--------
UBP20   (155) ------------------------------------------------
UBP21   (161) ------------------------------------------------
UBP23   (158) ------------------------------------K-------------
UBP25   (166) ------------------------------------Q-------------
UBP15   (195) ------------------------------------W-------------
UBP16   (198) ------------------------------------D-------------
UBP17   (195) ------------------------------------D-------------
UBP18   (198) ------------------------------------D-------------
UBP19   (198) ------------------------------------D-------------
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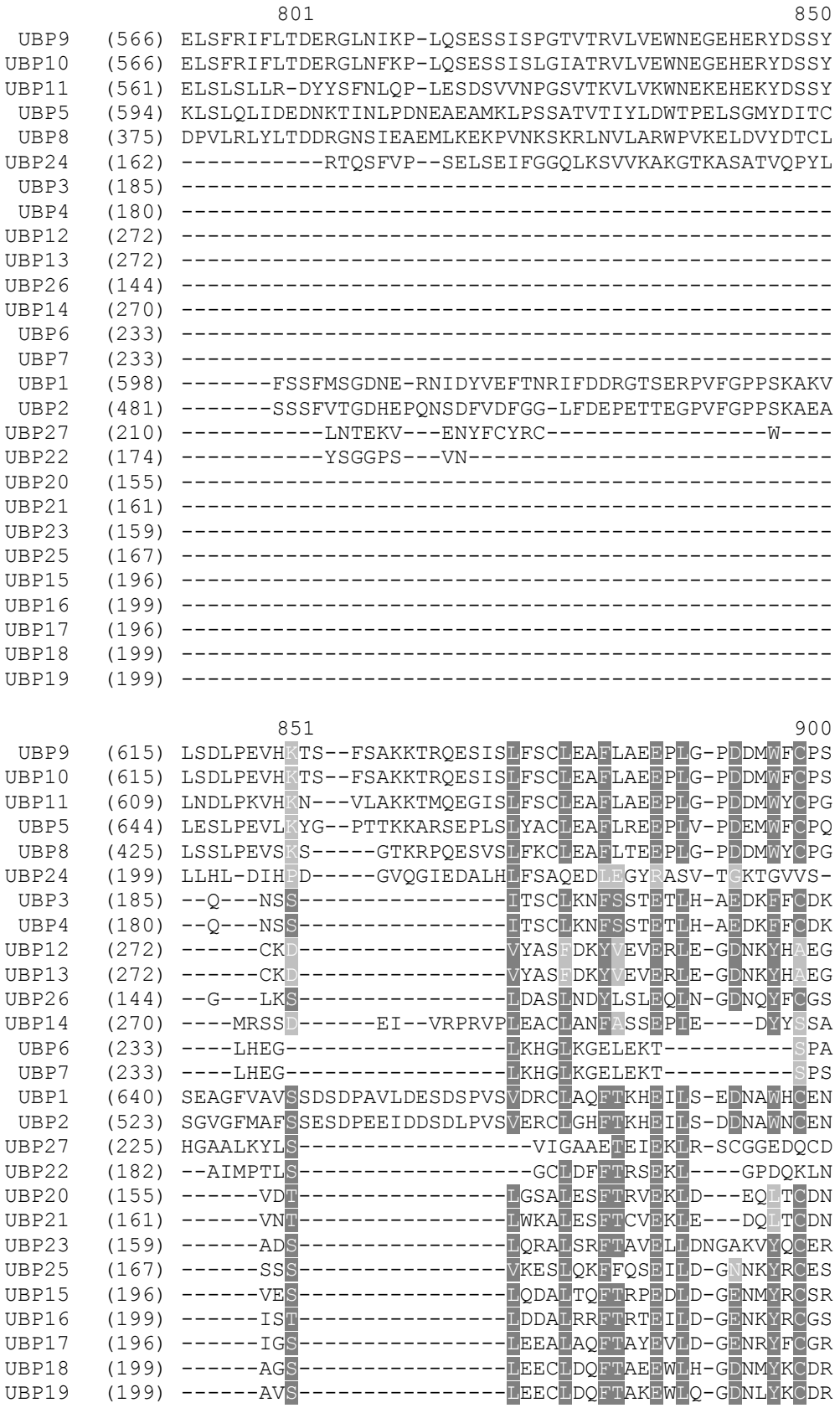
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UBP11 (561) ELSLSLLR-DYYSFNLQP-LESDSVVNPGSVTKVLVKWNEKEHEKYDSSY
UBP5 (594) KLSLQLIDEDNKTINLPDNEAEAMKLPSSATVTIYLDWTPELSGMYDITC
UBP8 (375) DPVLRLYLTDDRGNSIEAEMLKEKPVNKSKRLNVLARWPVKELDVYDTCL
UBP24 (162) ----------RTQSFVP--SELSEIFGGQLKSVVKAKGTKASATVQPYL
UBP3 (185) ----------
UBP4 (180) ----------
UBP12 (272) ----------
UBP13 (272) ----------
UBP26 (144) ----------
UBP14 (270) ----------
UBP6 (233) ----------
UBP7 (233) ----------
UBP1 (598) -------FSSFMSGDNE-RNIDYVEFTNRIFDDRGTSERPVFGPPSKAKV
UBP2 (481) -------SSSFVTGDHEPQNSDFVDFGG-LFDEPETTEGPVFGPPSKAEA
UBP27 (210) ----------LNTEKV---ENYFCYRC----------W----
UBP22 (174) ----------YSGGPS---VN----------
UBP20 (155) ----------
UBP21 (161) ----------
UBP23 (159) ----------
UBP25 (167) ----------
UBP15 (196) ----------
UBP16 (199) ----------
UBP17 (196) ----------
UBP18 (199) ----------
UBP19 (199) ----------
851 900
UBP9 (615) LSDLPEVHKTS--FSAKKTRQESISLFSCLEAFLAEEPLG-PDDMWFCPS
UBP10 (615) LSDLPEVHKTS--FSAKKTRQESISLFSCLEAFLAEEPLG-PDDMWFCPS
UBP11 (609) LNDLPKVHKN---VLAKKTMQEGISLFSCLEAFLAEEPLG-PDDMWYCPG
UBP5 (644) LESLPEVLKYG--PTTKKARSEPLSLYACLEAFLREEPLV-PDEMWFCPQ
UBP8 (425) LSSLPEVSKS-----GTKRPQESVSLFKCLEAFLTEEPLG-PDDMWYCPG
UBP24 (199) LLHL-DIHPD-----GVQGIEDALHLFSAQEDLEGYRASV-TGKTGVVS-
UBP3 (185) --Q---NSS----------ITSCLKNFSSTETLH-AEDKFFCDK
UBP4 (180) --Q---NSS----------ITSCLKNFSSTETLH-AEDKFFCDK
UBP12 (272) ------CKD----------VYASHDKYVEVERLE-GDNKYHAEG
UBP13 (272) ------CKD----------VYASHDKYVEVERLE-GDNKYHAEG
UBP26 (144) --G---LKS----------LDASLNDYLSLEQLN-GDNQYFCGS
UBP14 (270) ----MRSSD------EI--VRPRVPLEACLANFASSEPLE----DYYSSA
UBP6 (233) ----LHEG----------LKHGLKGELEKT----------SPA
UBP7 (233) ----LHEG----------LKHGLKGELEKT----------SPS
UBP1 (640) SEAGFVAVSSDSDPAVLDESDSPVSVDRCLAQFTKHEILS-EDNAWHCEN
UBP2 (523) SGVGFMAFSSESDPEEIDDSDLPVSVERCLGHFTKHEILS-DDNAWNCEN
UBP27 (225) HGAALKYLS----------VIGAAETEIEKLR-SCGGEDQCD
UBP22 (182) --AIMPTLS----------GCLDFFTRSEKL----GPDQKLN
UBP20 (155) ------VDT----------LGSALESFTRVEKLD---EQLTCDN
UBP21 (161) ------VNT----------LWKALESFTCVEKLE---DQLTCDN
UBP23 (159) ------ADS----------LQRALSRFTAVELLDNGAKVYQCER
UBP25 (167) ------SSS----------VKESLQKFFQSEILD-GNNKYRCES
UBP15 (196) ------VES----------LQDALTQFTRPEDLD-GENMYRCSR
UBP16 (199) ------IST----------LDDALRRFTRTEILD-GENKYRCGS
UBP17 (196) ------IGS----------LEEALAQFTAYEVLD-GENRYFCGR
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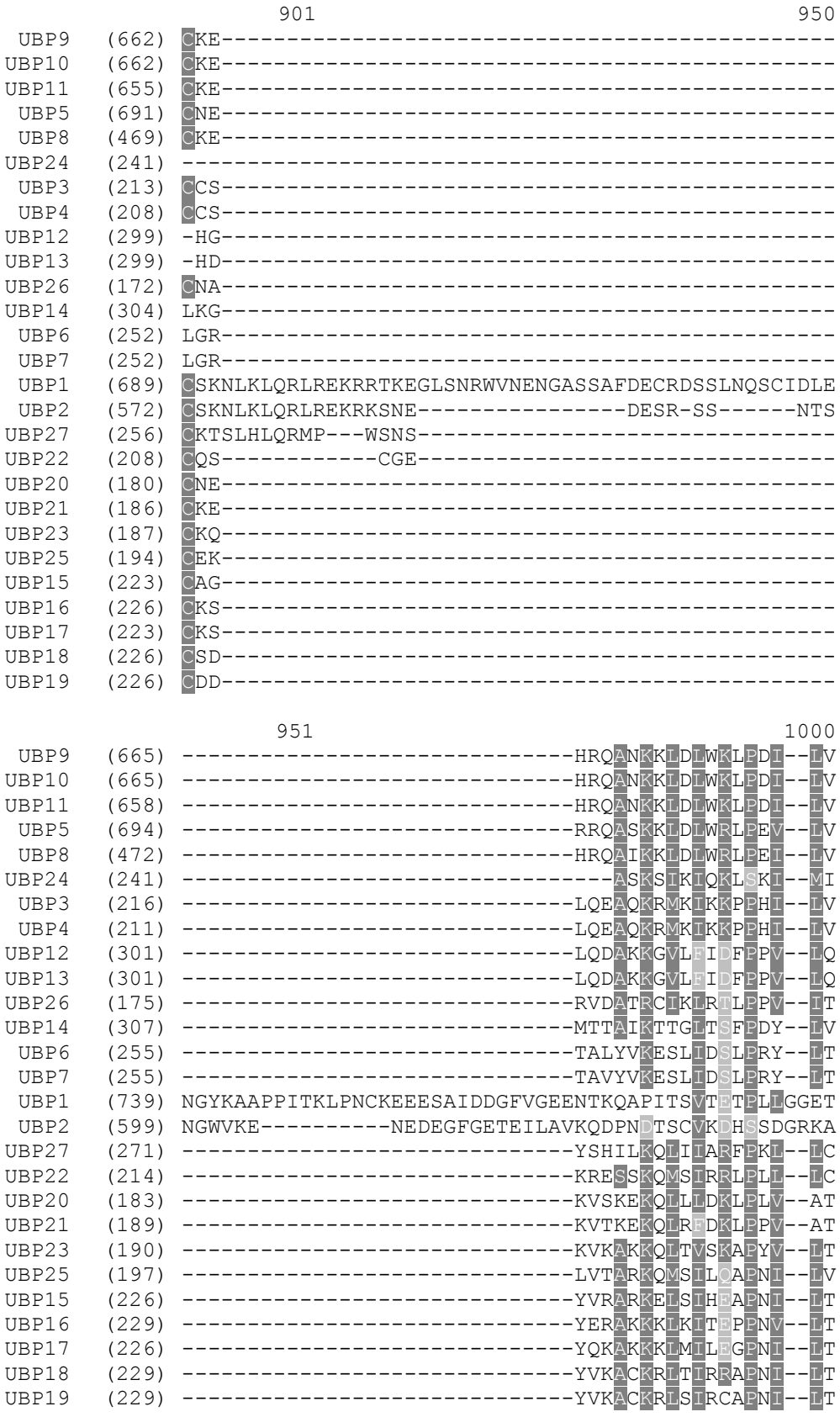
UBP19 (199) ------AVS----------LEECLDQFTAKEWLQ-GDNLYKCDR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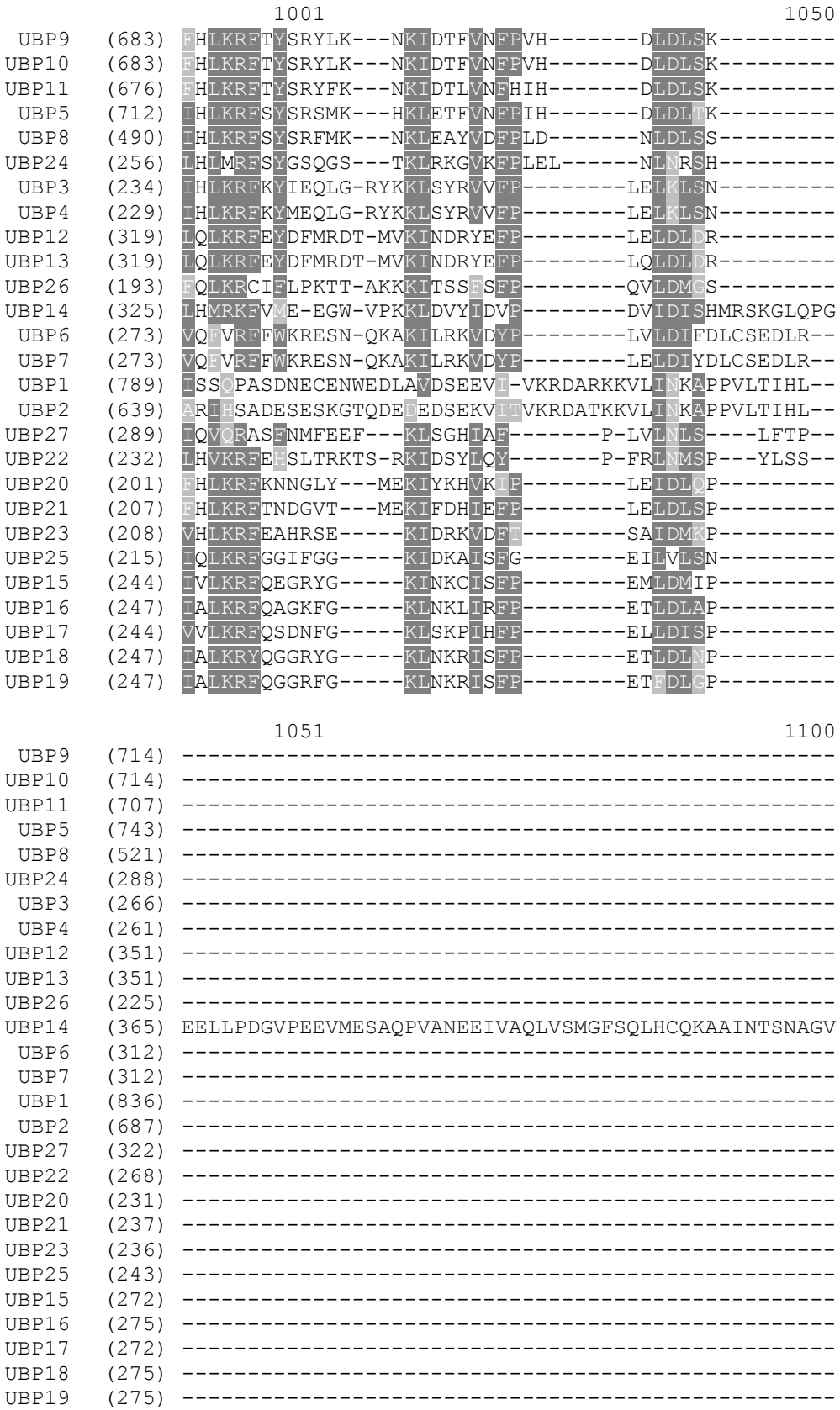
UBP9 (662) CKE
UBP10 (662) CKE
UBP11 (655) CKE
UBP5 (691) CNE
UBP8 (469) CKE
UBP24 (241)
UBP3 (213) CCS
UBP4 (208) CCS
UBP12 (299) -HG
UBP13 (299) -HD
UBP26 (172) CNA
UBP14 (304) LKG
UBP6 (252) LGR
UBP7 (252) LGR
UBP1 (689) CSKNLKLQRLREKRRTKEGLSNRWVNENGASSAFDECRDSSLNQSCIDLE
UBP2 (572) CSKNLKLQRLREKRKSNE----------DESR-SS------NTS
UBP27 (256) CKTSLHLQRMP---WSNS
UBP22 (208) CQS----------CGE
UBP20 (180) CNE
UBP21 (186) CKE
UBP23 (187) CKQ
UBP25 (194) CEK
UBP15 (223) CAG
UBP16 (226) CKS
UBP17 (223) CKS
UBP18 (226) CSD
UBP19 (226) CDD
951 1000
UBP9 (665) HRQANKKLDLWKLPDI--LV
UBP10 (665) HRQANKKLDLWKLPDI--LV
UBP11 (658) HRQANKKLDLWKLPDI--LV
UBP5 (694) RRQASKKLDLWRLPEV--LV
UBP8 (472) HRQAIKKLDLWRLPEI--LV
UBP24 (241) ASKSIKIQKLSKI--MI
UBP3 (216) LQEAQKRMKIKKPPHI--LV
UBP4 (211) LQEAQKRMKIKKPPHI--LV
UBP12 (301) LQDAKKGVLFIDFPPV--LQ
UBP13 (301) LQDAKKGVLFIDFPPV--LQ
UBP26 (175) RVDATRCIKLRILPPV--IT
UBP14 (307) MTTAIKTTGLTSFPDY--LV
UBP6 (255) TALYVKESLIDSLPRY--LT
UBP7 (255) TAVYVKESLIDSLPRY--LT
UBP1 (739) NGYKAAPPITKLPNCKEEESAIDDGFVGEENTKQAPITSVTETPLLGGET
UBP2 (599) NGWVKE----------NEDEGFGETEILAVKQDPNDTSCVKDHSSDGRKA
UBP27 (271) YSHILKQLIIARFPKL--LC
UBP22 (214) KRESSKQMSIRRLPLL--LC
UBP20 (183) KVSKEKQLLLDKLPLV--AT
UBP21 (189) KVTKEKQLRGDKLPPV--AT
UBP23 (190) KVKAKKQLTVSKAPYV--LT
UBP25 (197) LVTARKQMSILQAPNI--LV
UBP15 (226) YVRARKELSIHEAPNI--LT
UBP16 (229) YERAKKKLKITEPPNV--LT
UBP17 (226) YQKAKKKLMILEGPNI--LT
UBP18 (229) YVKACKRLTIRRAPNI--LT
UBP19 (229) YVKACKRLSIRCAPNI--LT


