

The Expanding Universe of Ubiquitin and Ubiquitin-Like Modifiers¹

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As we near a complete understanding of plant transcriptomes and their corresponding proteomes, a remaining frontier in plant biology will be the in-depth definition of the posttranslational modifications that help generate the diverse array of appropriately functioning proteins from more finite genomic information. It is now obvious that plant proteins are subject to a wide array of possible modifications that regulate their structure, activity, interactions, location, and/or half-life. These alterations are often genetically predetermined, transient, and highly dynamic, thus providing near unlimited layers of control across the life span of individual proteins. The continually expanding list of over 200 possibilities include the covalent addition of methyl, acetyl, phosphate, and glycosyl moieties, fatty acids, vitamin cofactors, nucleosides, and even other proteins.

The first polypeptide modifier to be discovered was ubiquitin (Ub), a highly conserved, 76-amino acid protein ubiquitously present in eukaryotes (Hershko and Ciechanover, 1998; Smalle and Vierstra, 2004; Vierstra, 2009). Via an intricate enzymatic cascade, Ub becomes attached to a multitude of targets through an isopeptide bond between its C-terminal Gly residue and one or more accessible Lys residues in its targets. This addition can then be reversed by a family of deubiquitylating proteases (DUBs) that uniquely recognize and cleave the bond linking the two moieties, thus generating a reaction cycle akin to those involving protein kinases and phosphatases.

Elucidation of the Ub enzymatic paradigm was followed by the discovery that it represents just one member from a constellation of peptide tags that become reversibly attached to other intracellular constituents, including lipids and prenyl groups, in some cases using parallel chemistries indicative of a common ancestor (Downes and Vierstra, 2005; Kerscher et al., 2006; Burroughs et al., 2012). In plants, these Ub-like modifiers (UBLs) currently include RUB (for related to ubiquitin, or Nedd8 in yeast [*Saccharomyces cerevisiae*] and animals), SUMO (for small ubiquitin-like modifier),

ATG8 (for autophagy8) and ATG12, MUB (for membrane-anchored ubiquitin-fold protein), UFM1 (for ubiquitin-fold modifier1), URM1 (for ubiquitin-related modifier1), HUB1 (for homology to ubiquitin1), and a diverse assortment of proteins that harbor structurally related folds fused translationally to other domains. The varied processes managed by these modifiers are extraordinary, ranging from cellular housekeeping, nutrient recycling, and sulfur chemistry to selective protein turnover, transcriptional regulation, chromatin remodeling, and RNA metabolism (Miura et al., 2007a; Hochstrasser, 2009; Vierstra, 2009; Li and Vierstra, 2012). In this review, I provide a glimpse into the importance of these modifiers in plant biology, an emerging appreciation for how these modifiers intersect to expand their influence, and how they might have evolved from prokaryotic progenitors. Specific examples supporting these themes are also well illustrated by the collection of papers and other reviews included in this Focus issue on "Ubiquitin in Plant Biology."

Ub AND THE UBL SUPERFAMILY

Ub and UBLs are characterized by a core β -grasp fold of approximately 70 amino acids that is phyletically widespread and of ancient origin (Burroughs et al., 2012). The fold contains secondary structure elements arranged in a $\beta\beta\alpha\beta\beta$ order and is so named because the resulting five-stranded β -sheet appears to grasp the α -helix cradled diagonally (Fig. 1). Often, a short α -helix intervenes between the β_4 and β_5 strands. This compact arrangement generates a highly stable tertiary structure that folds rapidly and is often resistant to environmental perturbation such as heat. Sometimes elaborating this fold are additional α -helices, loops, and N-terminal extensions that likely endow unique binding surfaces to individual members. Whereas the Ub/UBLs often share little sequence identity, remarkable conservation is seen within each family across eukaryotes (e.g. Ub is 96%–97% identical among the plant, yeast, and animal kingdoms), strongly suggesting that most surfaces of each β -grasp fold participate in essential interactions during the reaction cycle of each modifier. With the exception of HUB1 and MUB, a short C-terminal sequence protrudes from the β -grasp core that ends upon proteolytic maturation with a Gly residue essential to each Ub/UBL reaction scheme (Fig. 1).

¹ This work was supported by the U.S. National Science Foundation Arabidopsis 2010 Program (grant no. MCB-0929100) and Plant Genome Program (grant no. IOS-1232752), the Department of Energy Basic Energy Sciences Program (grant no. DE-FG02-88ER13968), and the U.S. Department of Agriculture National Institute of Food and Agriculture (grant no. 2008-02545).

