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Evolution and Functions of Plant U-Box Proteins: From Protein Quality Control to Signaling

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Abstract

Posttranslational modifications add complexity and diversity to cellular proteomes. One of the most prevalent modifications across eukaryotes is ubiquitination, which is orchestrated by E3 ubiquitin ligases. U-box-containing E3 ligases have massively expanded in the plant kingdom and have diversified into plant U-box proteins (PUBs). PUBs likely originated from two or three ancestral forms, fusing with diverse functional subdomains that resulted in neofunctionalization. Their emergence and diversification may reflect adaptations to stress during plant evolution, reflecting changes in the needs of plant proteomes to maintain cellular homeostasis. Through their close association with protein kinases, they are physically linked to cell signaling hubs and activate feedback loops by dynamically pairing with E2-ubiquitin-conjugating enzymes to generate distinct ubiquitin polymers that themselves act as signals. Here, we complement current knowledge

with comparative genomics to gain a deeper understanding of PUB function, focusing on their evolution and structural adaptations of key U-box residues, as well as their various roles in plant cells.

Contents

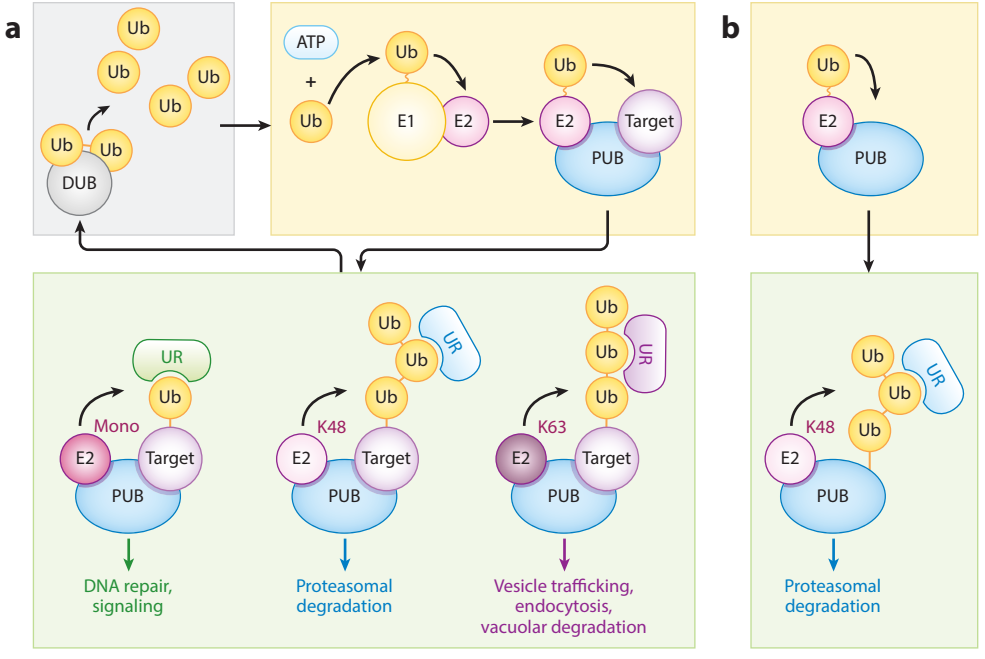
1. EVOLUTION OF THE UBIQUITIN MODIFICATION SYSTEM

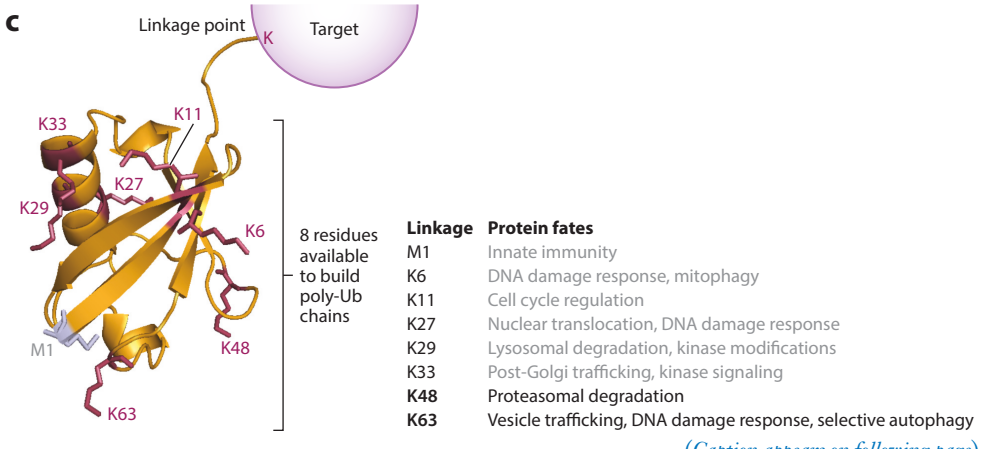
Ubiquitination: covalent attachment of ubiquitin, a 76-amino-acid-long and highly conserved protein modifier

Protein ubiquitination is a posttranslational modification with profound roles in cellular pathways across eukaryotes. Ubiquitin is attached to substrates by a three-enzyme cascade, involving first the E1 ubiquitin-activating enzyme (UBA), then an E2 ubiquitin-conjugating enzyme (UBC), and finally an E3 ubiquitin ligase (**Figure 1*a***). Ubiquitination is highly versatile owing to both its reversibility, mediated by ubiquitin-specific proteases known as deubiquitinases, and its diversity, due to the eight possible linkage types between ubiquitin moieties that result in structurally and functionally distinct chains (**Figure 1*a*,*c***). Ubiquitin is a highly conserved 76-amino-acid protein best known for its role as the so-called "kiss of death" leading to proteasomal degradation of

ubiquitin-labeled proteins, but it also has critical roles in vesicle trafficking, autophagy, DNA repair, and more (**Figure 1*c***).

Ubiquitin-like conjugation systems are thought to have emerged in prokaryotes, predating the evolution of complex posttranslational regulation mechanisms in eukaryotes (12). Ubiquitin itself likely evolved from sulfur transfer proteins involved in thiamine and molybdenum cofactor biosynthesis, THIAMINE BIOSYNTHESIS S (ThiS) and THEMOAD (MoaD), which possess the characteristic β-grasp fold of ubiquitin. Analogous to eukaryotic E1 enzymes binding ubiquitin, ThiS and MoaD form a thiocarboxylate with their C-terminal residues, catalyzed by UBA-like enzymes ThiF and MoaB (68, 147). ThiS and MoaD are closely related to eukaryotic UBIQUITIN-RELATED MODIFIER 1 (URM1), which also functions as a sulfur carrier by forming a thiocarboxylate that is catalyzed by the UBA-like ortholog UBA4, which mediates





Linkage	Protein fates
M1	Innate immunity
K6	DNA damage response, mitophagy
K11	Cell cycle regulation
K27	Nuclear translocation, DNA damage response
K29	Lysosomal degradation, kinase modifications
K33	Post-Golgi trafficking, kinase signaling
K48	Proteasomal degradation
K63	Vesicle trafficking, DNA damage response, selective autophagy