1001
1050
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UBP10 (683) FHLKRFTYSRYLK---NKIDTFVNFPVH-------DLDLSK---------
UBP11 (676) FHLKRFTYSRYFK---NKIDTLVNFHIH-------DLDLSK---------
UBP5 (712) IHLKRFSYSRSMK---HKLETFVNFPIH-------DLDLTK---------
UBP8 (490) IHLKRFSYSRFMK---NKLEAYVDFPLD-------NLDLSS---------
UBP24 (256) LHLMRFSYGSQGS---TKLRKGVKFPLEL------NLNRSH---------
UBP3 (234) IHLKRFKYIEQLG-RYKKLSYRVVFP--------LELKLSN---------
UBP4 (229) IHLKRFKYMEQLG-RYKKLSYRVVFP--------LELKLSN---------
UBP12 (319) LQLKRFEYDFMRDT-MVKINDRYEFP--------LELDLDR---------
UBP13 (319) LQLKRFEYDFMRDT-MVKINDRYEFP--------LQLDLDR---------
UBP26 (193) FQLKRCIFLPKTT-AKKKITSSFSFP--------QVLDMGS---------
UBP14 (325) LHMRKFVME-EGW-VPKKLDVYIDVP--------DVIDISHMRSKGLQPG
UBP6 (273) VQLVRFFWKRESN-QKAKILRKVDYP--------LVLDIFDLCSEDLR--
UBP7 (273) VQLVRFFWKRESN-QKAKILRKVDYP--------LELDIYDLCSEDLR--
UBP1 (789) ISSQPASDNECENWEDLAVDSEEVI-VKRDARKKVLINKAPPVLTIHL--
UBP2 (639) ARIHSADESESKGTQDEDEDSEKVITVKRDATKKVLINKAPPVLTIHL--
UBP27 (289) IQVQRASFNMFEEF---KLSGHIAF-------P-LVLNLS----LFTP--
UBP22 (232) LHVKRFEHSLTRKTS-RKIDSYLQY-------P-FRLNMSP---YLSS--
UBP20 (201) FHLKRFKNNGLY---MEKIYKHVKIP--------LEIDLQP---------
UBP21 (207) FHLKRFTNDGVT---MEKIFDHIEFP--------LELDLSP---------
UBP23 (208) VHLKRFEAHRSE-----KIDRKVDFT--------SAIDMKP---------
UBP25 (215) IQLKRFGGIFGG-----KIDKAISFG--------EILVLSN---------
UBP15 (244) IVLKRFQEGRYG-----KINKCISFP--------EMLDMIP---------
UBP16 (247) IALKRFQAGKFG-----KLNKLIRFP--------ETLDLAP---------
UBP17 (244) VVLKRFQSDNFG-----KLSKPIHFP--------ELLDISP---------
UBP18 (247) IALKRYQGGRYG-----KLNKRISFP--------ETLDLNP---------
UBP19 (247) IALKRFQGGRFG-----KLNKRISFP--------ETFDLGP---------
1051
1100
UBP9 (714) ----------
UBP10 (714) ----------
UBP11 (707) ----------
UBP5 (743) ----------
UBP8 (521) ----------
UBP24 (288) ----------
UBP3 (266) ----------
UBP4 (261) ----------
UBP12 (351) ----------
UBP13 (351) ----------
UBP26 (225) ----------
UBP14 (365) EELLPDGVPEEVMESAQPVANEEIVAQLVSMGFSQLHCQKAAINTSNAGV
UBP6 (312) ----------
UBP7 (312) ----------
UBP1 (836) ----------
UBP2 (687) ----------
UBP27 (322) ----------
UBP22 (268) ----------
UBP20 (231) ----------
UBP21 (237) ----------
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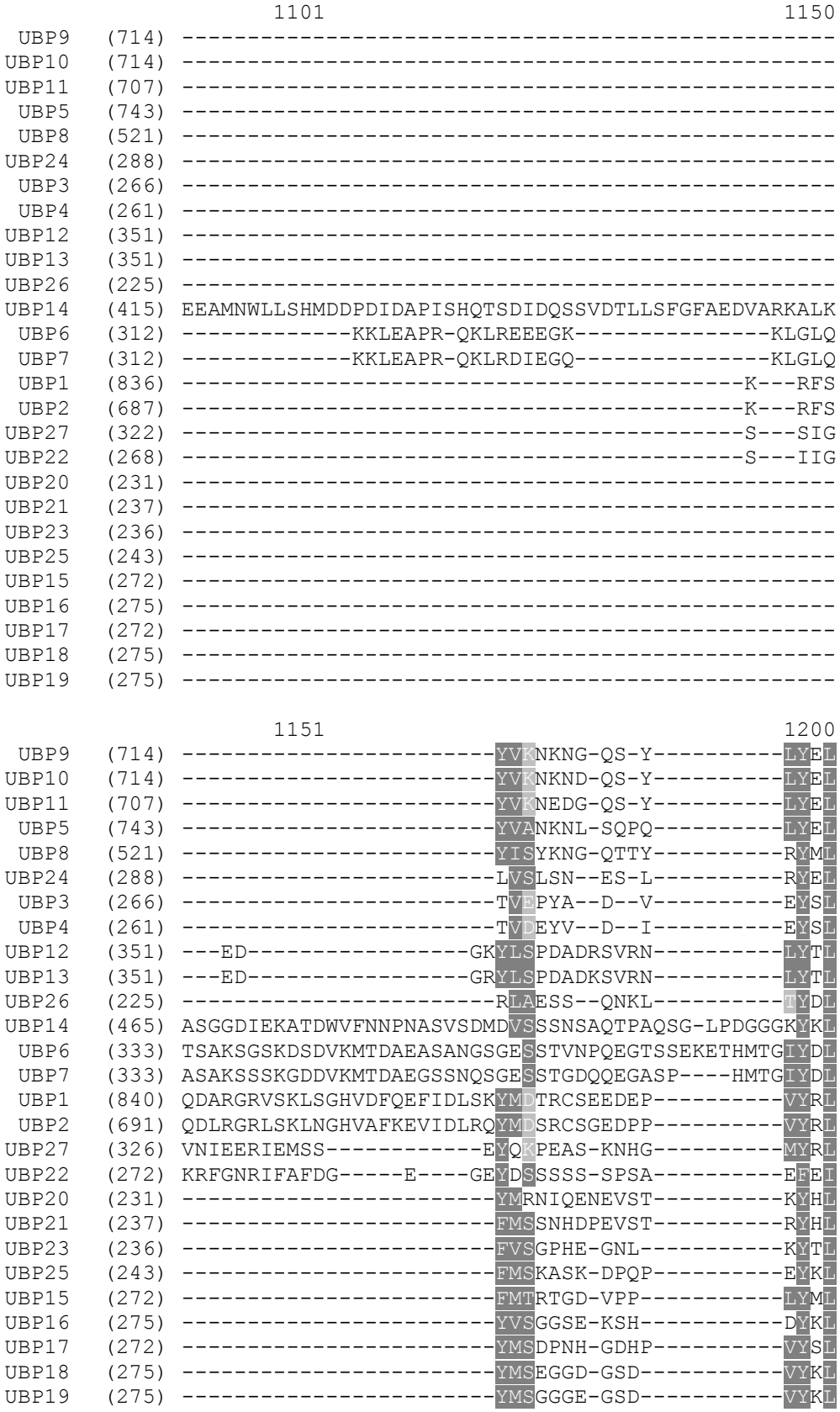
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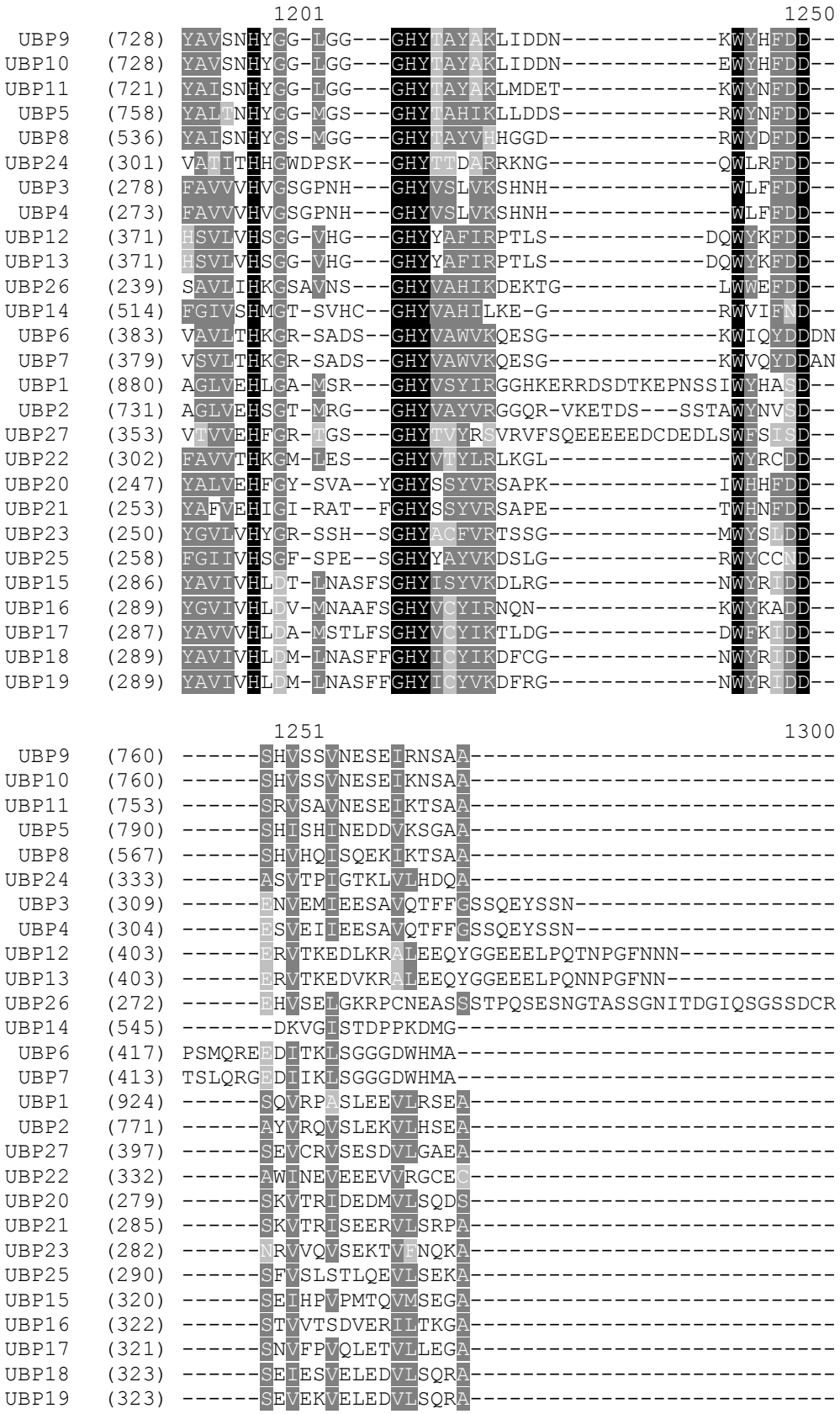
UBP8 (521)
UBP24 (288)
UBP3 (266)
UBP4 (261)
UBP12 (351)
UBP13 (351)
UBP26 (225)
UBP14 (415) EEAMNWLLSHMDDPDIDAPISHQTSDIDQSSVDTLLSFGFAEDVARKALK
UBP6 (312) KKLEAPR-QKLREEEGK KLGLQ
UBP7 (312) KKLEAPR-QKLRDIEGQ KLGLQ
UBP1 (836) K---RFS
UBP2 (687) K---RFS
UBP27 (322) S---SIG
UBP22 (268) S---IIG
UBP20 (231)
UBP21 (237)
UBP23 (236)
UBP25 (243)
UBP15 (272)
UBP16 (275)
UBP17 (272)
UBP18 (275)
UBP19 (275)
1151
1200
UBP9 (714) YVKNKNG-QS-Y LYEL
UBP10 (714) YVKNKND-QS-Y LYEL
UBP11 (707) YVKNEDG-QS-Y LYEL
UBP5 (743) YVANKNL-SQPQ LYEL
UBP8 (521) YISYKNG-QTTY RYML
UBP24 (288) LVSLSN--ES-L RYEL
UBP3 (266) TVEPYA--D--V EYSL
UBP4 (261) TVDEYV--D--I EYSL
UBP12 (351) ED GKYLSPDADRSVRN LYTL
UBP13 (351) ED GRYLSPDADKSVRN LYTL
UBP26 (225) RLAESS--QNKL TYDL
UBP14 (465) ASGGDIEKATDWVFNNPNASVSDMDVSSSNSAQTPAQSG-LPDGGGKYKL
UBP6 (333) TSAKSGSKDSDVKMTDAEASANGSGESSTVNPQEGTSSEKETHMTGIYDL
UBP7 (333) ASAKSSSKGDDVKMTDAEGSSNQSGESSTGDQQEGASP----HMTGIYDL
UBP1 (840) QDARGRVSKLSGHVDFQEFIDLSKYMDTRCSEEDEP VYRL
UBP2 (691) QDLRGRLSKLNGHVAFKEVIDLRQYMDSRCSGEDPP VYRL
UBP27 (326) VNIEERIEMSS EYQKPEAS-KNHG MYRL
UBP22 (272) KRFGNRIFAFDG E GEYDSSSSS-SPSA EFEI
UBP20 (231) YMRNIQENEVST KYHL
UBP21 (237) FMSSNHDPEVST RYHL
UBP23 (236) FVSGPHE-GNL KYTL
UBP25 (243) FMSKASK-DPQP EYKL
UBP15 (272) FMTRTGD-VPP LYML
UBP16 (275) YVSGGSE-KSH DYKL
UBP17 (272) YMSDPNH-GDHP VYSL
UBP18 (275) YMSEGGD-GSD VYKL
UBP19 (275) YMSGGGE-GSD VYKL