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www.plantphysiol.org/cgi/doi/10.1104/pp.112.200667

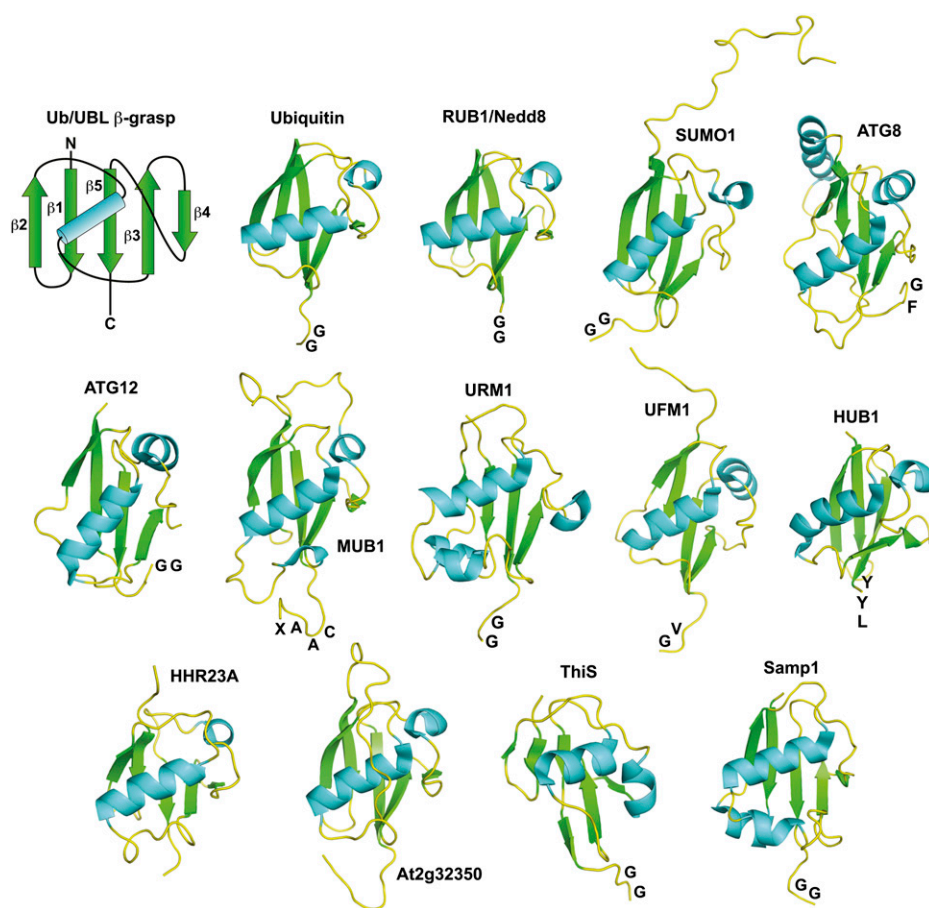


Figure 1. Three-dimensional ribbon diagrams of the Ub/UBL β -grasp folds from eukaryotes and prokaryotes. Shown are the processed forms with the exposed C-terminal amino acid indicated. The exceptions are HHR23A and At2g32350, which are synthesized as domain fusions. At top left is a schematic of the Ub/UBL β -grasp showing the arrangement of the α -helix and β -strand secondary structures. The Protein Database accession numbers and references for the structures are as follows: yeast ATG8, 2KWC (Kumeta et al., 2010); Arabidopsis ATG12, 1WZ3 (Suzuki et al., 2005); yeast HUB1, 1M94 (Ramelot et al., 2003); Arabidopsis MUB1, 1SE9 (Downes et al., 2006); Arabidopsis RUB1, 1BT0 (Rao-Naik et al., 1998); *H. volcanii* Samp1, 3PO0 (Jeong et al., 2011); human SUMO1, 1AR5 (Bayer et al., 1998); *E. coli* ThiS, 1F0Z (Wang et al., 2001); *Avena sativa* Ub, 1UBQ (Vijay-Kumar et al., 1987); UBL domain from human HHR23A, 1P98 (Mueller and Feigon, 2003); human UFM1, 1WXS (Sasakawa et al., 2006); yeast URM1, 2AX5 (Xu et al., 2006); and the UBL domain from Arabidopsis At2g32350, 2KAN.