(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

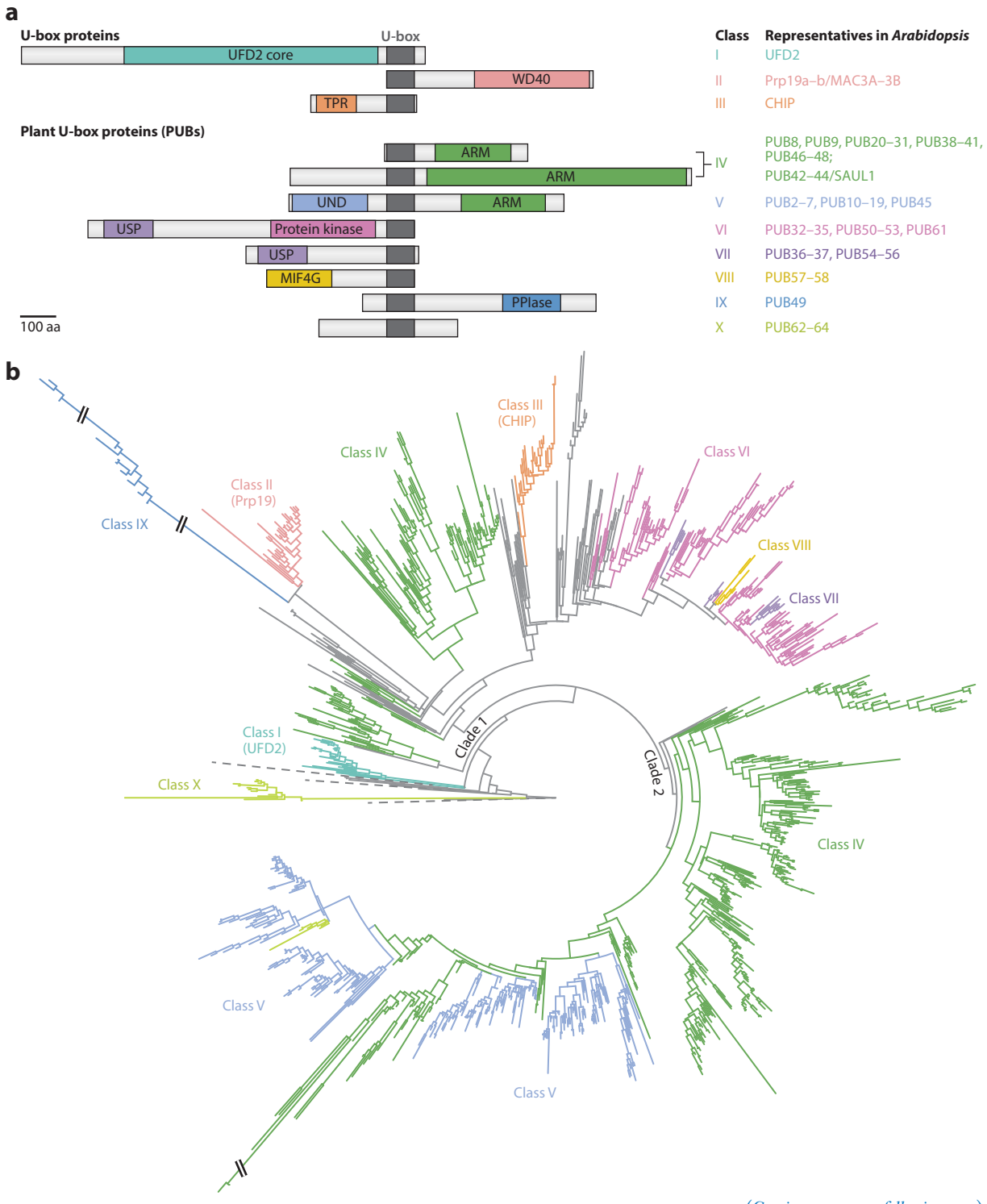
The ubiquitin modification cycle mediated by PUB E3 ligases. (*a*) Ubiquitination requires the concerted action of three proteins: an E1 UBA, an E2 UBC, and an E3 Ub ligase (here depicted as a PUB), which act sequentially to catalyze the attachment of Ub onto a target protein. In the first step, the E1 uses ATP to generate a thioester bond (~) between a cysteine in its active site and the carboxyl-terminal glycine (G76) of Ub. The E2 associates with the loaded E1, which passes Ub onto the E2, generating a second thioester bond between the cysteine in the E2's active site and the G76 of Ub. Once charged with Ub, the E2 associates with a PUB by docking to its U-box. The PUB E3 functions as a scaffold that guides the E2-Ub conjugate into close proximity of the target substrate, enabling the E2 to catalyze Ub transfer onto an exposed lysine. E2s are largely responsible for the type of Ub polymer that is built. The outcome is a conjugate in which the C-terminal glycine carboxyl group of Ub is linked by an isopeptide bond to an exposed ε-amino group of a lysine of the target. The Ub chains act as distinct signals that are decoded by Ub receptors responsible for mediating downstream events such as proteasomal degradation (K48 chains) or vacuolar degradation (K63 chains). Ub moieties are quickly released from chains by deubiquitinases. (*b*) A prevalent characteristic of most PUBs is the attachment of Ub onto themselves, a process called autoubiquitination. Autoubiquitination has been linked to a high PUB degradation rate in vivo; however, it is possible that autoubiquitination may result in other fates as well. (*c*) Ub is generally attached to lysine residues on substrate proteins. Ub itself has seven lysine residues (K6, K11, K27, K29, K33, K48, K63) that can also be ubiquitinated, as can its N-terminal methionine residue. The different types of polyubiquitin chains determine the fate of the modified substrate, as indicated (known functions in plants are in black and known functions in nonplant systems are in gray). The Ub structure was obtained from PDB ID 1UBQ. Abbreviations: ATP, adenosine triphosphate; DUB, deubiquitinase; G, glycine; K, lysine; PDB ID, Protein Data Bank identification; PUB, plant U-box protein; Ub, ubiquitin; UBA, ubiquitin-activating enzyme; UBC, ubiquitin-conjugating enzyme; UR, Ub receptor.

transfer RNA (tRNA) thiolation (70). URM1–UBA4 represents the most ancestral eukaryotic ubiquitin-like conjugation system and may have served as a starting point for the emergence of all other ubiquitin-like systems.

Accumulating evidence supports the function of these ancestral forms in responses to environmental changes, such as URM1 attachment in response to oxidative stress (102). In eukaryotes, these components have experienced massive functional diversification, which is reflected by their involvement in all aspects of complex eukaryotic physiology and their central roles in safeguarding cellular homeostasis during adverse conditions. The expansion of the ubiquitin modification system is best illustrated by E3 ligases, which are the specificity determinants for the ubiquitination reaction; for example, there are more than 1,400 in the model plant *Arabidopsis thaliana* (131). PLANT U-BOX PROTEINS (PUBs) are a group of E3 ligases of particular interest because they evolved into a large family of mostly plant-specific E3s that, as an accumulating body of studies shows, is significantly linked to stress responses.

2. THE U-BOX SHUFFLE

While the number of upstream E2 conjugating enzymes is relatively similar across eukaryotes (39 in *A. thaliana*, 40 in *Oryza sativa*, 35 in human), E3 ubiquitin ligases have massively expanded into large protein families in plants (131). The U-box E3s UBIQUITIN FUSION DEGRADATION 2 (UFD2), PRECURSOR RNA PROCESSING 19 (Prp19), and C TERMINUS OF HSC70-INTERACTING PROTEIN (CHIP) are present in most eukaryotes, suggesting that these are ancestral forms. However, U-box proteins diversified from these ancestral forms by fusing to a wide spectrum of accessory domains (**Figure 2*a***). In addition to the conserved functions carried out by UFD2, Prp19, and CHIP, novel domain combinations were accompanied by neofunctionalization. The U-box itself is a conserved domain, mainly dedicated to pairing with E2s (**Figure 1**). PUBs pair with specific E2s (129) and interact with



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Domain composition and phylogenetic analysis of U-box proteins. (*a*) Domain composition of broadly conserved U-box proteins (*upper*) and PUBs (*lower*). Identified domains in addition to the U-box include class I UFD2-core domain (ARM-type fold), class II WD40 repeats, class III TPR, class IV ARM repeats, class V UND, class VI USP, class VII S/T protein kinase, class VIII MIF4G (ARM-type fold), and class IX PPIase Class X PUBs do not contain an annotated domain in addition to the central U-box. Loci from each class in *Arabidopsis* are indicated on the right. (*b*) Phylogenetic analysis of PUBs in the plant lineage. To analyze the evolution of the U-box domain specifically, U-box domains of 1,121 PUB protein sequences from 20 species were isolated and aligned together with four RING finger domain sequences as an outgroup (*dashed line*). The alignment was then used to infer a phylogenetic tree using the maximum likelihood method supported by 2,000 bootstrap samples. Branches marked with break lines were shortened to fit figure size parameters. Linear phylograms of this tree, another tree based on full-length protein sequences, and the underlying sequence alignments are provided in **Supplemental Figures 1 and 2**. Abbreviations: ARM, Armadillo; CHIP, C TERMINUS OF HSC70-INTERACTING PROTEIN; MIF4G, MIDDLE DOMAIN OF EUKARYOTIC INITIATION FACTOR 4G; PPIase; peptidyl-prolyl isomerase; Prp19, PRECURSOR RNA PROCESSING 19; PUB, plant U-box protein; RING, REALLY INTERESTING NEW GENE; S, serine; T, threonine; TPR, tetratricopeptide repeat; UFD2, UBIQUITIN FUSION DEGRADATION 2; UND, U-box N-terminal domain; USP, Universal stress protein.

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substrate proteins to bring the ubiquitin-loaded E2 into close proximity of the substrate to mediate ubiquitin transfer, thus also conveying specificity to the ubiquitination process.

In 2001, 37 PUBs were annotated in the *Arabidopsis* genome and grouped into five classes based on phylogenetic analysis using available sequence information at that time (4). However, with the advent of genomics and improved algorithms, later studies uncovered additional PUBs that were not included in the earlier phylogeny, as well as subdomains that were missed in earlier domain predictions (123, 151). Here, we undertook a large-scale comparative genomics approach using sequence annotations from 20 species that span the plant lineage in order to gain a deeper understanding of PUB diversity and evolution. Based on our analysis, which is discussed in detail in Section 3, we propose that PUBs group into 10 classes based on their domain architecture. We therefore present a new classification of the PUB family, expanding on the previous groupings proposed 20 years ago (4).

Phylogenetic analysis: approach employed to study the evolutionary relationships between organisms or genes based on genetic sequences

Proteasome: a nuclear and cytosolic localized multisubunit complex of 750 kDa that recognizes and mediates the degradation of substrates that are modified with Lys48-linked ubiquitin chains

Proteostasis: process encompassing protein biogenesis, folding, trafficking, and degradation that ensures dynamic and balanced proteome composition

Nucleotide-binding, leucine-rich repeat proteins (NLRs): sensors of the immune system that directly or indirectly detect effectors

2.1. Class I: UFD2 Core Domain | UFD2—Chain Elongation and Quality Control

The prototype U-box protein UFD2 was first identified in a screen for *Saccharomyces cerevisiae* mutants that displayed defects in the degradation of a synthetic substrate (52) and was later shown to be able to extend short ubiquitin chains previously added by its partner E3 enzyme, UFD4 (63), earning it the name E4. More recently, UFD2 was shown to also function as an E3 and preferentially target unfolded protein segments in *Caenorhabditis elegans* muscle cells by cooperating with the UNC-45 chaperone (41).

UFD2 cooperates with CELL DIVISION CONTROL PROTEIN 48 (CDC48), an AAA ATPase that is able to unfold and separate polyubiquitin-tagged proteins from complexes or membranes and is involved in endoplasmic reticulum (ER)-associated degradation (40, 106). Although substrate ubiquitination and extraction from the ER is UFD2 independent, UFD2 increases the degree of ubiquitination and facilitates degradation by the proteasome (97, 122). Yeast cells lacking UFD2 activate the unfolded protein response, indicating a general role in proteostasis (78). Two reports have investigated the *Arabidopsis* ortholog of UFD2 [a.k.a. *MUTANT, SNC1-ENHANCING 3* (*MUSE3*)] as an immune regulator (45). *UFD2* complements the yeast *ufd2* mutant (45) and also interacts with the *Arabidopsis* ortholog of CDC48A (22), suggesting that in spite of its low sequence identity (23.1%) it is a functional ortholog. *UFD2* mutants display enhanced levels of the nucleotide-binding, leucine-rich repeat (NLR) immune sensors SUPPRESSOR OF npr1–1, CONSTITUTIVE 1 (SNC1) and RESISTANT TO PSEUDOMONAS SYRINGAE 2 (RPS2), which themselves are targeted by a cullin-REALLY INTERESTING

NEW GENE (RING) ligase (17, 37). This suggests that UFD2 displays a similar mode of function, acting as an E4 on substrates already primed with short ubiquitin chains. It is tempting to speculate that UFD2 also cooperates with RING E3s, such as SNC1-INFLUENCING PLANT E3 LIGASE REVERSE 1 (SNIPER1) and SNIPER2, which ubiquitinate multiple NLRs (146).