1201
1250
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UBP10 (728) YAVSNHYGG-LGG---GHYTAYAKLIDDN----------EWYHFDD--
UBP11 (721) YAISNHYGG-LGG---GHYTAYAKLMDET----------KWYNFDD--
UBP5 (758) YALTNHYGG-MGS---GHYTAHIKLLDDS----------RWYNFDD--
UBP8 (536) YAISNHYGS-MGG---GHYTAYVHHGGD----------RWYDFDD--
UBP24 (301) VATITHHGWDPSK---GHYTTDARRKNG----------QWLRFDD--
UBP3 (278) FAVVVHVGSGPNH---GHYVSLVKSHNH----------WLFFDD--
UBP4 (273) FAVVVHVGSGPNH---GHYVSLVKSHNH----------WLFFDD--
UBP12 (371) HSVLVHSGG-VHG---GHYYAFIRPTLS----------DQWYKFDD--
UBP13 (371) HSVLVHSGG-VHG---GHYYAFIRPTLS----------DQWYKFDD--
UBP26 (239) SAVLIHKGSAVNS---GHYVAHIKDEKTG----------LWWEFDD--
UBP14 (514) FGIVSHMGT-SVHC--GHYVAHILKE-G----------RWVIFND--
UBP6 (383) VAVLTHKGR-SADS--GHYVAWVKQESG----------KWIQYDDDN
UBP7 (379) VSVLTHKGR-SADS--GHYVAWVKQESG----------KWVQYDDAN
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UBP26 (272) ------EHVSEIGKRPCNEASSTPQSESNGTASSGNITDGIQSGSSDCR
UBP14 (545) -------DKVGISTDPPKDMG----------
UBP6 (417) PSMQREEDITKLSGGGDWHMA----------
UBP7 (413) TSLQRGEDIIKLSGGGDWHMA----------
UBP1 (924) ------SQVRPASLEEVLRSEA----------
UBP2 (771) ------AYVRQVSLEKVLHSEA----------
UBP27 (397) ------SEVCRVSESDVLGAEA----------
UBP22 (332) ------AWINEVEEEVVRGCEC----------
UBP20 (279) ------SKVTRIDEDMVLSQDS----------
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UBP25 (290) ------SFVSLSTLQEVLSEKA----------
UBP15 (320) ------SEIHPVPMTQVMSEGA----------
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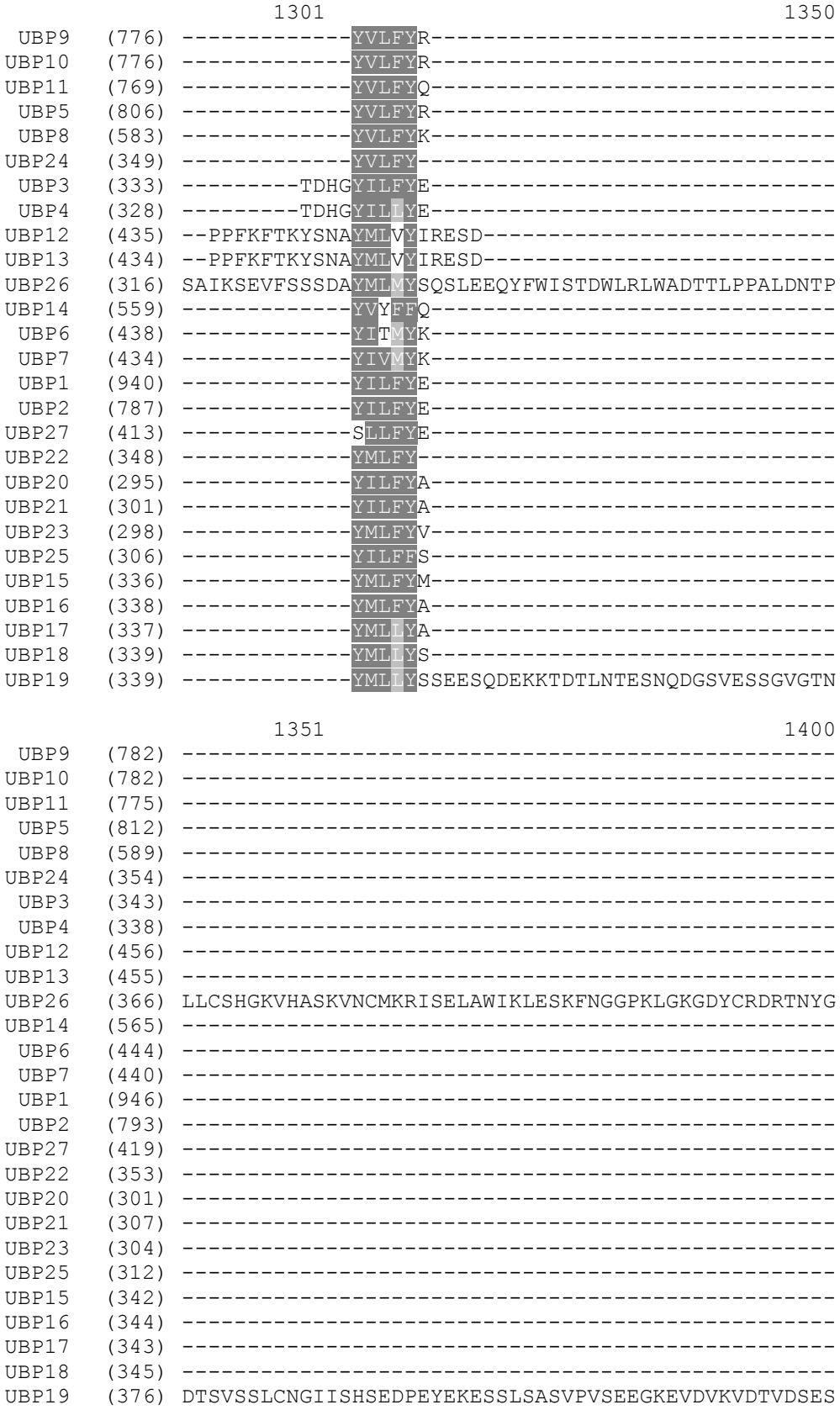
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SUPPLEMENTARY MATERIAL