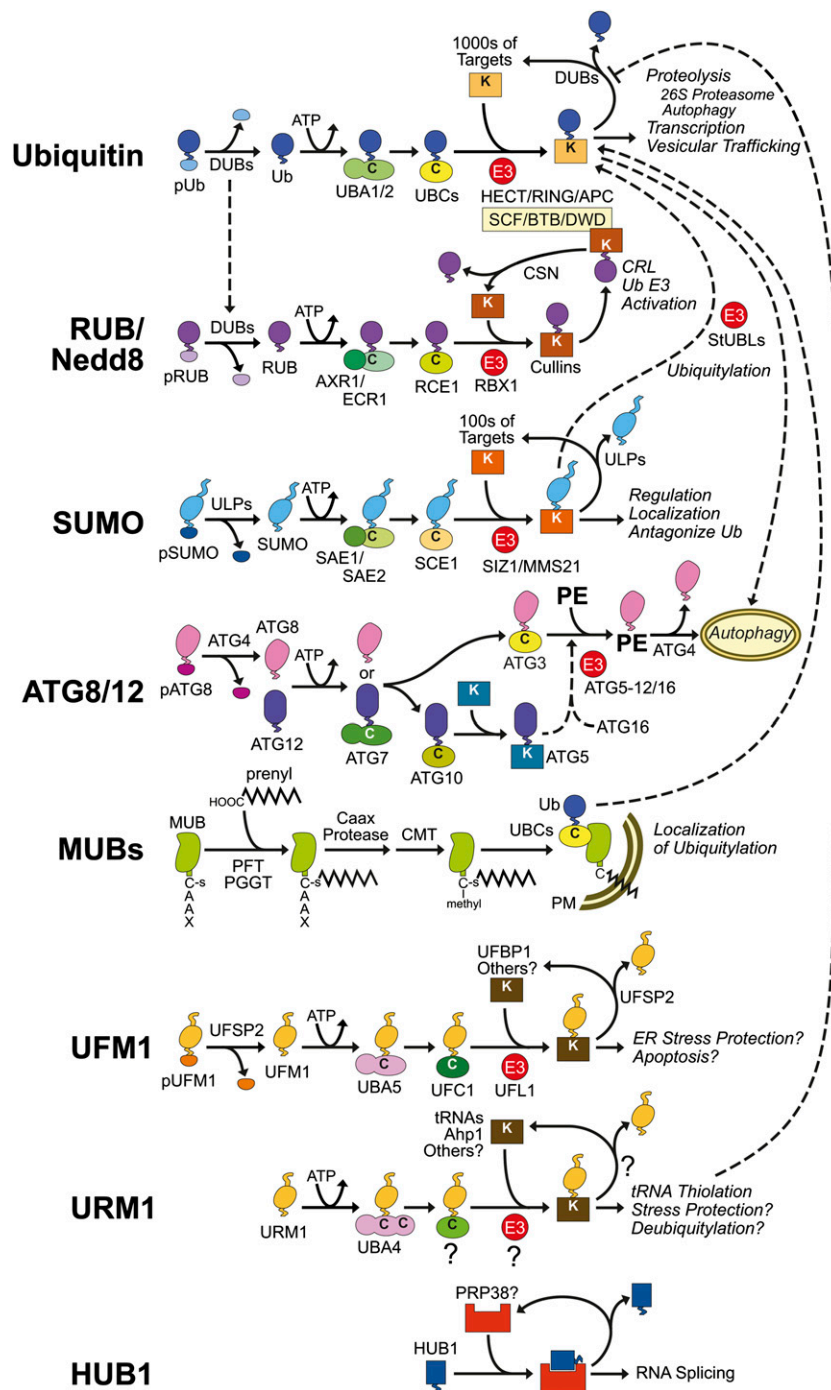
Ub and most UBLs enter an E1→E2→E3 reaction cascade that involves a signature high-energy thioester intermediate (Fig. 2). The cascade begins with activation of the Ub/UBL C-terminal Gly by an E1 that consumes ATP to first form an adenyl adduct followed by transfer of the Ub/UBL moiety onto an acceptor Cys sulfhydryl in the E1 (Smalle and Vierstra, 2004; Hochstrasser, 2009). This thioester product is then transferred to a unique Cys in an E2 via transthioleation before attachment to the target through an isopeptide bond. Although not universally required, the E3 has the crucial role of identifying appropriate targets and then spatially arranging Ub/UBL transfer. Most often, the ϵ -amine group in accessible Lys residues becomes conjugated to the C-terminal Gly, but at least for Ub, instances have been reported where the free N-terminal amino group or internal Cys, Ser, and Thr residues are acceptors (Iwai and Tokunaga, 2009; Shimizu et al., 2010; Okumoto et al., 2011). Like Ub, conjugation of most UBLs is transient and can be reversed by unique proteases that release the isopeptide-linked UBL moiety (Kerscher et al., 2006). These “deconjugases” are highly specific for the corresponding tag and fail to recognize or poorly recognize even closely related members (e.g. Ub and RUB), and in some cases they are highly selective for the modified target as well (Fig. 2).

Interestingly, the appearance of Ub/UBL-specific deconjugases likely influenced by facultative evolution the unique arrangement of the corresponding *UBQ/UBL* genes, which often express precursors with C-terminal extensions of random sequence and, in the case of Ub and RUB, as head-to-tail polymers with varying numbers of monomers (Callis et al., 1990, 1995; Doelling et al., 2002; Kurepa et al., 2003). These novel genes presumably arose by mutation or unequal crossover but were genetically benign to Ub/UBL function given that the cognate deconjugases could easily correct the defect proteolytically following translation of the aberrant transcripts.