2.2. Class II: WD40 | Prp19—Splicing and More

Prp19 is arguably the most studied U-box protein and is essential for cell survival in yeast and mice (13). It is the founding member of the Prp19 complex (Prp19C), a multifunctional protein complex involved in various biological processes that include premessenger RNA (premRNA) splicing and the DNA damage response (13). Ubiquitination by Prp19 promotes both splicing and DNA repair (13). As one example, Prp19 ubiquitinates Prp3, a core protein of the tri-small nuclear ribonucleoproteins, resulting in enhanced interaction between Prp3 and the spliceosome machinery (101, 117). *Arabidopsis* Prp19C components, including partially redundant Prp19a and Prp19b [a.k.a. *MOS4-ASSOCIATED COMPLEX 3A* (*MAC3A*)*/PUB59* and *MAC3B/PUB60*], copurify with MODIFIER OF SNC1, 4 (MOS4), an ortholog of the premRNA processing factor SPF27/BCAS2 and a potential core component of the spliceosome (94). In agreement with its proposed function, *Arabidopsis prp19a prp19b* double mutants exhibit significantly higher levels of intron retention compared to wild type (50). In addition, *S. cerevisiae* Prp19 participates in transcription and was shown to be recruited to transcribed genes by the Prp19C, which interacts with RNA polymerase II (13). In nonplant systems, the Prp19C also interacts with the TREX complex, coupling transcription elongation to nuclear mRNA export (13). *Arabidopsis prp19a prp19b* double mutants were first identified as regulators of immunity (94), potentially because of impaired stress-induced transcriptional reprogramming. Along the same lines, *Arabidopsis* Prp19a and Prp19b were recently shown to also contribute to the control of microRNA levels through modulating miRNA precursor transcription, processing, and stability (73).

2.3. Class III: Tetratricopeptide | CHIP—Bridging Protein Folding and Degradation

CHIP was originally identified as a cochaperone for HSP70 and HSP90 in a screen for novel tetratricopeptide repeat (TPR)-containing proteins in mammals (6). Both HSP70 and HSP90 are core chaperones that mediate proper folding of proteins within global protein homeostasis (29). CHIP is a major link between chaperone-mediated protein folding (biosynthesis) and protein degradation, acting as both a cochaperone and an E3 ligase (29). Its primary role is to divert chaperone complexes toward protein degradation during protein quality control, instead of supporting protein folding. Accordingly, *Arabidopsis* CHIP recognizes the C terminus of HSP70–4, likely through its conserved EEVD motif (69). The substrate-binding domain of HSP70 recognizes exposed hydrophobic regions, and in *Arabidopsis*, HSP70–4 cooperates with CHIP to mediate proteasomal degradation of chloroplast-destined precursor proteins (69). However, even though mutant plants do not display a general developmental phenotype in *Arabidopsis*, CHIP also plays a more general role in plant resilience against a wide spectrum of stresses (159). Moreover, together with NEIGHBOR OF BRCA1 (NBR1)-mediated selective autophagy, CHIP additively protects plants against proteotoxicity (159).

2.4. Class IV: Armadillo | Dedicated to Stress

The most prominent group of PUBs possess C-terminal Armadillo (ARM) repeats and have experienced the strongest expansion, ranging from 16% of all PUBs in the green alga *Klebsormidium*

microRNA: small single-stranded noncoding RNA molecule containing about 22 nucleotides that functions in RNA silencing to regulate gene expression

HSP70 and HSP90: molecular chaperones that assist the conformational folding and unfolding of proteins and assembly of complexes

Armadillo (ARM) repeat: composed of a pair of α-helices forming a hairpin that in tandem form an α-solenoid structure; mediates protein–protein interactions

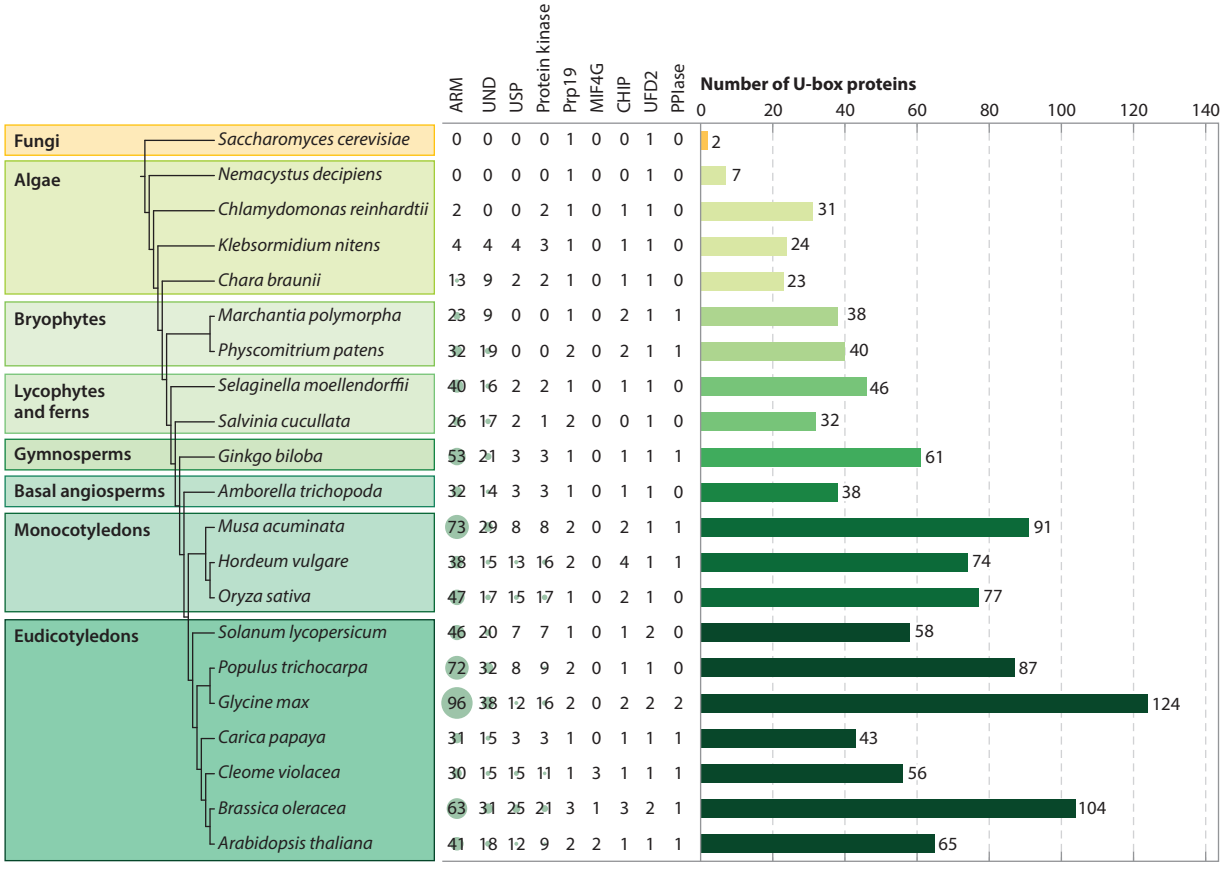

Figure 3

Diversification and expansion of U-box domain combinations within the major branches of the green lineage. The number of U-box proteins encoded by selected fungal, algal, bryophyte, lycophyte, fern, gymnosperm, and angiosperm genomes are indicated on the right as a histogram, and the number of those U-box proteins that contain additional domains are indicated in the middle as a bubble plot. See **Supplemental Methods** for further details. Abbreviations: ARM, Armadillo; CHIP, C TERMINUS OF HSC70-INTERACTING PROTEIN; MIF4G, MIDDLE DOMAIN OF EUKARYOTIC INITIATION FACTOR 4G; PPIase, peptidyl-prolyl isomerase; Prp19, PRECURSOR RNA PROCESSING 19; UFD2, UBIQUITIN FUSION DEGRADATION 2; UND, U-box N-terminal domain; USP, Universal stress protein.

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nitens to 81% in the monocotyledonous *Musa acuminata* (banana), as well as the dicotyledonous *Populus trichocarpa* (poplar) (**Figure 3**). Class IV PUBs may contain 4 to 13 predicted ARMs, which are dedicated to mediating protein–protein interactions. Class IV PUBs have surfaced as hubs of stress responses by integrating protein kinase–mediated signaling with ubiquitination. Their wide range of roles will be discussed in later sections together with class V.

2.5. Class V: U-Box N-Terminal Domain | The Big Unknown, a Potential HeLo Domain?

In *Arabidopsis*, 18 different PUBs that harbor ARMs additionally possess a U-box N-terminal domain (UND). Although one study showed that the UND is able to determine target specificity (112), its purpose has remained cryptic. Domain prediction analyses revealed a subdomain in

PUB5, PUB13, PUB16, and PUB18, with similarities to a four-helical bundle (4HB) found in the N terminus of the human MIXED-LINEAGE KINASE DOMAIN-LIKE PROTEIN (MLKL) (**Figure 2*a***). MLKL executes necroptosis, a regulated cell death that promotes the release of proinflammatory molecules and supports the activation of a robust immune response (96). Three MLKLs are conserved in *Arabidopsis* with overlapping functions in disease resistance (87). Human MLKL consists of a C-terminal pseudokinase that upon phosphorylation relays activation to the N-terminal 4HB executioner domain, triggering oligomerization, insertion into the plasma membrane, and cell death (96). The structures of *Arabidopsis* MLKL3 and MLKL2 revealed a 4HB related to pore-forming HeLo domains, which mediated tetramerization (87). With the exception of SPOTTED LEAF 11 (SPL11) in rice and the *Arabidopsis* ortholog PUB13 (74, 152, 158), which are both predicted to harbor a 4HB, cell death phenotypes have not been reported for other *PUB* mutants. Therefore, the function of a putative 4HB in the UND may be limited to mediating oligomerization.