Functional characterization of the Arabidopsis ubiquitin-specific protease gene family reveals specific role and redundancy of individual members in development

Liu *et al.*

Full legends of Supplementary Figures

Figure S1. Alignment of 27 UBP proteins.

Identicalt amino acids are shaded in black while conserved ones are shaded in gray. Numbers above the alignment indicate the amino acid position in the consensus sequence.

Figure S2. Phylogenetic analysis of 27 *UBP*s gene family of *Arabidopsis*.

The phylogenetic tree of 27 UBPs. The 0.1 scale represents 10% change. Bootstrap values are shown in percentages at nodes. The 27 proteins can be subdivided into 14 subfamilies shown in distinct colors. Abbreviations: aa, amino acids; UBP, ubiquitin specific protease; ZnF, zinc finger; MYND, myeloid, Nervy, and DEAF-1; DUSP, domain in ubiquitin-specific proteases; UBQ, ubiquitin homologues; MATH, meprin and TRAF homology; UBA, ubiquitin-associated.

Figure S3. Gene expression pattern of *UBP15* subfamily.

Organs from 1 to 18 are cauline leaves, rosette leaves, and pistil one day before pollination, pistil one day after pollination, silique three days after pollination, silique eight days after pollination, stem, sepal, stamen, petal, seed, cultured cell, root dark, root white light, hypocotyl dark, hypocotyl white light, cotyledon dark and cotyledon white light.

Figure S4. Phylogenetic analysis of AtUBP15 subfamily and its homologues in rice (*Oryza sativa*).

The phylogenetic tree of 9 UBPs in *Arabidopsis* or rice. Bootstrap values are shown in percentages at nodes.

Figure S5. Two month old lines of wild type, two *ubp15* mutants and *UBP15*-overexpression line.

Two-month-old wild type, *ubp15-1*, *ubp15-2*, *UBP15*-overexpression line and rescued line. Mutants are weak, with early flowering time and more secondary stems while *UBP15*-overexpression line showed opposite phenotype with late flower and strong apical dominance. Bar = 1 cm.

Figure S6. Comparison of palisade cell number in transverse sections across the lamina of rosette leaf. Error bars represent standard deviation of three biological repeats.

Figure S7. Real-time PCR confirms the microarray results.

Seven down-regulated genes and two up-regulated genes are randomly picked for real-time PCR analysis which confirms microarray result. At1g42970/*GAPDH* served as an internal control.

Figure S8. Comparison of the ninth rosette leaf of wild type and mutants as well as transgenic lines.

(a) Mature ninth rosette leaf of wild type, *ubp15-1*, *ubp15-2*, *UBP15*-overexpression line and *ubp15 ubp16*. Bar=1 cm.

(b) Comparison of adaxial epidermal cell numbers of transverse sections in three regions of the ninth rosette leaf.

Figure S9. Characterization of the *UBP18* and *UBP19* genes.

(a) Gene structure of *UBP18* (upper panel) and *UBP19* (bottom panel).

(b) *ubp19* exhibits recessive embryo lethality. The upper silique is *ubp19* heterozygote and the lower is wild type. Arrows refer to those abnormal homozygote embryos. Bar=1 mm.

(c) Microscopic examination of embryos of wild type (left) and *ubp19* (right). Development of *ubp19* stagnates at global stage. Bar=10 μm.

Figure S10. Tissue expression pattern of *UBP19*. The expression pattern of *UBP19* was determined using *pUBP19:GUS* transgenic lines.

(a) 12-day-old seedling of the *pUBP19:GUS* line. Figure in the right bottom is six-fold enlarged view of the root tip region.

(b) Mature rosette leaf of the *pUBP19:GUS* line.

(c) Inflorescence of the *pUBP19:GUS* line. Figure in the left bottom is an enlarged flower.

(d) Silique of the *pUBP19:GUS* line. All bar=1 mm.