UB AND ITS CENTRAL ROLE IN PROTEIN TURNOVER

Besides its founding status, both its depth of influence and striking genomic diversity easily place Ub at the top of the Ub/UBL pantheon. This β -grasp protein (Fig. 1) is synthesized by a complex assemblage of *UBQ* genes that express the full-length sequence fused either with other proteins or with varying numbers of Ub monomers concatenated head-to-tail, with the last monomer capped at its C terminus by extra amino acids. Following translation, the fusions are rapidly processed by DUBs to release functional Ub monomers

Figure 2. Schematic diagrams of the enzymology, targets, and function(s) of individual Ub/UBL pathways and known interactions among them (dashed lines). Where available, the protein names for the various components are indicated with the Arabidopsis nomenclature used if known. Question marks indicate components that might exist but have not been identified.



with an exposed C-terminal Gly. In Arabidopsis (*Arabidopsis thaliana*), for example, 15 *UBQ* genes exist, with one expressing six Ub units linked in frame (Callis et al., 1995). Its E1→E2→E3 cascade is generated by a hierarchical organization of activities that ultimately reflects the myriad likely targets (Vierstra, 2009; Hua and Vierstra, 2011). Whereas Arabidopsis encodes for only two E1s and 37 E2s, it can theoretically express over 1,500 different E3s (Kraft et al., 2005; Stone et al., 2005; Hua and Vierstra, 2011). Remarkably, the vast number of predicted E3s in Arabidopsis is surpassed only by

the number of transcription factors, thus illustrating the importance of ubiquitylation to plant biology.

A range of outcomes is possible for the conjugation cascade, including monoubiquitylation, the attachment of multiple Ubs to different Lys residues, or the assembly of poly-Ub chains in which any of the seven Ub Lys residues provides the site to link additional Ubs (Saracco et al., 2009; Komander and Rape, 2012). The architecture of the poly-Ub chains imbues additional information related to the fate of the modified protein. As examples, Lys-48 and Lys-11-linked chains commit proteins to

degradation, while Lys-63 linked chains direct non-proteolytic outcomes related to DNA repair and endocytosis (Komander and Rape, 2012). The bound Ubs are then recognized by over 16 structurally distinct Ub recognition motifs, depending on the process (Harper and Schulman, 2006; Fatimababy et al., 2010). Numerous DUBs also exist to reverse ubiquitylation, with as many as 70 identified thus far in *Arabidopsis* (Yan et al., 2000; Vierstra, 2009). Whereas some DUBs help in general Ub recycling and poly-Ub disassembly (Doelling et al., 2001; Book et al., 2010), others uniquely act on specific ubiquitylated targets.

Proteomic studies in turn have identified at least 1,000 *Arabidopsis* proteins modified with Ub (Maor et al., 2007; Saracco et al., 2009; D.Y. Kim and R.D. Vierstra, unpublished data), strongly suggesting that many of the approximately 1,500 potential E3s are paired with specific targets. The lists of substrates include numerous nuclear proteins involved in chromatin structure, transcription, and RNA processing, enzymes that control rate-limiting steps in metabolism, ribosomal proteins and factors that effect translation, and transporters at the plasma membrane. Alignments of Ub attachment sites mapped by mass spectrometry from human cell cultures and *Arabidopsis* failed to identify even weak consensus sequences, except for an overrepresentation of acidic residues (Kim et al., 2011, D.Y. Kim and R.D. Vierstra, unpublished data), strongly suggesting that the information that directs Ub ligation is outside the primary sequence context of the affected Lys(s).

Based on both genetic and biochemical studies, E3s represent the key factors that determine substrate specificity. To date, three main E3 types are known in plants based on their mechanisms of action and subunit organization: HECT (for homology to E6-AP C terminus), RING (for really interesting new gene)/U-box, and the CRL (for Cullin-RING ligases) E3s, with the CRLs further divided into four subtypes (Downes et al., 2003; Mudgil et al., 2004; Stone et al., 2005; Lee et al., 2008; Lima et al., 2010; Hua and Vierstra, 2011). The assembly, activity, and specificity of each CRL is defined by the combinatorial assembly of adaptors that recruit appropriate substrates and the RBX1 protein that tethers the activated Ub-E2 intermediate onto one of several Cullin scaffolds. This assembly is dynamically controlled by a modification cycle requiring another β -grasp protein, RUB (see below). Substrate recognition by the adaptors is accomplished by a diverse array of protein-protein-binding domains that often look and act like "catcher's mitts," including familiar domains such as Leu-rich, Kelch, tetratricopeptide, and armadillo repeats, and MATH, ankryin, NPH3, Jumonji C, and Tubby domains (Gagne et al., 2002; Figueroa et al., 2005; Gingerich et al., 2005; Stone et al., 2005; Hua et al., 2011).