Universal stress proteins (USPs): linked to stress responses via unknown molecular mechanism; some are able to bind ATP and may act as switches

2.6. Class VI: Protein Kinase | Two Posttranslational Modifications in One Pot

An interesting group of PUBs contain integrated receptor-like cytoplasmic kinase (RLCK) domains, raising the exciting possibility that they can catalyze the transfer of both phosphoryl groups and ubiquitin moieties to substrates. The Ser/Thr kinase domains are of the INTERLEUKIN-1 RECEPTOR ASSOCIATED KINASE (IRAK) type, belonging to the RLCK-IXb subfamily predicted to have emerged about 1,500 million years ago (26). Class VI/RLCK-IXb PUBs are conserved across the plant lineage and among the few RLCKs found in chlorophytes, suggesting that they may represent ancestors of the expanded receptor-like kinase (RLK) superfamily in streptophytes (26) and that they may have conserved roles in canonical signaling pathways. The RLCK-IXb subfamily contains 20 members in *Arabidopsis*, 9 of which contain a U-box domain (**Figure 2*a***). Intriguingly, several class VI/RLCK-IXb PUBs also contain an N-terminal universal stress protein (USP) (18), suggesting that they might function as stress sensor and executor proteins capable of receiving information through their USP domain and transducing that information through their RLCK and U-box domains. It is possible that the RLCK domain autophosphorylates the U-box domain or transphosphorylates a docked E2 enzyme or another protein target, thus regulating their function. Conversely, it is also possible that binding targets via the N-terminal and/or RLCK domains positions them for ubiquitination by the U-box-docked E2.

While all residues important for E2 binding appear to be conserved, some class VI/RLCK-IXb PUBs are predicted to be pseudokinases (71). Although they lack one or more of the canonical residues required for catalysis, pseudokinases are functional molecules that through conformational switching are able to allosterically regulate catalysis (116). Notably, the RLCK-IXb family is related to HOPZ-ETI-DEFICIENT 1 (ZED1)-RELATED KINASE (ZRK) pseudokinases, belonging to RLCK subgroup XII-2 (71). During infection of *Arabidopsis* by the bacterial pathogen *Pseudomonas syringae*, ZED1 and ZRKs bind to both the bacterial effector protein HopZ1a and its immune receptor HopZ1a-ASSOCIATED RESISTANCE 1 (ZAR1) (71). Given its similarity to immune signal–transducing RLCKs, HopZ1a may accidentally target ZED1, which potentially acts as a nonfunctional decoy that binds the invading bacterial protein and triggers immune signaling (71, 114). However, we are not aware of any experimental study on class VI/RLCK-IXb PUBs, and whether they act in signaling or the pseudokinases act as decoys remain untested hypotheses.

2.7. Class VII: Universal Stress Proteins—Potential Stress Switches

The Rossmann fold mediates binding to nucleotides and is found in a large group of proteins of ancient origin that includes USPs in bacteria, archaea, plants, and metazoans (18). In bacteria,

some USPs can bind adenosine triphosphate (ATP) and play diverse roles in signaling pathways via their integrated functional domains (18). There are 44 USPs encoded in *Arabidopsis*, all predicted to possess ATP-binding sites, of which 12 contain a U-box domain. Elucidation of the structure of the USP domain–containing protein At3g01520 revealed conservation of residues in an ATP-binding loop (56). Like other USPs, At3g01520 forms a dimer (56), which, in combination with its potential ATP-binding features, opens the possibility that USPs function as molecular switches. When fused to kinase domains, USPs may also regulate phosphorylation activity. In support of this role, reactive oxygen species are able to induce oligomerization of the USP At3g53990 (54). In addition, another *Arabidopsis* USP protein, HYPOXIA RESPONSIVE UNIVERSAL STRESS PROTEIN 1 (HRU1), coordinates oxygen sensing during anoxia, potentially through its interaction with the GTPase ROP2 and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase RBOHD (36).

2.8. Additional Uncharacterized Domains

PUB49 is the only *Arabidopsis* U-box protein that possesses a peptidyl-prolyl *cis-trans* isomerase (PPIase) domain (145), comprising class IX (**Figure 2*a***). PPIases catalyze the *cis-trans* isomerization of the peptide bond preceding a proline residue and are essential for correct folding of proteins. *Cis-trans* isomerization can also change protein conformation, acting as a switch. The related but not U-box-containing protein CYP2 was demonstrated to catalyze the *cis-trans* isomerization of the transcriptional repressor IAA11 of the auxin response in rice to facilitate its degradation by the proteasome (51). A further domain found in combination with a U-box domain is the MIDDLE DOMAIN OF EUKARYOTIC TRANSLATION INITIATION FACTOR 4G (MIF4G), which shares a common ARM repeat-type fold but can differ in sequence. These PUBs comprise class VIII. The MIF4G domain is found in several proteins of RNA metabolism, including the EUKARYOTIC TRANSLATION INITIATION FACTOR 4 GAMMA (eIF4G). MIF4G of the human eIF4G coordinates the assembly of the translation initiation machinery (88). However, additional domains found in eIF4G are absent in class VIII proteins PUB57 and PUB58, and it remains unclear what function PUB57/PUB58 or PUB49 plays in plants. Finally, class X PUBs (PUB62–PUB64) do not have a known additional domain. Although nothing is known about their function, they are interesting from an evolutionary point of view, as described below.

3. EVOLUTION OF THE U-BOX GENE FAMILY

3.1. Emergence of Plant U-box Proteins in the Kingdom Plantae

Sequence profile analyses suggest that the U-box evolved from the RING zinc-finger domain, losing the metal-chelating residues while adopting a new set of amino acids that stabilize the domain structure (3). In spite of these substitutions, the U-box and RING domains have maintained a structural homology throughout evolution. UFD2 and Prp19 are the only U-box E3s present in all eukaryotes, as well as the only U-box proteins present in the baker's yeast *S. cerevisiae* (**Figure 2*b***; **Supplemental Figure 1**). It is therefore likely that UFD2 and Prp19 are the ancestral U-boxes and that their emergence predates the divergence of the last eukaryotic common ancestor. However, it currently remains unclear which of the two emerged first from a RING E3 ligase.

The recent surge in sequencing and annotation of nonmodel organisms provides exciting opportunities to understand the evolution of protein families. To complement available studies, which often focus on single species, and to obtain a better understanding of PUB evolution, we carried out phylogenetic analyses using 1,121 amino acid sequences from 20 plant species spanning all major branches of the plant lineage (**Supplemental Figure 1 and Supplemental**

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Methods). In addition to the phylogeny based on full-length sequences, we also generated one based on the U-box domains alone (**Figure 2*b***; **Supplemental Figure 2**). As it is the defining feature of PUBs, we focused on the phylogeny based on U-box domains, which is largely consistent with that of the full-length sequences and best reflects the evolution of this family without the bias introduced by the additional fused domains or their orientation at N- or C-termini.

The branch that contains the UFD2 U-boxes (class I) is closest to the root of the tree, composed of 4 RING domains, and included as an outgroup that represents U-box ancestors (**Figure 2*b***). Given that UFD2-like U-box proteins are conserved across all eukaryotes, they may represent the ancestral class of PUBs. Surprisingly, U-boxes from class X PUBs, harboring no additional known domains, were also next to the root, suggesting that they are of ancient origin.

Based on the phylogeny, PUBs then split into two large clades. Clade 1 contains two PUB subfamilies with ancient domain combinations: WD40-containing Prp19-like (class II) U-box proteins and TPR domain–containing CHIP-like class III proteins. Interestingly, neither conserved UFD2-like, Prp19-like, nor CHIP-like PUBs diversified in plants. While they can be found in the vast majority of plant species, they exclusively occur in single or low copy numbers (**Figures 2*a*** and **3**), possibly reflecting their key roles in essential eukaryotic (and not plant-specific) functions. CHIP-like U-box proteins, which share a common ancestor with Prp19 (89), are absent in the brown alga *Nemacystus decipiens*, the fungus *S. cerevisiae*, and the fern *Salvinia cucullata*. However, as they are also found in humans and choanoflagellates—including *Monosiga brevicollis*, possibly the closest known relative of metazoans—it is likely that CHIP was repeatedly lost in the course of evolution, at least in brown algae and ferns (**Figures 2*b*** and **3**).

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Outgroup: distantly related group of sequences that serves as a reference to determine evolutionary relationships

Choanoflagellates: free-living unicellular and colonial flagellate eukaryotes considered to be the closest living relatives of animals

Terrestrialization: colonization of the land habitat out of the sea by plants; one of the key events in the history of life

3.2. Diversification of Plant U-Box Protein Classes

Following the evolution of ancestral PUBs, new domain combinations emerged and expanded into large subfamilies, resulting in U-box protein family sizes ranging from a total of 7 genes in *N. decipiens* to 126 in the eudicot *Glycine max*. Similar to F-box proteins (98), PUBs may have undergone several expansion waves during green plant evolution (**Figure 3**). The first massive expansion becomes evident by the large number of PUBs in green algae compared to fungi and brown algae. A second expansion may have occurred during and after plant terrestrialization in specific subfamilies (**Supplemental Figure 3**). PUBs group into ten different domain classes, which mostly correspond with the phylogeny (**Figure 2*a*,*b***; **Supplemental Figure 2**). The rise of new domain combinations also showcases a burst of PUB neofunctionalization in plants (**Figure 2*a***).