Supplementary Tables

Table S1 Microarray data of 27 *UBP* genes expression in various organs (Submitted as a separated Excel file)

Table S2 Primers designed to identify the genotype of T-DNA insertion lines

T-DNA insertion lines	Forward (FP) and reverse (RP) primers
SALK_086190	FP: 5'-TGCGTGAAGGAATTCAGATCCA-3'
	RP: 5'-TTCAGCGTTATATCTAAAAGAATTG-3'
SALK_064103	FP: 5'-GCTTCGCTTACGTTATACCACGC-3'

	RP: 5’-CTGAAGCCTCGGGAGTTGGTT-3’
SALK_059858	FP: 5’-CGGGTCTTCTCCGCTACACCT-3’
	RP: 5’-CCTTTGGTGGTTGCAGATTCG-3’
SALK_112950	FP: 5’-TGTGCACAACACCATTTGCCT-3’
	RP: 5’-TCTCTCCCTTGTGCAGGCTCTT-3’
SALK_043210	FP: 5’-TGTGATTGGGTTTGGTTTGGG-3’
	RP: 5’-TCTCTTGACCTGCTTGGCTGA-3’
SALK_088398	FP: 5’-GTGCTGCTACTGCTGCTTCCC-3’
	RP: 5’-CAACAGCAGCTAAATCAAAAAGG -3’
SALK_108832	FP: 5’-AAAATGTGGTCCAAGTGGATGG-3’
	RP: 5’-TGAGAAGGAAACTCACATGACTGGA-3’
SALK_014223	FP: 5’-CCAATTACAGTGCGTTCCAAGC-3’
	RP: 5’-TGGCCAACTTTGTTAGATGTTTCA-3’
SALK_034744	FP: 5’-GACCAAGGGGATTCCAAATGC-3’
	RP: 5’-TTTCTGGTTGCAGGGCCAATA-3’
SALK_149329	FP: 5’-AGCGGGAAATCCACATATGCC-3’
	RP: 5’-CCTTTCCAATGGTTTTCAGGC-3’
SALK_088692	FP: 5’-CGTAAGCAGCCGAGGTCTTGA-3’
	RP: 5’-TCCAAGCGGTTGAATGTGCTT-3’
SALK_141485	FP: 5’-TGCAATGATGCTAATTGGATCAAGA -3’
	RP: 5’-TTTTATTATGCTTCTGTTCCTTTTT -3’

SALK_093503	FP: 5’-AGCATCAGGAAGGTGGCCATT -3’
	RP: 5’-TCGGTTACCATTTCCTTCCATTG -3’
SALK_043515	FP: 5’-CACTAGGAAACCAGTGCCTTCG -3’
	RP: 5’- ACACTTTGGGCCCCTGTCACT -3’
SALK_128312	FP: 5’-CCCTCCACAACAGTTCCCTTG-3’
	RP: 5’-TTGGAATGGAGTCAAGTTACCGC-3’
SALK_024054	FP: 5’-CGCACTATGAACCCCAACACC -3’
	RP: 5’-GAAAGGTTGGATGCTTGTTTTG -3’
SALK_130784	FP: 5’-GCTTTTGTTGGAACAGATGTCAA-3’
	RP: 5’-TCCTCATGTAGGAAGAGGTAGCCA-3’
SALK_132368	FP: 5’-CCAAGCTTCTCAGCCACCCTT-3’
	RP: 5’-TGTTGGCAGGCTAATGGTGAAA-3’
SALK_050151	FP: 5’-GCCGAAAAGGAGTATCGTTCCA-3’
	RP: 5’-CAAGGTAGATGCCATTGCCCA-3’
SALK_012863	FP: 5’-GGAGGCAAATTAAAAAGACAGCGA-3’
	RP: 5’-ATGCACCAATCTCCCACCAGA-3’
SALK_018601	FP: 5’-ATGGTGAACCGGAGCTTTTCC-3’
	RP: 5’-CCAGGTTAAATGCCTGAGGTGTG-3’
SALK_015611	FP: 5’-TCACACCTCAGGCATTTAACC-3’
	RP: 5’-TTGTGGAAACAGGTATTGTCTC-3’
SALK_023552	FP: 5’-TACGCAAATGAAAGACCATGA-3’

	RP: 5’- TGGGTTTGAGAAGCTGGTCGT -3’
SALK_087726	FP: 5’-GGTGAATCATATGGGTTTTGCTTT-3’
	RP: 5’- TTTGAACCAATCTCCATCAAGGG-3’
SALK_113300	FP: 5’-TGCTTCTTTATGCAAGGTGAATGA-3’
	RP: 5’-CATACTCCCTCCGTTTTCACAA-3’
SALK_009641	FP: 5’-AAAGGCAAGGGGAGGAGAATC-3’
	RP: 5’-GAAGCTCGGGAAAATGGATGG-3’
SALK_101685	FP: 5’-TGAGCATCCTCCTGTCTTCCA -3’
	RP: 5’-TTTTTCACATTGTTACCCAAAAA -3’
SALK_126252	FP: 5’- TGAGCATCCTCCTGTCTTCCA -3’
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SALK_084566	FP: 5’-TCGGCGATGGTCTCTATCGAA -3’
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SALK_121772	FP: 5’-TGTAACCTCGATCCCTCAGCATC-3’
	RP: 5’-TTGCCAAATGGGATGAGGAAA -3’
SALK_001531	FP: 5’-CCTTCCCAGTAACCGAGGCTCT-3’
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SALK_088458	FP: 5’-CGGAGAAAACCAACCAAGCAA -3’
	RP: 5’-ACAGCTATTGCCGGTGTAGCG -3’
SALK_111336	FP: 5’-TGAACGTTGCAAATTCATTCGAT -3’
	RP: 5’-CCGATGCGCCTAACAAGATTTC -3’
SALK_024392	FP: 5’-TTGTGGAAACACCCCACAAAA -3’
	RP: 5’-TTGGCTTCGTCTATGGGCTGA -3’
SALK_067020	FP: 5’-TTCTCAAAACATTCGCAGTGGC -3’
	RP: 5’-AATAGACCGTGCTGTTGGGCA -3’
SALK_027968	FP: 5’-TTTCAAATCAAATAAGCTAAAAAG -3’
	RP: 5’-TGGCTTGTCAAATTGAAATTTTTG -3’

Table S3 Trascriptome analysis of *ubp15-1* (Submitted as a separate Excel file)

W3, wild type Cy3 labeled; W5, wild type Cy5 labeled; M3, mutant Cy3 labeled; M5, mutant Cy5 labeled.

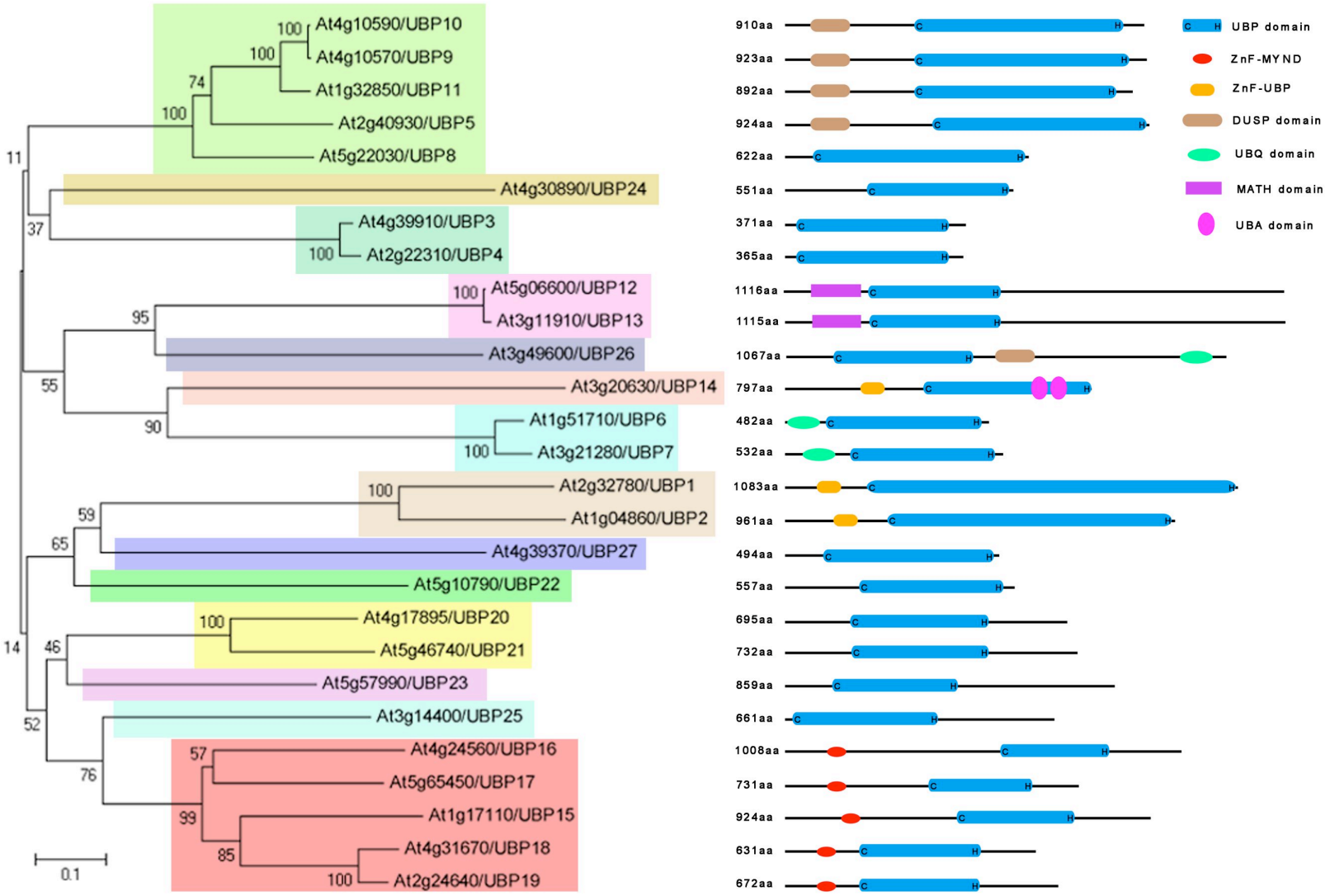
Table S4 Microarray data for selected genes with various functions

AGI Code	Fold Change	Gene Description and Putative Function	GO Category
		Proteins related to cell cycle	
At2g23430	1.72	ICK1	Negative regulation of cell division and promoter of endoreduplication.
At1g77880	0.3	Cyclin-like F box domain containing protein	
		Proteins regulating flowering	
At1g26310	1.98	Floral homeotic gene CAL	Positive regulation of flower development
At5g65080	0.31	MAF5	Negative regulation of flower development, vernalization response
		Transcription factors	
At1g74080	4.26	MYB122	Encodes a putative transcription factor
At3g07650	2.39	CONSTANS gene family	Negative regulation of long-day photoperiodism, flowering
At4g17980	2.32	No apical meristem (NAM) family protein	Transcription factor activity
At3g20810	2.05	jmjC domain-containing transcription factor	Transcription factor activity
At3g23250	2.03	MYB15	Transcription factor activity ,response to kinds of hormone
At1g21910	1.92	ERF/AP2 transcription factor	Transcription factor activity, TINY-like protein
At2g31220	1.86	Basic helix-loop-helix (bHLH) family protein	Transcription factor activity
At2g19810	0.56	Zinc finger (CCCH-type) family protein	Transcription factor activity
At5g57660	0.51	B-box type zinc finger family protein	Transcription factor activity
At1g71030	0.42	MYB family transcription factor	Transcription factor activity, response to kinds of hormone, mainly in leaves.