In line with the vast number of targets, the consequences of Ub addition are highly diverse. The most common is to commit ubiquitylated proteins to breakdown by the 26S proteasome, a 2.5-MD proteolytic machine structurally conserved among eukaryotes

(Shibahara et al., 2002; Finley, 2009; Book et al., 2010). It is assembled from two particles, a 28-subunit 20S core protease (CP) and an 18-or-more-subunit 19S regulatory particle that caps one or both ends of the CP. The CP is a hollow cylinder that houses the protease active sites internally; this chamber is accessed by a gated axial channel that restricts entry to only those proteins that are deliberately unfolded and threaded inside (Finley, 2009). The regulatory particle has a set of Ub-binding receptors (RPN1, RPN10, and RPN13) that recognize appropriate substrates, particularly those modified with Lys-48 and Lys-11 poly-Ub chains, several associated DUBs that rescue the Ub moieties before target degradation, and a hexameric ring of AAA-ATPase activities that opens the CP gate and unfolds and imports substrates. A number of accessory proteins present at substoichiometric levels are also essential to the assembly of the 26S proteasome, recruitment of targets, and regulation of its proteolytic activity (Finley, 2009; Book et al., 2010).

Through the ubiquitin/26S proteasome system (UPS), Ub controls many facets of plant growth, development, and cytoplasmic and nuclear housekeeping. Examples include signaling by most, if not all, of the major plant hormones, the cell cycle, circadian rhythms, photomorphogenesis, floral and leaf homeosis, self-incompatibility, defense against pathogens, and survival during environmental stress (Dreher and Callis, 2007; Vierstra, 2009; Hua and Vierstra, 2011). Notably, Ub E3s have been shown to serve as receptors for the hormones auxin (Tan et al., 2007) and jasmonic acid (Sheard et al., 2011) as well as to provide numerous control points in the pathways required for the synthesis and/or perception of ethylene, abscisic acid, salicylic acid, strigolactone, and GA (Dreher and Callis, 2007; Santner and Estelle, 2010; Hua and Vierstra, 2011). The continuing arms race between plant pathogens and their hosts also exploits the UPS. While the host synthesizes E3s to ward off infection by ubiquitylating and removing pathogenic factors injected inside, the pathogen retaliates by injecting proteins and small molecule inhibitors that disregulate ubiquitylation and the UPS to favor pathogen invasion (Vierstra, 2009).

Additional roles for Ub outside of the 26S proteasome are also prominent and include the regulation of various DNA functions and trafficking proteins to their appropriate destinations. Studies with yeast and mammalian cells revealed that reversible ubiquitylation of core and linker histones is part of the code that controls nucleosome assembly and thus the accessibility of genes to transcriptional regulators (Weake and Workman, 2008), while the modification of various DNA repair factors such as proliferating cell nuclear antigen appears critical for fixing DNA breaks induced by genotoxic stress (Ulrich and Walden, 2010). H2B ubiquitylation/deubiquitylation is driven in *Arabidopsis* by the RING E3 BRE1 (or HUB1) and the UBP26 DUB, with null mutants displaying severe problems in the cell cycle, ploidy, transcription, and small interfering RNA-mediated gene silencing (Fleury et al., 2007; Liu et al.,

2007; Sridhar et al., 2007). Moreover, the action of at least some transcription factors may require cycles of ubiquitylation to promote each round of transcriptional initiation (Geng et al., 2012). A number of membrane-bound receptors and transporters are also strongly ubiquitylated upon ligand engagement (Mukhopadhyay and Riezman, 2007). This modification involves either monoubiquitylation or polyubiquitylation via Lys-63-linked chains and appears to control signaling by cycling the receptors to internal compartments and/or directing their breakdown by the vacuole/lysosome. In this regard, the Arabidopsis DUB AMSH3 may be particularly important for ESCRT-mediated protein sorting (Katsiarimpa et al., 2011). As will be described below, ubiquitylation of supramolecular complexes and organelles too large for the proteasome may be widely used by plants to direct autophagic turnover (Li and Vierstra, 2012).