The two most prominent groups of U-box proteins diverged from the above-described large clade 1 that contains Prp19 and CHIP. The second large clade is plant specific and harbors exclusively PUBs with ARM repeats, either without (class IV) or with an additional UND domain (class V) (**Figure 2*b***). The latter seems to have evolved from class IV PUBs on various occasions. The only exception within this large clade 2 is a class X U-box-only protein from *Arabidopsis thaliana*, which is likely to have recently lost its ARM domain(s) and was named PUB64. However, ARM-PUBs are not specific to clade 2. Based on the phylogeny, they independently evolved several times also in clade 1, either as single genes or even in a larger cluster (**Figure 2*b***).

The second-largest PUB subfamily contains a U-box and a USP domain with (class VI) or without (class VII) an additional protein kinase domain. The U-box-based phylogenetic tree suggests that this subfamily has evolved within clade 1 from CHIP. Class VI and VII PUBs are present in the green alga *K. nitens* and absent in bryophytes, but they expanded significantly in flowering plants (**Figure 2*a*,*b***). Most plant species have a combination of USP with the kinase. However, Brassicaceae also includes PUBs that are only fused to the USP, suggesting that they lost the kinase domain (**Figures 2*a*** and **3**). Also specific to the Brassicaceae family are MIF4G-PUBs

from class VIII, which cluster with USP and kinase PUBs (class VI and class VII), suggesting a common ancestor (**Figure 3**).

Interestingly, algae show a divergent domain composition compared to land plants (**Figure 3**). This is illustrated by *Chlamydomonas reinhardtii* domain fusions, where U-box proteins are commonly found in combination with coiled-coil regions and ankyrin repeat domains but possess only two class IV PUBs (85). The proportion of PUBs with divergent domains is lower in *Chara braunii*, which instead possesses a much higher proportion of class IV and V PUBs (**Figure 3**).

In summary, the evolutionary history of U-box proteins shows the two major hallmarks of the plant E3 ubiquitin ligase superfamily: a general expansion in land plants compared to nonplant lineages and diversification by the adoption of a wide array of domains, which often mediate protein–protein interactions. Together, this evolutionary dynamic may reflect adaptations required to cope with challenges of the terrestrial and sessile lifestyle.

Supplemental Material >

4. STRUCTURAL CONSIDERATION OF U-BOX FUNCTION

The term U-box (UFD2-homology domain) was first coined to describe the approximately 100 conserved residues at the C terminus of UFD2 (63). Sequence profile analysis suggests that the U-box evolved from the RING domain, losing the characteristic histidine (His) and cysteine (Cys) Zn^{2+}-chelating residues, while adopting a new set of amino acids that stabilize the domain structure (3) (e.g., RBX1; **Supplemental Figure 4*a***). However, RING and U-box domains share a similar pattern of hydrophobic and polar amino acids (3, 100) (**Supplemental Figure 4*b***). The first structure of the yeast Prp19 U-box revealed that the conserved zinc-binding residues maintaining the cross-brace arrangement in the RING domain are replaced by hydrogen-bonding networks in the U-box (100). Soon after, the first structure of a plant U-box, that of PUB14, was determined (1). The structure confirmed a conserved arrangement of hydrogen-bonding residues and hydrophobic cores that stabilized the U-box (1) (**Supplemental Figure 4*b***).

4.1. U-Box Structure and E2 Interaction

The main role of the U-box is to bind the E2-ubiquitin conjugate to facilitate ubiquitin transfer. The U-box engages E2s via hydrophobic residues located on loop 1 (L1) and L2, as well as on the N-terminal α-helix1 (H1), as illustrated by the models for UFD2 with UBC8 (1, 3, 7) (**Figure 4*a***). More specifically, residues Pro264, Ile265, and Leu267 in L1; Pro299 in L2; and Trp290 are predicted to mediate interaction with UBC8 (**Figure 4*a***).

So far, all characterized E2-E3 pairings are mediated by low-affinity hydrophobic interactions with dissociation constants (K_D) in the lower μM range, which has also now been shown in plants for the soybean PUB13-E2 complex (77). Comparison of the yeast Prp19 U-box domain with known RING-E2 complex structures indicates that both U-boxes share interaction surfaces with RING domains that include these residues (100).

U-box superimposition of the ancestral UFD2s from *Arabidopsis*, *S. cerevisiae*, and human shows a close alignment, supporting their evolutionary relatedness (99, 126) (**Figure 4*b***). Structure models also support the importance of the highly conserved isoleucine (Ile) and proline (Pro) for E2 pairing with UFD2, PUB13, PUB14, and PUB22 (**Figure 4*c***).

Variations in the sequences around these key residues cause slight changes in L1-L2-H1 arrangement, resulting in distinct E2 binding specificities and affinities. A comparison of U-boxes from canonical PUBs Prp19 and PUB22, as well as that of the less conserved PUB62, reveals several structural differences (23) (**Figure 4*c***). First, Prp19 shows only slight changes in L1 alignment, while there are more pronounced changes between all U-boxes in L2. PUB22 displays

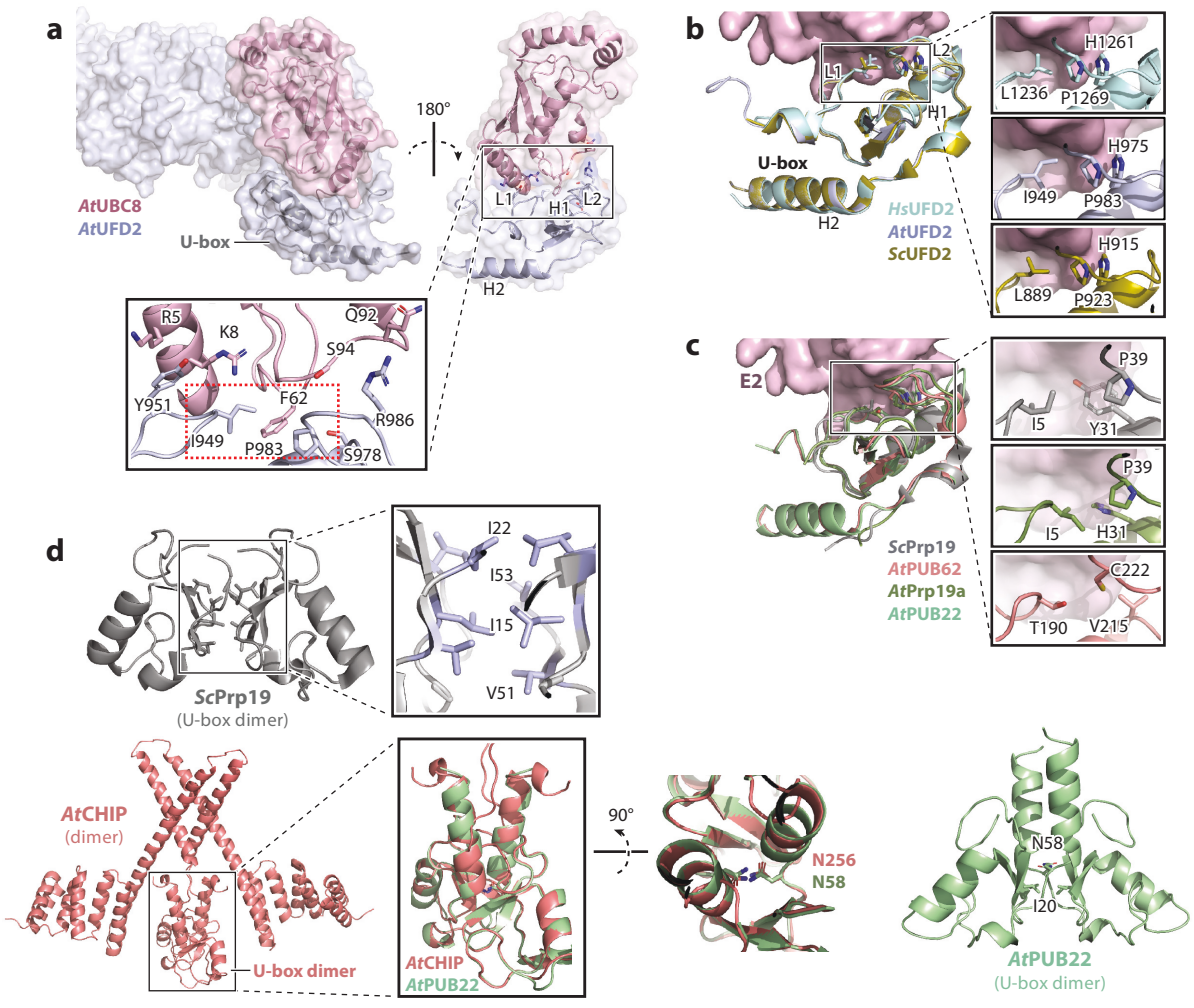

Figure 4

Structure analysis of plant U-boxes. (*a–c*) Structure analysis of the U-box and E2 interface. (*a*) The U-box domain of the *At*UFD2 pairs with *At*UBC8 via its L1, L2, and H1. *At*UBC8 (PDB ID 4X57) was modeled with *At*UFD2 (generated model with UBC8 was simulated via PyMOL based on PDB ID 3L1Z as a reference E2) (*b*) Superimposition of modeled *At*UFD2 U-box with *Hs*UFD2 (PDB ID 3L1X) and *Sc*UFD2 (PDB ID 2QIZ) orthologs, suggesting conserved interaction modes. Magnified views highlight residues mediating the interaction between UFD2 from different species and the E2, suggesting that they are conserved. (*c*) Superimposition of U-box domains from *Arabidopsis* PUBs and *Sc*Prp19 (PDB ID 2BAY) as a reference structure suggests conserved structure between homologs. Key residues mediating interaction with the E2 are highlighted in the magnified views. (*a–c*) *At*PUB22, *At*PUB62, *At*UFD2, *At*Prp19a, and *At*CHIP models were generated and validated by I-TASSER (149) and Phyre^2 (55). All models were adjusted and visualized via PyMOL. (*d*) U-box domain mediates dimerization via surfaces distal from those required for E2 docking. Dimerization interface of U-box is shown for *Sc*Prp19, *At*PUB22, and *At*CHIP. ScPrp19 dimer analyzed based on PDB ID 2BAY; *At*PUB22 and *At*CHIP were analyzed based on generated dimer model that was simulated based on *Sc*Prp19 superposition. Abbreviations: At, *Arabidopsis thaliana*; CHIP, C TERMINUS OF HSC70-INTERACTING PROTEIN; H1, α-helix1; *Hs*, *Homo sapiens*; I-TASSER, iterative threading assembly refinement; L1, loop 1; L2, loop 2; PDB ID, Protein Data Bank identification; Phyre^2, protein homology/analogy recognition engine v. 2.0; Prp19, PRECURSOR RNA PROCESSING 19; PUB, plant U-box protein; *Sc*, *Saccharomyces cerevisiae*; UBC, ubiquitin-conjugating enzyme; UFD2, UBIQUITIN FUSION DEGRADATION 2.