At4g16780	0.42	Homeobox-leucine zipper protein 4	Transcription factor activity, response to cytokinin stimulus
At3g04070	0.45	No apical meristem (NAM) family protein	Transcription factor activity
		Biosynthetic metabolism	
At5g38710	4.81	Proline oxidase	Glutamate biosynthesis, proline catabolic process, located in MT
At4g21990	3.31	APS REDUCTASE 3	Sulfate assimilation, located in chloroplast
At3g12430	3.24	3'-5' exonuclease	Nucleic binding, 3'-5' exonuclease activity
At5g06290	2.53	2-cys peroxiredoxin	Located in chloroplast, antioxidant activity
At3g24420	2.48	Hydrolase, alpha/beta fold family protein	Located in endomembrane system
At5g61440	2.04	Thioredoxin family protein	Thiol-disulfide exchange intermediate activity, located in chloroplast
At1g51760	1.96	IAA-amino acid hydrolase 3	IAA-Ala conjugate hydrolase activity
At1g76130	1.92	Alpha-amylase	Carbohydrate metabolic process,located in extracellular region
At4g19170	0.54	Putative nine-cis-epoxycarotenoid dioxygenase	Located in plastoglobule
At5g35970	0.48	DNA helicase-like	DNA-binding protein, located in chloroplast
At1g73480	0.47	Alpha/beta fold family hydrolase	Aromatic compound metabolism, located in chloroplast
At1g77760	0.47	Nitrate reductase 1 (NR1)	
At3g49160	0.43	Pyruvate kinase -like protein	Pyruvate kinase activity, involved in glycolysis
At1g77760	0.43	Nitrate reductase	Response to light stimulus, nitric oxide biosynthetic process
At5g24470	0.42	APRR5	Circadian rhythm, photomorphogenesis
At5g11330	0.41	Monooxygenase family protein	Electron transport, metabolic process, Located in endomembrane system

At1g32900	0.39	Starch synthase	Transferase activity, transferring glycosyl groups, located in chloroplast
At5g48490	0.28	Lipid transfer protein (LTP) family protein	Lipid binding and transport, located in endomembrane system
		Polysaccharide metabolism	
At5g25980	4.53	Glycosyl hydrolase family 1	Hydrolase activity, hydrolyzing O-glycosyl compounds
At1g55850	3.57	Cellulose synthase family protein	Polysaccharide biosynthetic process, cell wall biosynthetic process
At3g44990	2.52	Xyloglucan: xyloglucosyl transferase	Hydrolase activity, acting on glycosyl bonds, located in chloroplast
At2g32290	2.3	Putative 1,4-alpha-D-glucan maltohydrolase	Polysaccharide catabolic process
At3g21750	2.17	UDP-glucosyl transferase family protein	Transferring glycosyl groups
		Signal transduction	
At5g39670	3.03	Calcium-binding EF hand family protein	
At1g14320	2.52	Wilm's tumor suppressor protein-related	Involved in translation
At5g35735	2.04	Auxin-responsive family protein	Dopamine beta-monooxygenase activity, located in membrane
At1g61370	2.1	S-locus lectin protein kinase family protein	Protein amino acid phosphorylation, located in endomembrane system
At1g51760	1.96	IAA amino acid hydrolase (IAR3)	Proteolysis, located in endomembrane system
At5g67030	0.55	Zeaxanthin epoxidase	Abscisic acid biosynthetic process
At5g45830	0.52	Tumor-related protein like	
At4g12980	0.51	Auxin-responsive family protein,putative	Dopamine beta-monooxygenase activity, located in membrane
At4g28950	0.47	ROP GTPase gene family protein	Protein transport, small GTPase mediated signal transduction
At5g45820	0.26	CBL-interacting protein kinase 20 (CIPK20)	Kinase activity

		Photosynthesis	
At3g17040	0.5	Tetratricopeptide repeat (TPR)-containing protein	Chloroplast precursor
At3g59400	0.37	GUN4	Chlorophyll biosynthetic process, located in chloroplast
At1g44446	0.34	Chlorophyll a oxygenase/chlorophyll b synthase	Chlorophyll biosynthetic process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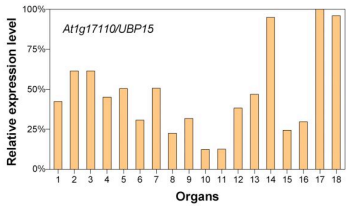
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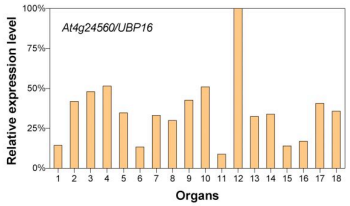
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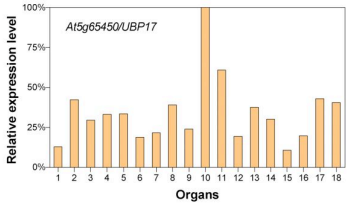
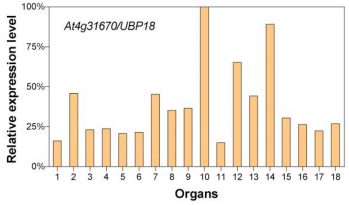
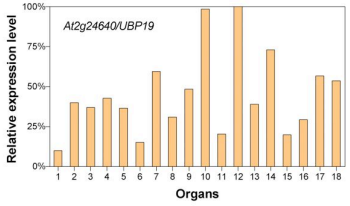
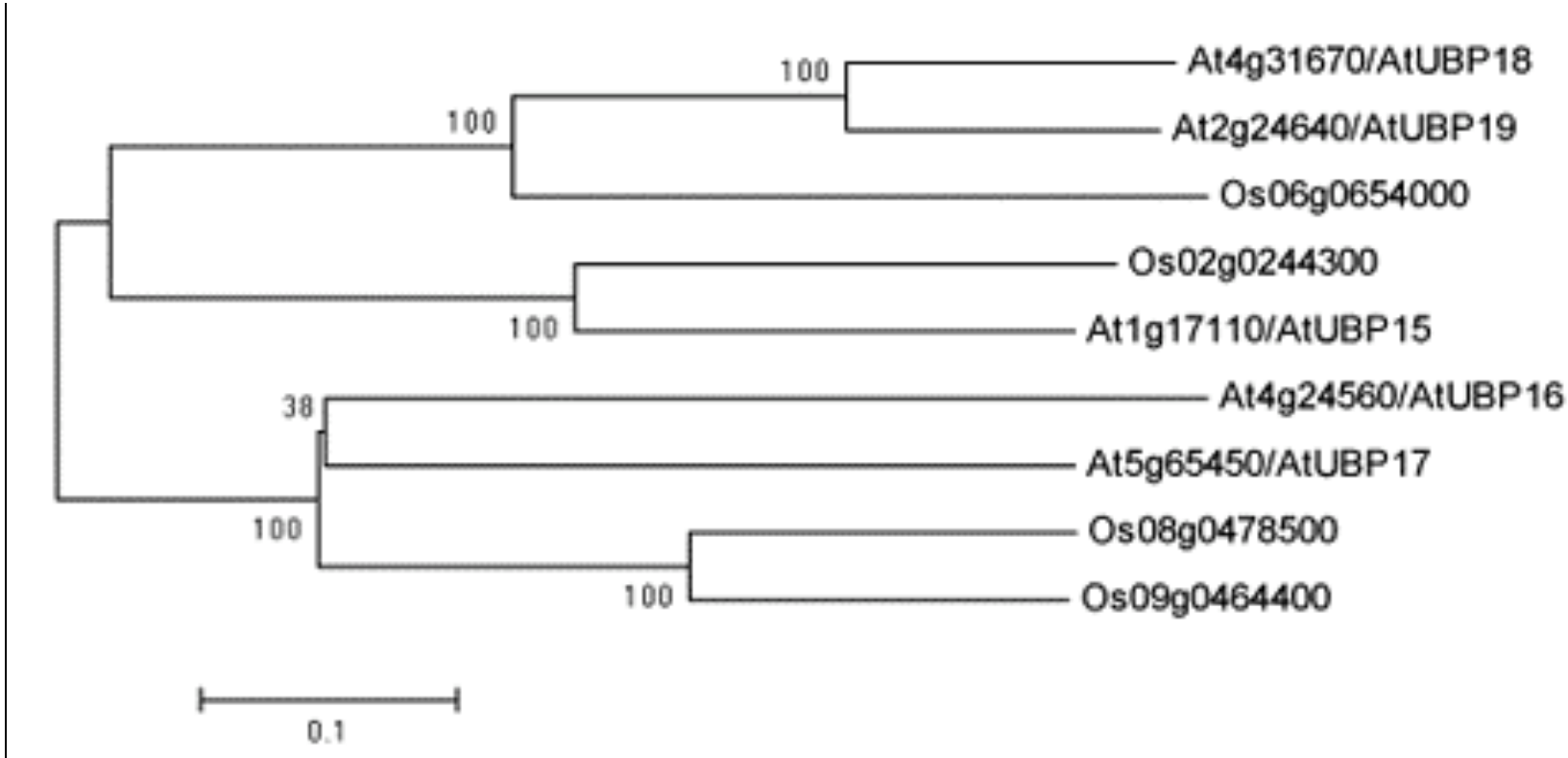
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Organs

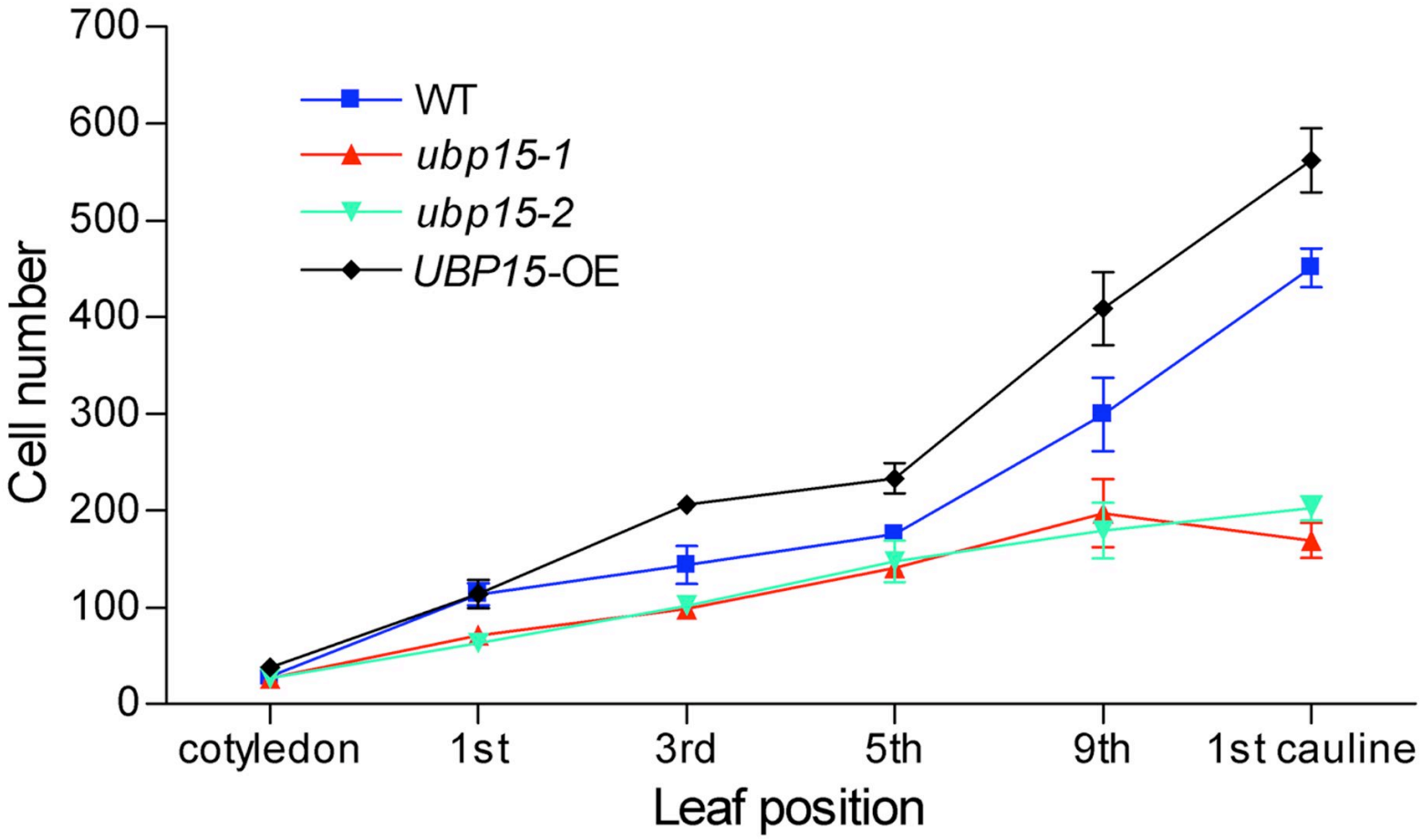

At5g65450/UBP17
Relative expression level
100%
75%
50%
25%
0%
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Organs