REGULATION OF CRL Ub E3S BY RUB

RUB (or Nedd8 in yeast and animals) was first discovered as part of a novel gene (*UBQ7* in Arabidopsis) that expresses Ub appended in frame to a closely related polypeptide (55% amino acid sequence identity) that is released upon translation (Callis et al., 1995). X-ray crystallography studies of a free RUB revealed a β -grasp fold that is nearly identical to that of Ub, including a C-terminal extension ending in a di-Gly (Fig. 1). Subsequent genetic analyses of Arabidopsis RUBs (designated RUB1–RUB3) uncovered a distinct but chemically similar conjugation pathway with the surprising finding that its main, if not exclusive, targets are the Cullin subunits from CRL E3s, thus intimately connecting rubylation to ubiquitylation (del Pozo and Estelle, 1999; Hakenjos et al., 2011; Fig. 2). In fact, it is now clear that RUB is part of two dueling cycles that dynamically navigate CRL complexes through assembly, conjugation, and disassembly steps as they ubiquitylate substrates (Hotton and Callis, 2008; Hua and Vierstra, 2011).

One cycle employs CAND1 (for Cullin-associated Nedd8-dissociated1) as a clamp to maintain a pool of disassembled CRL complexes by binding reversibly to the Cullin-RBX1 subcomplex in a way that inhibits the association of Cullin-RBX1 with substrate adaptors in the absence of cognate targets. Possibly in the presence of a substrate-occupied adaptor, the Cullin scaffold becomes rubeinated at a particular Lys by an E1→E2→E3 reaction cascade involving the heterodimeric E1 encoded by the *AXR1/AXR1-LIKE* and *ECR1* genes and the RCE1 E2. The RBX1 subunit in association with the Cullin is thought to provide the E3 activity that directs RUB transfer. The conjugated RUB occludes CAND1 binding to the Cullin, which in turn allows the CRL to enter a second cycle, where the substrate-bound adaptor binds to Cullin-RBX1 followed by substrate ubiquitylation. After release of the appropriately ubiquitylated protein, the eight-subunit COP9/signalosome (CSN) enters to remove the RUB moiety using the

deconjugase activity provided by the CSN5 subunit (Lyapina et al., 2001; Zhang et al., 2008). Finally, the CRL is disassembled and CAND1 rebinds to Cullin-RBX1.

Paradoxically, the opposing CAND1/RUB cycles are both essential for proper CRL activity, likely by maintaining a revolving pool of uncommitted Cullin-RBX1 scaffolds despite the presence of hundreds of waiting substrate adaptors (Hua and Vierstra, 2011). Accordingly, Arabidopsis mutants missing any components in the RUB scheme are lethal while weaker alleles have pleiotropic effects on plant growth and development and compromised UPS substrate turnover (del Pozo et al., 2002; Schwechheimer et al., 2002; Feng et al., 2004; Zhang et al., 2008).

Recent proteomic studies ectopically expressing tagged versions of RUB/Nedd8 in Arabidopsis and mammals reported other potential targets of rubeination besides Cullins, including numerous ribosomal subunits (Jones et al., 2008; Xirodimas et al., 2008; Hakenjos et al., 2011). Whether these represent authentic conjugates remains to be confirmed, given the observations that human Nedd8 will inappropriately enter the ubiquitylation system when in excess or when Ub is depleted (Kim et al., 2011).

SUMO AND NUCLEAR ACTIVITIES ASSOCIATED WITH PLANT STRESS DEFENSE

SUMO was first discovered in animals from studies on the nuclear pore protein RanGAP, which upon sequencing was found to have two N termini but only one C terminus, indicative of one protein attached to another (Matunis et al., 1996). The appended protein was named SUMO for its weak sequence homology to Ub (12% identity) and was later confirmed as a UBL by its β -grasp fold and analogous conjugation chemistry. However, SUMO differs from other UBLs by a long N-terminal extension that appears unstructured in solution (Fig. 1). Plants and animals typically express several SUMO isoforms (four in Arabidopsis) with evidence for subfunctionalization (Kurepa et al., 2003; van den Burg et al., 2010). The sumoylation pathway in Arabidopsis includes a heterodimeric E1 encoded by *SAE1a/b* and *SAE2* genes, a single E2 SCE1, at least two E3s encoded by the *SIZ1* and *HYP2/MMS21* loci, and a family of desumoylating proteases (Fig. 2; Kurepa et al., 2003; Colby et al., 2006; Miura et al., 2007a). These desumoylating proteases not only disassemble SUMO conjugates, they are needed to generate mature SUMO by trimming extra amino acids beyond the C-terminal di-Gly. Whereas most targets bear a single SUMO moiety, some can be modified with multiple SUMOs or poly-SUMO chains via iterative cycles of conjugation analogous to ubiquitylation (Miller et al., 2010; Wilkinson and Henley, 2010). Based on alignments of known SUMO attachment sites, sumoylation appears to employ several loose consensus motifs; one prevalent in plants, yeast, and mammals encompasses a Ψ KXE motif (where Ψ is a large hydrophobic residue and K is the Lys where SUMO is linked), and two others

identified thus far in mammals contain extended motifs rich in phosphorylated or negatively charged residues (Miller et al., 2010; Wilkinson and Henley, 2010).