an elongated L2 that creates an additional interaction surface with the E2. Close-ups of the PUB22 and Prp19 interface with the E2 suggest that the conserved Ile and Pro take up similar positions, while different residues on H1 (Prp19 His31 and PUB22 Trp40) contact the E2, and may result in different E2-E3 affinities (**Figure 4*c***). By contrast, the less conserved U-box of PUB62 does not have any of the canonical residues yet forms a similar scaffold that results in hydrophobic contacts that are predicted in the interaction interface.

Oligomer: molecule consisting of several similar or identical repeating units (monomers)

Conformational restriction: process of reducing the number of potential conformations (positions) of a molecule or molecule segments

4.2. Homo- and Heterooligomerization of Plant U-Box Proteins

In addition to receiving the ubiquitin-loaded E2, the U-box can also mediate dimerization using a surface distal to the one mediating E2 docking (**Figure 4*d***), as was initially suggested for PUB14 by chemical shift analysis (1). Elucidation of the yeast Prp19 structure revealed that the U-box dimerizes within a tetramer stalk, formed by coiled coils (128). Interruption of U-box dimerization by mutating the corresponding residues in the hydrophobic interface of human Prp19 impaired activity (23). In *Arabidopsis*, PUB22 was shown to homo- and heterodimerize, and sequence comparison between dimerizing Prp19 from yeast and the murine CHIP showed that key hydrophobic residues on the N-terminal portion of the U-box, as well as a hydrogen-bonding asparagine (Asn), were conserved in CHIP and PUB22, and required for in vivo interaction (32, 128, 154) (**Figure 4*d***). Dimerization can contribute to autoubiquitination in *trans* between PUB protomers (32, 118). ARM repeats can also mediate oligomerization, as in the case of PUB10 (53), opening the possibility of higher-order oligomers, similar to Prp19 (23, 128).

Dimerization can also contribute to the priming of the E2-ubiquitin conjugate by favoring a nucleophilic attack by an available lysine (Lys) on the substrate onto the active site thioester bond (9). The priming mechanism can require the formation of a dimer, in which each protomer contacts ubiquitin to position it for catalysis (104). In line with this, an E2 mutant that irreversibly binds ubiquitin displays stronger interaction with PUB22, suggesting that ubiquitin contributes to E2-E3 pairing (127). However, yeast and human UFD2s are monomeric and utilize an allosteric mechanism for conformational restriction (105). Interestingly, PUB22 is active as both a monomer and a dimer/oligomer, opening the possibility that PUBs employ a dimerization-independent mechanism to increase reactivity of the ubiquitin-E2 conjugate (31). Therefore, dimerization of PUBs has various functions, which include facilitating autoubiquitination, potentially priming the E2-ubiquitin conjugate, and other roles that may determine E2-E3 pairing. The exact impact of hetero- and homodimerization on PUB activity still requires further analysis.

5. HUBS OF CELLULAR SIGNALING

Cell surface receptors perceive a diverse range of cellular cues that impact plant physiology, development, and stress acclimation. Along with other posttranslational modifications that directly regulate enzyme activity and localization, ubiquitination plays a central role in many signaling pathways, ultimately reshaping the proteomic landscape of a cell (38, 86).

5.1. E3 Ligase–Protein Kinase Modules

Many recent studies have underlined the coupling of kinase signaling with ubiquitin-mediated responses, and an interdependent phosphorylation-ubiquitination circuitry has started to take shape (124). The PUB-kinase connection was first observed through the interaction of the PUB ARM REPEAT CONTAINING1 (ARC1) with the *S*-LOCUS RECEPTOR KINASE (SRK) during the self-incompatibility response in *Brassica napus* (28, 39) (**Supplemental Table 1**). Later work suggested a more general link between PUBs and kinases, showing that ARC1 and the

Supplemental Material >

closely related PUB13/PUB14 could interact with various *S*-locus receptors in yeast two-hybrid assays (110) (**Supplemental Table 1**). In the years that followed, a surge of studies showed that PUBs interact with kinases, namely cytoplasmic protein kinases (24, 32, 92, 141) and RLKs (15, 25, 30, 59, 76, 79, 83, 91, 134, 157, 158) involved in diverse pathways (**Supplemental Table 1**).

As a means to dampen signaling, ligand-bound membrane receptor complexes can be endocytosed and degraded in the vacuole. For example, several immune receptors are endocytosed following the perception of immunogenic ligands (90), as is SRK following pollination (47). Receptor ubiquitination is likely involved in this process, potentially acting to recruit vesicular traffic machinery. A first clue came when it was discovered that PUB12 and PUB13 ubiquitinate the immune receptor FLAGELLIN-SENSING2 (FLS2) (83). However, it was later shown that PUB13 also associates with and ubiquitinates the hormone receptor BRASSINOSTEROID INSENSITIVE1 (BRI1), contributing to its endocytosis following activation (157). In addition, the PUB13 ortholog in rice, SPL11, has been shown to destabilize the RLK SPL11 CELL-DEATH SUPPRESSOR2 (SDS2) (30). It is thus possible that PUB12/PUB13 and their orthologs contribute broadly to the endocytosis of RLKs. Indeed, PUB12 and PUB13 also interact with various additional RLKs, including the LYSM-CONTAINING RECEPTOR LIKE KINASE 5 (LYK5) (76), and PUB13 and PUB14 interact with additional SRKs (110) (**Supplemental Table 1**). By contrast, PUB22–PUB24 interact with the cytoplasmic mitogen-activated protein kinase MPK3 but do not mediate its ubiquitination under tested conditions (32). Instead, PUB22 ubiquitination results in vacuolar degradation of exocyst component Exo70B2 (and potentially Exo70B1) to dampen secretion (118). This mechanism may be broader, as the exocyst complex mediates the tethering of post-Golgi vesicles to the plasma membrane during secretion, and Exo70B1/Exo70B2 were recently shown to mediate the delivery of FLS2 (138). In addition to these PUB-mediated degradation mechanisms, it is important to note that kinase stability is regulated by additional pathways (150), highlighting the importance of their degradation for cellular homeostasis.

However, PUBs can also positively regulate signal propagation. For example, the *Arabidopsis pub4* mutant is impaired in responses triggered by several immunogenic elicitors (24, 25) and interacts with the receptor CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) (25), as well as the RLCK BOTRYTIS-INDUCED KINASE1 (BIK1), a major immune regulator (24). BIK1 is a convergent substrate of multiple receptors and is rate-limiting for immune signaling in *Arabidopsis*. Detailed analyses suggest that PUB4 contributes to the degradation of inactive BIK1 but supports accumulation of activated BIK1, ultimately contributing to signal competence (24). BIK1 is also ubiquitinated and regulated by PUB25 and PUB26, which specifically target inactive BIK1 (133). The emerging picture suggests that PUBs form a complex with nonactivated kinases to precisely regulate their accumulation. As the aforementioned kinases do not seem to be inherently unstable proteins, it is therefore likely that PUBs modify substrates in a context-dependent manner, influenced by specific phosphorylation codes, E2 pairing, and other regulatory components (**Supplemental Table 1**).

5.2. Regulation of Plant U-box Protein Activity by Kinases

For many of the so-far identified PUB-kinase modules, which largely involve class IV and V PUBs with ARM repeats, the E3s are phosphorylated upon activation of the interacting kinase. This includes PUB4, PUB13, PUB22, PUB25, and PUB26, which are all phosphorylated on residues located in predicted disordered stretches connecting the U-box and ARM domains (24, 32, 124, 141, 157). However, the impact of phosphorylation on E3 activity varies. For instance, phosphorylation of PUB13 on Ser344 increases its association with BRI1 and is required for its ubiquitination

(157). This is reminiscent of the rice ortholog SPL11, which only interacts with active SDS2 (30). By contrast, phosphorylation of PUB25 and PUB26 at orthologous sites in the linker region (Thr95 and Thr94, respectively) increases their ubiquitination activity (133). The closely related PUB22 is modified by MPK3 on the orthologous linker residue Thr88 as well as Thr62 in the U-box domain (32). Phosphorylation of both residues increases PUB22 stability; however, phosphorylation of Thr62 regulates autoubiquitination. Autoubiquitination is a trait common to most E3 ligases and may lead to inherent proteasome-dependent instability (**Figure 1*b***). Thr62 is located distal to the E2 interaction surface, and its phosphorylation inhibits oligomerization and thus autoubiquitination (**Figure 4*b***), resulting in PUB22 accumulation, engagement of targets, and the dampening of immune signaling (32). PUB25 is additionally phosphorylated in a nearby residue (Ser63) and may also be stabilized in response to immune stimulation (133). Similarly, PUB11 turnover is decreased in response to drought and abscisic acid (ABA) treatment (15), while PUB18 needs to be incubated in cell extracts to be activated (112). Therefore, the activity of both PUB11 and PUB18 may also require in vivo posttranslational regulation.