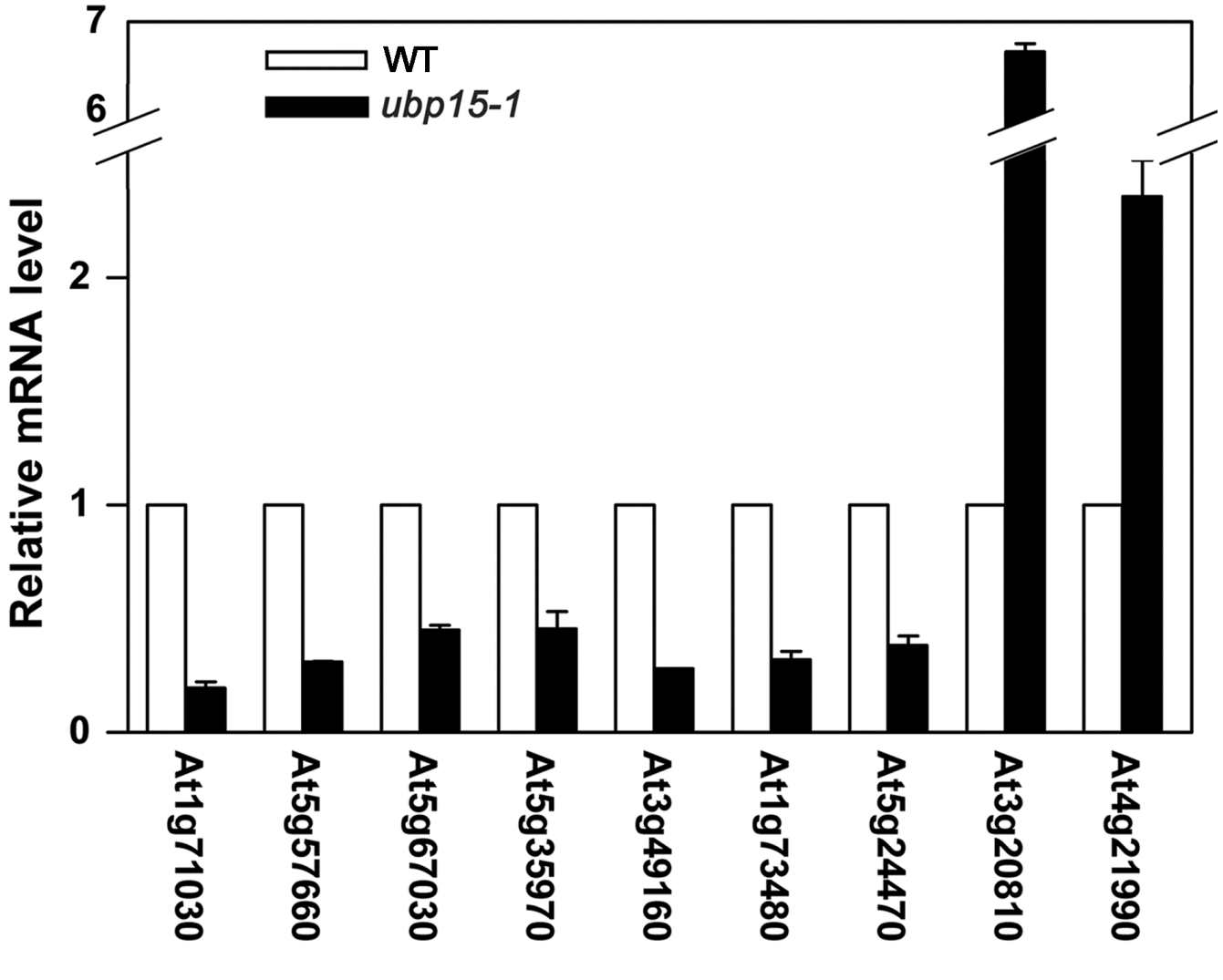

At4g31670/UBP18
Relative expression level
100%
75%
50%
25%
0%
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Organs

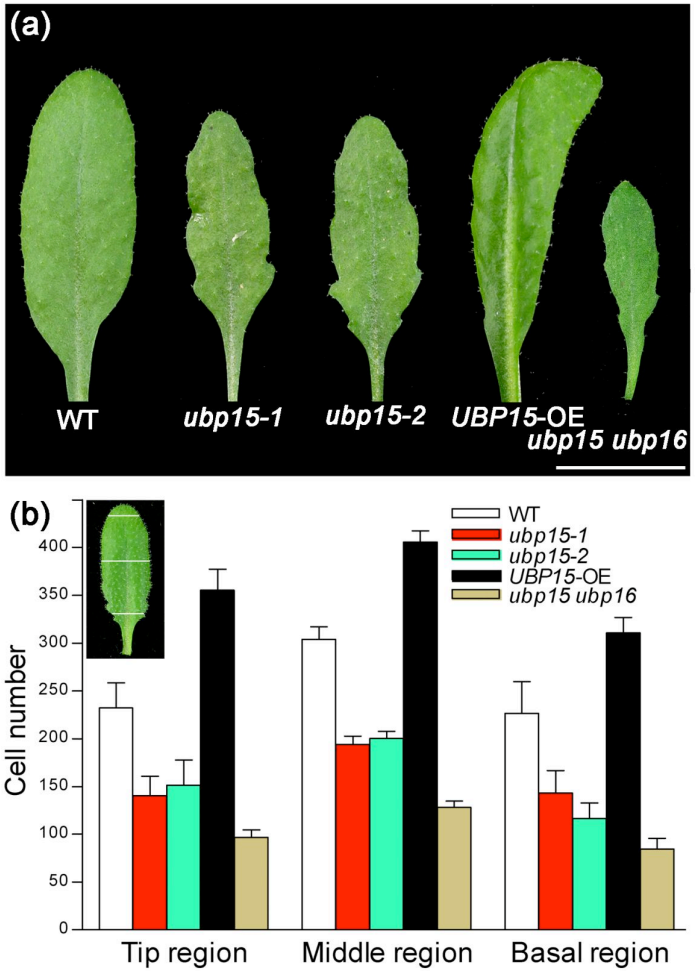

At2g24640/UBP19
Relative expression level
100%
75%
50%
25%
0%
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Organs

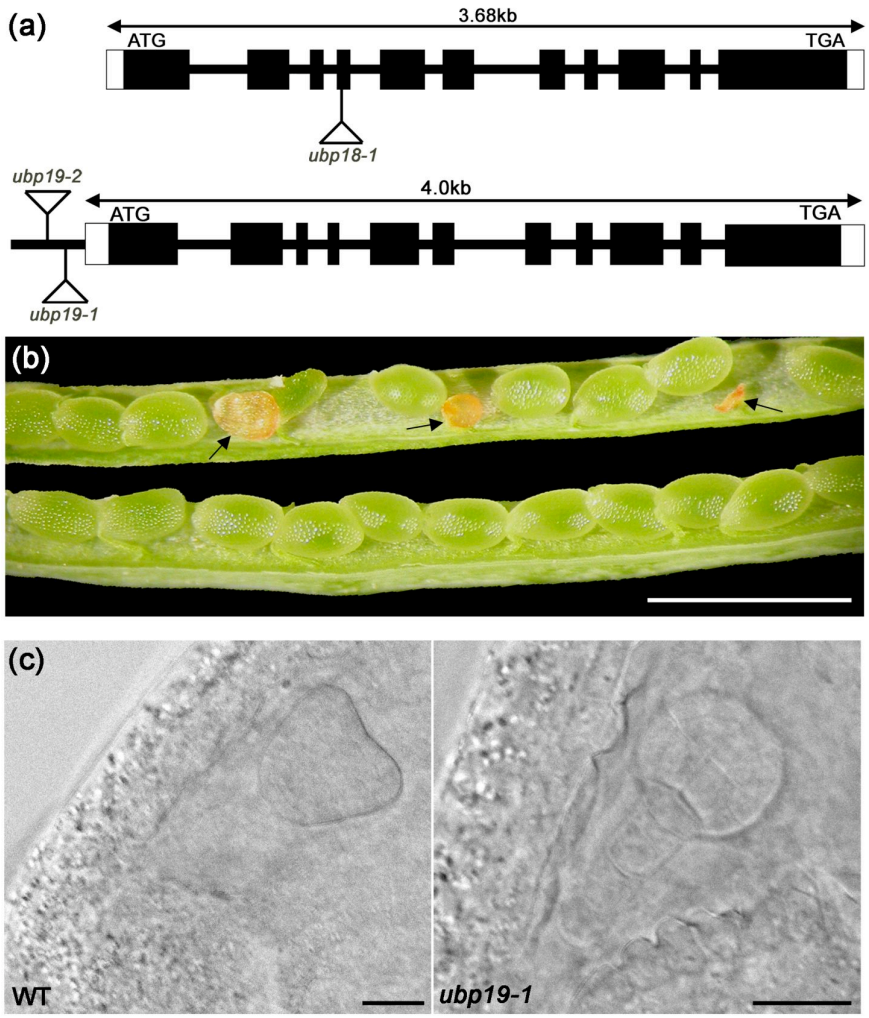

100
100
100
100
38
100
100
At4g31670/AtUBP18
At2g24640/AtUBP19
Os06g0654000
Os02g0244300
At1g17110/AtUBP15
At4g24560/AtUBP16
At5g65450/AtUBP17
Os08g0478500
Os09g0464400
0.1