The functions of sumoylation are diverse and mostly nonproteolytic and include controls on localization, interaction, and activity of the modified protein (Miura et al., 2007a; Wilkinson and Henley, 2010). One unique situation has also been reported in mammalian cells, where SUMO addition protects the I κ B α protein from Ub addition by blocking accessible Lys residues (Desterro et al., 1998). Some of these effects are mediated by a collection of binding partners bearing SUMO-interacting motifs.

Particularly important to plants is the role of SUMO in stress defense. The first connection came from the discovery that sumoylation is strongly up-regulated by various types of stress, including heat shock, freezing, drought, ethanol, oxidants, amino acid analogs, and pathogen exposure (Kurepa et al., 2003; Saracco et al., 2007; Conti et al., 2008). In fact, a dramatic rise in SUMO conjugates can be detected within minutes after subjecting Arabidopsis seedlings to a mild heat shock (37°C), making it one of the fastest stress responses observed, and is so strong that it consumes almost all available free SUMO. This stress-induced sumoylation is reversible, such that a few hours after the stress most conjugates are disassembled and the free SUMO pool is regenerated, and has a “memory” that prevents a second burst if too close in time to the first (Kurepa et al., 2003; Saracco et al., 2009; Miller et al., 2012).

The second connection of SUMO to stress came from phenotypic analysis of Arabidopsis pathway mutants. Whereas null mutants for the two main SUMOs (SUMO1 and -2), the E1 subunit SAE2, and the E2 SCE1 are embryo lethal (Saracco et al., 2007), mutants missing the SIZ1 E3 display pleiotropic stress phenotypes, including a hypersensitivity to heat, cold, drought, salt, and low phosphate and nitrogen, a hyposensitivity to pathogens, and altered signaling by the stress hormones abscisic acid and salicylic acid (Miura et al., 2005, 2007b, 2009; Lee et al., 2007; Conti et al., 2008; van den Burg et al., 2010; Park et al., 2011).

At present, it is unclear how SUMO helps plants survive adverse environments. Clues have come from that fact that most sumoylated proteins are nuclear (Saracco et al., 2007) and recent proteomic and interactome analyses that uncovered a deep catalog of SUMO targets and SUMO-interacting proteins (Elrouby and Coupland, 2010; Miller et al., 2010). Included in the list of approximately 350 conjugates are a “who’s who” of key transcriptome regulators and chromatin modifiers, including histones H1 and H2B, histone acetyl- and SET-domain-containing histone-methyl transferases, SAGA complex subunits, proteins involved in RNA-directed DNA methylation, SWI/SNF chromatin remodelers, DNA repair components, TOPLESS-type corepressors, numerous transcription factors required for developmental- and/or stress-induced gene expression, and nuclear pore proteins. Collectively, these targets imply that SUMO is crucial to chromatin architecture,

transcription, cytoplasm/nuclear partitioning, and RNA retention, especially during stress (Miller et al., 2010; Meier, 2012). Subsequent quantitative proteomic studies on heat, oxidative, and ethanol stress discovered that proteins involved in RNA biology are some of the most robustly sumoylated targets during stress (as much as 15-fold within 30 min of heat shock), suggesting that RNA-related processes are particularly affected (Miller et al., 2012). From studies of individual SUMO targets outside of plants, it is noteworthy that only a small proportion of a substrate needs to be sumoylated to achieve maximal effect (Miura et al., 2007a; Wilkinson and Henley, 2010). This enigma implies that sumoylation does not activate/inactivate proteins per se but may drive cycles critical for their functions (e.g. assembly/disassembly of transcription complexes or nuclear/cytoplasm shuttling).

Surprisingly, proteomic studies with yeast, mammalian cell cultures, and Arabidopsis also discovered that a subset of sumoylated proteins becomes ubiquitylated during stress, with evidence that Ub can be directly linked to the SUMO moieties (Uzunova et al., 2007; Miller et al., 2010, 2012; Tatham et al., 2011). This dual modification implies a cross talk between the Ub and SUMO systems along with the added possibility that the bound SUMOs act as secondary degrons for directing UPS-mediated turnover. In yeast and metazoans, this Ub addition is driven by a family of SUMO-targeted Ub ligases containing both a SUMO-interacting motif and a RING motif (Sun et al., 2007; Uzunova et al., 2007). Possible SUMO-targeted Ub ligase orthologs are apparent in various plant genomes, including at least three in Arabidopsis.