Overall, it seems likely that phosphorylation regulates PUB activity broadly, but through varying mechanisms. PUBs are also regulated by binding partners—as shown for PUB25 and PUB26, which are negatively regulated by heteromeric G protein complex proteins (75, 133, 142), and for PUB13, which is regulated by the small GTPase RabA4B (2)—and are also almost certainly regulated by the dynamic pairing with E2s.

5.3. Pairing with E2 Ubiquitin-Conjugating Enzymes

Proteins can be mono-, multi-mono-, or polyubiquitinated, each affecting protein fates in different ways. While E3 ligases are typically thought to be target specificity determinants, it is the E2 UBCs that largely dictate the type of ubiquitin chain that will be generated (**Figure 1*a***). The ubiquitin C terminus can be attached to Lys residues as well as N-terminal methionine (Met) residues (67) (**Figure 1*c***). As ubiquitin itself contains seven Lys residues, differently linked chains can be built depending on which Lys is used to build the chain. The type of linkage determines the topology of the ubiquitin polymer, which is then decoded by ubiquitin receptors that mediate distinct downstream processes, ranging from proteasomal degradation to endocytosis or DNA repair (**Figure 1*a***). In most cases, the E2 enzyme determines the linkage specificity, and therefore it is possible that E3s may pair with multiple E2s in a context-specific manner (**Figure 1*a***).

Studies using in vitro autoubiquitination activity had previously shown a pairing specificity between E2 and E3s (21, 65, 66). A pairwise screen to identify E2s that interact with PUB22 in vivo detected 11 UBCs belonging to 4 different groups, out of 37 tested *Arabidopsis* E2s (127). Interaction specificity was dictated by both the U-box as well as the ARM repeats. Further analyses with a subset of E2s showed that PUB22 and the closely related PUB20 and PUB24, as well as the UND-containing PUB4, interact with UBC35 in vivo. Because UBC35 is dedicated to building Lys63-linked chains, these E3s most likely mediate the modification of substrates with this chain type (107, 127). By contrast, PUB13, which was shown to control FLS2 levels and mediate BRI1 internalization, did not interact with UBC35 (127), suggesting that it may pair with other E2s to mediate endocytosis or may require activation. It is conceivable that the pairing between E2s and PUBs is dynamic and changes depending on the cellular status. For example, following immune elicitation by flg22 treatment, interaction between PUB22 and UBC35 is induced, while pairing is inhibited with the highly processive UBC30, which does not have a defined linkage-building activity (127). Notably, *Arabidopsis* mutants lacking UBC35 and its closest homolog UBC36 (*ubc35 ubc36*) are compromised in surface-receptor- and NLR-mediated responses (95, 127, 135), further highlighting their importance to plant immune responses.

Given the different roles of each type of ubiquitin chain, it is important to determine the physiological E2 components. Group VI UBCs, such as UBC8, are widely used to determine E3 activity because they are highly processive and promiscuous. However, caution in the interpretation of in vitro assays is advised, since E2-E3 pairs that are active in vitro do not necessarily interact in vivo (127).

Biotroph: parasitic organism that maintains live host cells to derive nutrients from them

Mutualist: interacting organism with a mutual net benefit for host and microorganism

Necrotroph: parasitic organism that kills the host cells to feed on the dead tissue

6. THE ROLE OF PLANT U-BOX PROTEINS IN THE MAINTENANCE OF CELLULAR HOMEOSTASIS

The change from aquatic to terrestrial habitats required major adaptations to cope with new environments, such as limited availability of water and large changes in temperature and light, in addition to being exposed to a new diversity of microbes (24). Pathogen attack and abiotic stress cause cellular imbalances affecting speed, fidelity, and capacity of protein biogenesis and degradation systems. Proteasomal and vacuolar degradation pathways are critical to buffer these imbalances, and ubiquitination is the main signal that marks substrates for degradation. These conditions may have posed evolutionary pressures that prompted PUB diversification to contribute to stress management by modulating signaling pathways and responses during pathogen attack or abiotic stresses.

6.1. Roles During the Immune Response

The rice *SPL11* E3 was the first identified PUB with a role in immunity, based on the mutants' enhanced resistance to rice blast and bacterial blight, as well as spontaneous cell death, which is a hallmark of autoimmunity (152). The discovery of additional PUBs that negatively regulate the immune response in *Arabidopsis* and other species followed (79, 83, 91, 125, 133). Loss-of-function mutants of *PUB22*, *PUB23*, and *PUB24* show enhanced signaling triggered by immunogenic elicitors, suggesting a connection to RLK-mediated pathways (125). Notably, the triple mutant *pub22 pub23 pub24* displays broad resistance against pathogens with distinct infection strategies, including the hemibiotrophic bacteria *Pseudomonas syringae* pv. *tomato*, the oomycete *Hyaloperonospora arabidopsidis* (a biotroph) (125), and the fungus *Piriformospora indica* (a mutualist) (48), as well as the fungus *Fusarium oxysporum* (a necrotroph) (16), which is surprising given the antagonistic defense responses of *Arabidopsis* plants towards biotrophic and necrotrophic pathogens (11). In contrast to *pub12 pub13* (74, 158) and *pub4* (25) (class IV), *pub22 pub23 pub24* (125) and *pub25 pub26* (133) (class V) mutants do not accumulate high levels of salicylic acid and they grow normally, suggesting different roles. Further differences include the production of reactive oxygen species triggered by pathogen-associated molecular patterns (PAMPs), which is enhanced in all of the above-mentioned PUB mutants, but only *pub22 pub23 pub24* displays prolonged reactive oxygen species production (85, 127, 134). These observations underline the complementary but still distinct roles in dampening immune signaling.

Maintaining the link to the plasma membrane, PUB ligases also play a role in the regulation of cell death during immune responses activated by the detection of virulence factors known as effectors through NLR sensors or surface receptor proteins. The tomato Cf-9 is a transmembrane receptor that contains extracellular leucine-rich repeats and a short cytoplasmic tail (35). Cf-9 confers resistance to *Cladosporium fulvum* races expressing the Avr9 effector. Silencing of the *Nicotiana benthamiana* PUB CMPG1 impaired cell death triggered by Cf-9/Avr9 (35), as well as by plasma membrane–located Cf-4/Avr4, Pto/AvrPto, and the oomycete cellulose-binding elicitor lectin (CBEL) (33). Similarly, PUB17 was also required for Cf-9/Avr9- and Cf-4/Avr4-triggered cell death (148). By contrast, CMPG1 was dispensable for responses activated by the nucleocytosolic NLRs R3a, R2, and Rx (33).

6.2. Targeting of Plant U-Box Proteins by Pathogen Effectors

As part of their virulence strategy, plant pathogens secrete effector proteins into host cells to evade detection or interfere with immune signaling (8). Substantiating their central role in the immune response, PUBs are targeted by pathogenic effectors, including Avr3a (10) and Avr1d (77) from *Phytophthora infestans*, as well as XopP from *Xanthomonas oryzae* (46). All three effectors inhibit autoubiquitination by interacting with the U-box, thereby stabilizing PUBs, many of which may be regulated through autoubiquitination and degradation (10, 15, 32, 77, 133), and in addition also stabilizing their substrates (10, 46, 77). The effectors Avr3a and Avr1d target CMPG1 in *N. benthamiana* and PUB13 in soybean, respectively. Silencing of the corresponding *PUBs* increased resistance against both *P. infestans* (10) and *Phytophthora sojae* (77). Both effectors carry a signal peptide followed by a classical RxLR motif and an effector domain (144). A model of the interacting UBC8 with the soybean PUB13 shows the residues mediating pairing (**Figure 5**). Comparison with the structure of Avr1d in complex with the U-box of PUB13 revealed that the effector targets the same conserved residues (77) (**Figure 5**). Indeed, Avr1d uses a hydrophobic groove that engages L1 and L2 of PUB13 to bind with 400–500 times higher affinity and, thus, outcompete binding of host E2s (**Figure 5**). Also interesting are the structural similarities between Avr1d (77), *P. infestans* Avr3a (143), as well as *Phytophthora capsici* Avr3a (2LC2), suggesting a conserved strategy for E3 inhibition. Because Avr1d engages conserved features of the U-box for docking (**Figure 5**), it will be interesting to determine its U-box binding specificity. By contrast, the *X. oryzae* effector XopP binds to the rice PUB44 U-box, but not the closely related PUB45 or PUB46. The unique residues Leu86 and His94 play defining roles and are predicted to be located on the α-helix2, which is distal and on the opposite side of the E2-interacting surface, suggesting an alternative mechanism of inhibition (46). For instance, XopP may change the orientation of the U-box to ARM repeats, inhibiting auto- and substrate ubiquitination (46).