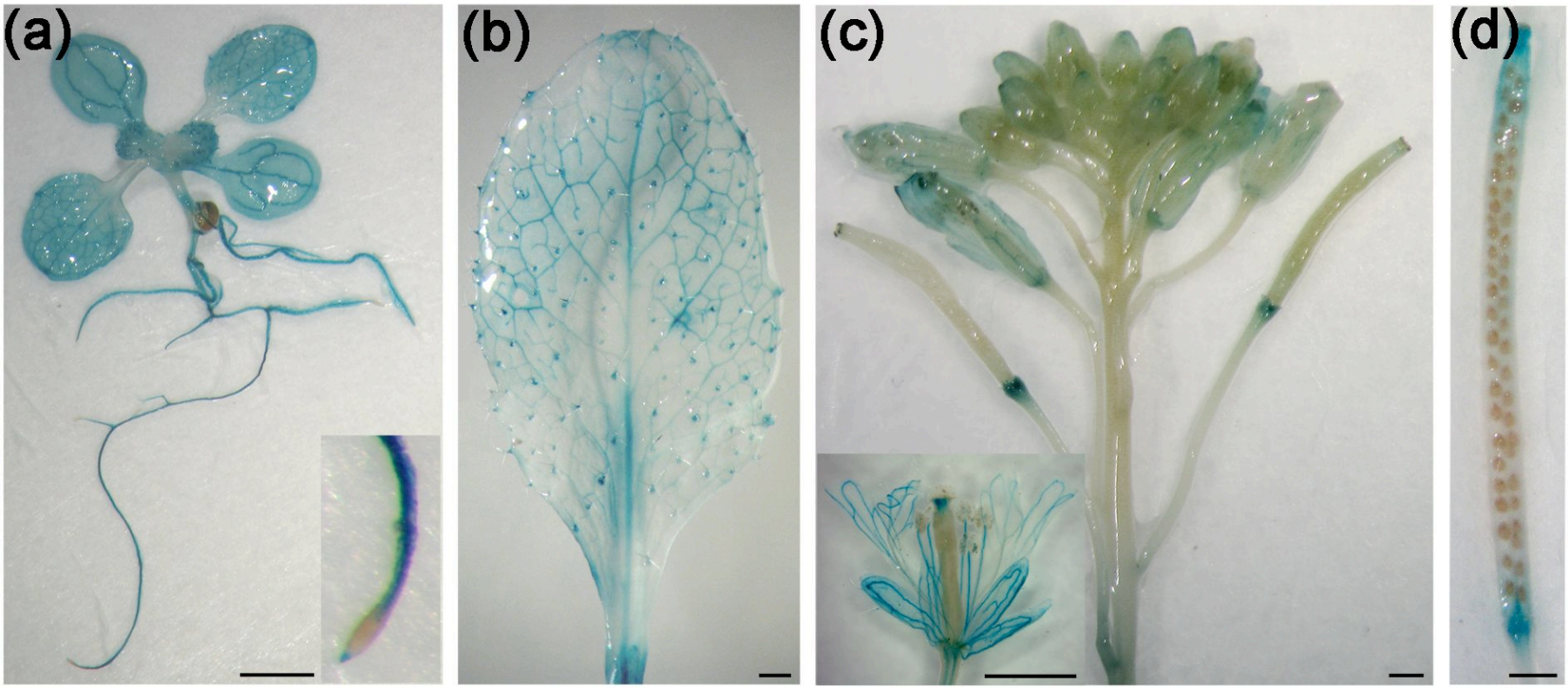

WT
ubp15-1
ubp15-2
UBP15-OE
Rescued


700
600
500
400
300
200
100
0
Cell number
WT
ubp15-1
ubp15-2
UBP15-OE
cotyledon
1st
3rd
5th
9th
1st cauline
Leaf position


Relative mRNA level
7
6
2
1
0
WT
ubp15-1
At1g71030
At5g57660
At5g67030
At5g35970
At3g49160
At1g73480
At5g24470
At3g20810
At4g21990


(a)
WT
ubp15-1
ubp15-2
UBP15-OE
ubp15 ubp16
(b)
WT
ubp15-1
ubp15-2
UBP15-OE
ubp15 ubp16
Cell number
0
50
100
150
200
250
300
350
400
Tip region
Middle region
Basal region


(a)
3.68kb
ATG
TGA
ubp18-1
ubp19-2
4.0kb
ATG
TGA
ubp19-1
(b)
(c)
WT
ubp19-1


(a)
(b)
(c)
(d)

Supplementary experimental procedures

Construction of double and triple mutants

Double and triple mutants were obtained by crossing and the homozygote of the progeny was confirmed by PCR analysis. The *ubp15 ubp16*, *ubp15 ubp17* and *ubp16 ubp17* double mutants were obtained by crossing *ubp15-1* (or *ubp15-2*) with *ubp16-1* or *ubp17-1,* or by crossing *ubp16-1* with *ubp17-1*. Triple mutant *ubp15 ubp16 ubp17* was obtained by crossing *ubp15-1* or *ubp15-2* with double mutant *ubp16 ubp17*; homozygotes of F2 generation were confirmed by PCR analysis.

Measurement of the length of seedlings' root, silique, as well as width and length of rosette leaf

To obtain the root length of seedlings, wild type, *ubp15-1* and *ubp15-2* plants were grown on plates vertically. The length of the root of 20 randomly-selected seedlings (either wild type or mutants) was determined. Twenty randomly selected mature siliques (older than 8 d after pollination) of wild type or mutants were measured. Mature rosette leaves from the first true leaf to the last true leaf were dissected from wild type or *ubp15-1*, and the width and length of the leaves were measured.

Measurement of the flowering time and rosette leaf number

The flowering time of wild type, *ubp15-1*, and the *UBP15*-overexpressing line was measured as number of days from seed germination to the opening of the first flower. Rosette leaf numbers were counted at the bolting time.

Microarray analysis and quantitative RT-PCR

We use early emerged 9th rosette leaves of the wild type and *ubp15-1* as samples. Total RNA was isolated using RNAwiz reagent (Ambion) and purified by the RNeasy kit (Qiagen). For each sample, 50 μg of total RNA was labeled with aminoallyl-dUTP (aa-dUTP, Sigma-Aldrich) by reverse transcription. The aminoallyl- dUTP-labeled cDNAs were purified using a Microcon YM-30 filter (Millipore) and resuspended in 0.1 M $NaHCO_3$. The purified cDNAs were then fluorescently labeled by conjugating monofunctional Cy3 or Cy5 dye (Amersham) to the aminoallyl functional groups. Pair-wise combinations of two samples were used to simultaneously hybridize to each microarray slide, and four independent biological replicates were performed (two replicates with dye exchange). Hybridized slides were scanned with a GenePix 4000B scanner (Axon), and independent TIFF images for Cy3 and Cy5 channels were used for subsequent analysis by GenePix Pro5.0 software package.

To confirm the microarray result, real-time PCR was performed using the ABI SYBR Green PCR master mix in a volume of 20 μL on an ABI 7900 system. The PCR mixture consisted of 0.3 μL of cDNA, 0.6 μM primers, and 1X master mix. In every real-time PCR run, At1g42970/*GAPDH* was used as an internal control to normalize for variation in the amount of cDNA templates. The primers sequences used for those constitutive expressed genes based on t-test were At1g42970/*GAPDH*, 5'-TCTTTCCCTGCTCAATGCTCCTC-3' and 5'-TTTCGCCACTGTCTCTC CTCTAAC-3'. Seven down-regulated genes and two up-regulated genes were selected for measurement by quantitative RT-PCR. Primers used to quantify those genes are At1g71030/*MYB*, 5'-CATTTGCCTGACCTAAACATTG-3' and 5'-

AAGCGTTTCTTGACCTGTTGA-3'; At5g57660/*TRANSCRIPT FACTOR*, 5'-GGCTCATCCACCACCGTT-3' and 5'-GGGAGAGGCTCTGTTTTCGTC-3'; At5g67030/*ABA1*, 5'-GGGCTTGGTCCTCTGTCTT-3' and 5'–GTGAGTCTGCAACTAGGTGGC3'; At5g35970/*HELICASE-LIKE*, 5'-CCACAGGGCTCGGAGGTAT-3' and 5'-TCGTAAGTAAGGGCATCGGC-3'; At3g49160/*PYRUVATE KINASE FAMILY PROTEIN*, 5'-TCCAGCAGGTCTCACATAAACAA-3' and 5'-CTGCTGCTAAGAGATGTGACCG-3'; At1g73480/*HYDROLASE*, 5'-AATGGCGGTGGAAACAATG-3' and 5'-ACGACGCGAAACGGAAGGAG-3'; At5g24470/*APRR5*, 5-TACCCTACGCCAACCCCTAT-3' and 5'-ATGTGATTGCCTATTGCACTATGT-3'; At3g20810/*TRANSCRIPTION FACTOR,* 5'-CCTCAATGCTGTTGCTGGTAA-3' and 5'-TGGGCAAGATAAGTAGGCTCC-3'; At4g21990/*APR3*, 5'-CTGTCAACACGCTTCACGC-3' and 5'-TCTTTCCGGTTCTCTAACTTCATC-3'. To determine the specificity of the primers, the amplified products were subjected to melt curve analysis using the machine's standard method. Cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min. The reported values are averages of six independent trials (two biological replicates and three technical replicates). We calculated relative expression levels as follows. We first normalized transcript levels of those genes relative to a standard (*GAPDH)* using the formula $\Delta C_T = C_{T\ (gene\ X)\ \text{mean of technical repeat}} - C_{T\ (GAPDH)\ \text{mean of technical repeat}}$ either in wild type or *ubp15-1*. Then we got $\Delta Ct_{\ (\text{wild type})}$ and $\Delta Ct_{\ (ubp15\text{-}1)}$ of each gene. Wild type was used as the standard for the comparison of expression levels. We then

calculated relative expression levels using the equation $2^{-[\Delta CT\ (ubp15\text{-}1)\ -\Delta CT\ (wild\ type)]}$. We next calculated the C_T average of two biological repeats.

Creation of *pUBP19:GUS* construct and generation of its transgenic lines

For detecting the tissue expression pattern of *UBP19* by GUS staining, we inserted a *BamH*I/*Kpn*I fragment of the *UBP19* promoter (1.2kb) into the pCAMBIA1381Z and transformed the construct into wild type background by means of *Agrobacterium*-mediated transformation. Transgenic lines were selected by hygromycin (20 μg/mL; Roche).

GUS activity assay

pUBP19:GUS transgenic line were grown on MS plates. Seedlings were washed gently with 100 mM sodium phosphate buffer, pH 7.0, and then stained for 4 h at 37°C in 2 mM X-glucuronide dissolved in 0.1 mM potassium ferricyanide, 0.1 mM potassium ferrocyanide, 10% Triton X-100, 100 μg/ml chloramphenicol, and 500 mM sodium phosphate buffer, pH 7.0 (Byrne et al., 2003; Weigel and Glazebrook, 2002). After that, tissue was washed in 100 mM sodium phosphate buffer followed by 95% and 70% ethanol at room temperature. Microscopy was performed by a dissection microscope (Leica MZFLIII) with a 10x objective, and images were captured by Canon digital camera (Power Shot S70).

Supplementary References

Byrne, M.E., Groover, A.T., Fontana, J.R., and Martienssen, R.A. (2003) Phyllotactic pattern and stem cell fate are determined by the Arabidopsis homeobox gene BELLRINGER. *Development* **130,** 3941-3950.

Weigel, D., and Glazebrook, J. (2002) *Arabidopsis A laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.