The effector RipAC from *Ralstonia solanacearum* targets the plant E3 ubiquitin ligase PUB4 to inhibit RLK-triggered immunity (24). Targeting PUBs that act as negative regulators, as in the case of *Phytophthora* effectors, and positive ones, as with *Xanthomonas* and *Ralstonia*, may reflect

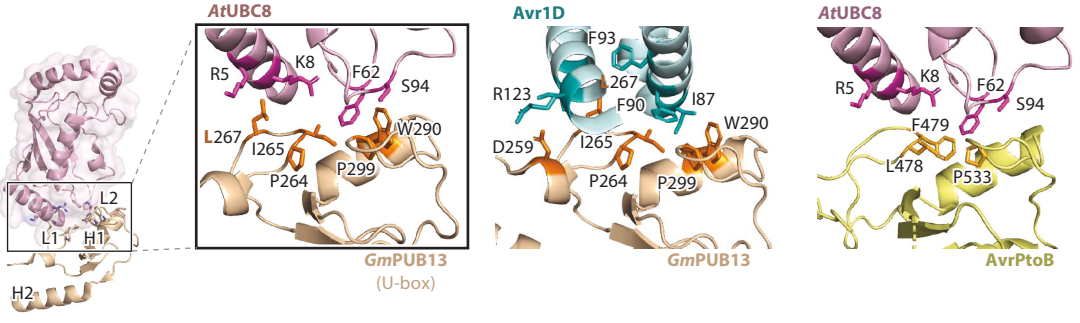

Figure 5

Pathogen effectors can target or mimic U-box domains. The *Phytophthora infestans* effector Avr1D (*teal*) binds the U-box domain (*orange*) of *Gm*PUB13 by engaging similar residues as *At*UBC8 (*pink*). Residues predicted to mediate the interaction are highlighted in darker colors. *At*UBC8 (PDB ID 4X57) was modeled with the E3 *Gm*PUB13 (PDB ID 7C96) and the *Pseudomonas syringae* effector AvrPtoB (PDB ID 2FD4). Generated models of *Gm*PUB13 and AvrPtoB with *At*UBC8 were simulated via PyMOL based on PDB ID 3L1Z as a reference E2. Also shown is the structure of *Gm*PUB13-Avr1D (PDB ID 7C96). Abbreviations: *At*, *Arabidopsis thaliana*; *Gm*, *Glycine max*; H1, α-helix1; H2, α-helix2; L1, loop 1; L2, loop 2; PDB ID, Protein Data Bank identification; PUB, plant U-box protein; UBC8, ubiquitin-conjugating enzyme 8.

the different lifestyles of these pathogens. CMPG1 is stabilized during later stages of infection, in which an increased immune response may help the necrotrophic *Phytophthora* kill the host cell (10). By contrast, the hemibiotrophs *Xanthomonas* and *Ralstonia* aim to inhibit immune signaling (46).

The U-box domain itself is also mimicked by effectors such as AvrPtoB from *P. syringae*, which hijacks the host ubiquitination machinery to mediate the degradation of key components of the immune system, including the master regulator of salicylic acid NONEXPRESSOR OF PATHOGENESIS-RELATED 1 (NPR1), the immune receptor CERK1, and the exocyst subunit Exo70B1 (14, 34, 49, 139). In AvrPtoB, the conserved U-box Ile is replaced by Leu478 and Phe479, which may result in a higher affinity pairing (**Figure 5**). In vitro analyses indicate that the resulting E2-AvrPtoB pairing is highly active (49, 65, 108).

In line with these observations, plasma membrane–associated SAUL1 (PUB44), which functions as a positive regulator of surface receptor signaling (27), is monitored by the NLR SUPPRESSORS OF CHS1–2, 3 (SOC3) in *Arabidopsis* (121). In addition, the PUB17/PUB18, and potentially PUB22–PUB24, substrate Exo70B1 is associated with the truncated NLR TIR-NBS (TN2) (156), suggesting that effectors target not only PUBs but also their substrates, underlining that these are important nodes in plant immunity.

6.3. The Stress Response–Development Nexus

Many additional examples reveal roles for PUBs beyond immune signaling. Although a significant proportion of studies have highlighted the role of PUBs in stress responses, connections to developmental pathways are starting to be revealed. For example, in addition to regulating immune responses (133) and freezing tolerance (141), *PUB25* and *PUB26* also repress the duration of cell proliferation and the change into postmitotic cell expansion in petal development (72). In addition, *PUB4*, which contributes to the accumulation of BIK1 and immune homeostasis (24), is connected to CLAVATA3 (CLV3)-mediated stem cell maintenance and participates in shoot apical meristem size in a manner similar to the known CLV-related genes (60, 61). The mutants of *PUB4* are less sensitive to the peptide hormone CLV3, as reflected by reduced inhibition of root cell proliferation and columella stem cell maintenance (61), and other hormone-related defects (61, 132, 142). Of note, in a genome-wide study involving 451 *A. thaliana* accessions, PUB4 had the strongest effect on the adaptive trade-offs between seed production and abiotic stress resistance in contrasting environments. It additionally displayed signatures of balancing selection, suggesting that it plays an important role in balancing growth and stress responses (129).

This opens the possibility that other PUBs, which may have primarily evolved to cope with different stresses, are potentially able to integrate both stress and developmental responses. PUBs are conserved in economically valuable crops such as rapeseed (*Brassica oleracea*) (42), banana (*M. acuminata*) (43), wheat (*Triticum aestivum*) (57), cotton (*Gossypium hirsutum*) (84), tomato (115), and soybean (*G. max*) (137, 153), holding biotechnological promise.

Hormones shape plant physiology and allow plants to adapt to changing environments. ABA is a vital phytohormone controlling development and is especially important for responses to environmental stresses including drought and salinity (93). ABA signaling in *Arabidopsis* is controlled by a derepression mechanism, whereby the ABA-bound coreceptor complex inhibits repressive phosphatases (93). One of these phosphatases is ABA-INSENSITIVE 1 (ABI1), which is targeted by PUB12 and PUB13, leading to its degradation (64). PUB12 and PUB13 additionally target the brassinosteroid (BR) receptor BRI1 (157). BRs are steroid hormones essential for plant growth and development, but recent studies have started to reveal that they have additional roles in stress (103) and the integration between stress and development by acting antagonistically to immunity

(82). However, the biological functions of PUB13 are likely to be more complex than anticipated, reflected by additional phenotypes including salicylic acid–dependent spontaneous cell death and early flowering (74), roles that are conserved in rice homologs (80, 130, 152). Therefore, PUB12 and PUB13 engage various pathways, potentially contributing to their coordination during different conditions. Further links to BR signaling include PUB30 (class IV), which interacts with BRI1 KINASE INHIBITOR1 (BKI1), which associates with BRI1 to inhibit the formation of a complex with its coreceptor BRI1-ASSOCIATED KINASE1 (BAK1) (140, 155). Further downstream, PUB40 (class IV) mediates degradation of BR-RESPONSIVE TRANSCRIPTION FACTOR1 (BZR1) in *Arabidopsis* roots (58). The role of PUBs in regulating BR signaling is conserved across species: Inactivation of the rice homolog of *Arabidopsis* PUB30, TUD1/PUB75, affected growth and reduced sensitivity to brassinolide (44), while rice PUB24 (the homolog of *Arabidopsis* PUB44/SAUL1) negatively regulates BR responses by targeting the rice ortholog of BZR1 (92).

Adding complexity to their functions, PUBs also negatively regulate drought and ABA responses in *Arabidopsis* and other species (15, 19, 20, 81, 110, 112, 113, 119, 120, 136, 137). For example, *PUB22/PUB23* (class IV) and *PUB18/PUB19* (class V) mutants are more tolerant to drought (20, 113). In contrast to *pub18 pub19* phenotypes, *pub22 pub23* drought tolerance does not require ABA, again highlighting differences and complementarity between class IV and class V PUBs (20, 81, 113). Interestingly, PUB22/PUB23 and PUB18/PUB19 target Exo70B2 and Exo70B1, respectively, and mutant plants are more sensitive to drought (112, 118). Similarly, *B. napus* ARC1 is proposed to target Exo70A1 during the self-incompatibility response (62, 109). Secretion contributes to reshaping of the plasma membrane protein composition (e.g., receptor kinases, transporters, NADPH oxidases). This highlights that PUBs regulate cellular processes of general importance in plants, putting them at the interface between stress and development.

SUMMARY POINTS

1. UFD2, Prp19, and CHIP are ancestral U-box proteins that are likely to carry out functions common to all eukaryotes. During the course of evolution, the U-box fused with new domains, resulting in the emergence of plant U-box proteins (PUBs), which are specific to the green lineage.
2. The emergence of PUBs was accompanied by neofunctionalization from ancestral U-box proteins and a significant expansion.
3. The surge of PUB expansion events at specific stages of evolution, such as during terrestrialization, coupled with the large body of evidence linking them to stress management suggests that they evolved to cope with new challenges posed by these new environments.
4. PUBs have adopted different roles to contribute to signal transduction and the maintenance of cellular homeostasis, often acting as hubs that initiate feedback loops. This is highlighted by their close association with kinases involved in stress signaling and development.
5. Dual functions in stress and developmental pathways may allow PUBs to balance stress responses with developmental trade-offs.
6. PUBs cooperate with sets of E2s and are therefore likely able to generate different types of chains. PUB-E2 pairing is dynamic and changes depending on the cell status.

FUTURE ISSUES

1. To obtain deeper insight into PUB activity and to better understand the ubiquitination process, structural information will be pivotal to dissecting the diverse mechanisms underlying substrate recognition and E2 pairing. Recent advances in machine learning algorithms to predict protein structures provide the unprecedented ability to make structural hypotheses and inform experimental validation (5, 111).
2. The study of PUBs, which has led to identification of substrates and the pathways that they participate in, as well as the elucidation of some aspects of their regulation, holds promise for biotechnological applications. On one hand, it is possible to directly capitalize on valuable genetic traits conferred by PUBs, and on the other hand, PUBs can be exploited as platforms to mediate the targeted degradation of proteins of interest through genetic engineering.
3. PUBs have evolved unconventional, and therefore highly interesting, domain combinations. Of particular interest are class VI PUBs, which are predicted to contain both a U-box and a kinase domain, combining the capacity to execute ubiquitination as well as phosphorylation. The USP domain in class VI PUBs may additionally act as a stress switch. Understanding how these different activities integrate mechanistically will be an exciting advancement.
4. A central question involves what type of chains are generated by different E2s and how they affect the fate of the modified substrate. Is there a division of labor between E2s, in which one E2 primes and a second elongates the ubiquitin chain with specific linkage?

DISCLOSURE STATEMENT

N.S. has an equity interest in and serves on the Scientific Advisory Board of Oerth Bio, a company that designs targeted protein modulators for plant health applications. The other authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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157. Revealed that PUB12 and PUB13 mediate BRI1 endocytosis in addition to degradation of FLS2.

159. Shows that CHIP cooperates with the autophagy receptor NBR1 to maintain proteostasis via distinct pathways.

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Errata

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