ROLES OF ATG8 AND ATG12 IN DIRECTING AUTOPHAGIC RECYCLING

Autophagy, or “self-eating,” represents a major recycling route in eukaryotes whereby cytoplasmic constituents and organelles are sequestered in small double membrane-bound vesicles, which are then deposited into the vacuole (or lysosome in animals) for eventual breakdown by resident hydrolases (Bassham, 2009; Li and Vierstra, 2012). Even though autophagy was described in morphological terms decades ago, only recently have the underpinning mechanisms become apparent. Particularly informative were genetic screens of yeast and *Pichia pastoris* designed to discover loci required for autophagic transport (Klionsky, 2007). In addition to identifying various kinase signaling components required to direct autophagy during nutrient starvation and factors that promote hemifusion of the resulting vesicles with the tonoplast, these screens unexpectedly discovered a pair of UBLs named ATG8 and ATG12 whose conjugation is essential for autophagy (Ohsumi, 2001). Both ATG8 and ATG12 are faintly similar to Ub (8% and 5% amino acid sequence identity for the Arabidopsis versions [Doelling et al., 2002]) but contain obvious β -grasp folds that are decorated with additional helices, loops, and N-terminal

extensions (Fig. 1). ATG12 is translated in its mature form with a protruding Gly and thus does not need processing by a deconjugase. ATG8, on the other hand, is typically synthesized with a variable C-terminal extension that must be removed by the ATG4 protease to expose its Gly.

Both ATG8 and ATG12 are activated by the same E1 (ATG7), but the thioester intermediates are transferred to separate E2s, ATG3 and ATG10, respectively (Fig. 2). ATG12 is then conjugated through an isopeptide bond to a single protein target, ATG5, without the assistance of an E3. In a unique twist, ATG8 is attached instead to the amine group of the lipid phosphatidylethanolamine (PE) and becomes membrane bound (Ohsumi, 2001; Klionsky, 2007). The E3 driving PE conjugation is composed of the ATG12-ATG5 conjugate in association with the dimeric ATG16 protein, thus generating an unusual scheme in which one UBL conjugate directs modification by another UBL (Fujioka et al., 2008; Chung et al., 2010). Toward the end of autophagic vesicle assembly, the ATG12-ATG5 conjugate dissociates from the membrane, while the ATG8 moiety lining the outer surface is delipidated by ATG4 and released. The ATG8-PE adduct bound to the inner surface enters the vacuole and is eventually consumed.

Why the ATG autophagic system culminates in generating the ATG8-PE adduct remained a puzzle until recently. A key breakthrough was the realization that ATG8, upon coating autophagic membranes, provides a docking platform to recruit other factors needed to build the engulfing vesicle and encapsulate cargo (Noda et al., 2010; Johansen and Lamark, 2011). Humans alone express almost 70 different ATG8-interacting proteins (Behrends et al., 2010). These factors are loosely defined by a signature ATG8-interacting motif (AIM) with a consensus core W/Y/F-XX-L/I/V sequence that tightly binds a hydrophobic cleft in ATG8 (Noda et al., 2008). Importantly, a number of AIM proteins have been identified that bind specific protein cargo and organelles, thus revising our appreciation of autophagy from one committed to bulk degradation to one that selectively recycles. Two AIM proteins of particular interest in mammals are SQSTM1 (for sequestosome1) and NBR1 (for neighbor of BRAC1-1), which have the capacity to bind ubiquitylated proteins through ubiquitin-associated (UBA) domains (Johansen and Lamark, 2011). This connection appears to provide an alternative recycling route for ubiquitylated proteins too large for the 26S proteasome, such as protein aggregates, large macromolecular complexes, and even organelles and invading pathogens following ubiquitylation of their surface proteins.

Descriptions of ATG components in Arabidopsis revealed that a remarkably similar autophagic system exists in plants (Thompson and Vierstra, 2005; Bassham, 2009; Li and Vierstra, 2012), including the presence of an NBR1 ortholog (but not SQSTM1) that connects the autophagic and ubiquitylation systems (Svenning et al., 2011; Zientara-Rytter et al., 2011). Genetic analyses showed that although ATG-mediated

autophagy is not essential to plants, it is important for natural senescence, survival during nutrient starvation, and pathogen defense (Doelling et al., 2002; Hanaoka et al., 2002; Liu et al., 2005; Thompson et al., 2005; Hofius et al., 2009; Lenz et al., 2011). ATG-mediated autophagy also has been connected to chloroplast and mitochondria turnover (Wada et al., 2009; F. Li and R.D. Vierstra, unpublished data), the removal of oxidized or aggregated proteins (Toyooka et al., 2006; Xiong et al., 2007), the clearing of cytotoxic heme metabolites (Vanhee et al., 2011), and possibly the elimination of defective ribosomes and proteasomes (Hillwig et al., 2011; R.M. Marshal and R.D. Vierstra, unpublished data).

The main organizational difference is the fact that many plant ATG components are encoded by small gene families as opposed to single genes in yeast. For example, Arabidopsis expresses a pair of ATG12 proteins and nine ATG8 isoforms that have subfunctionalized expression patterns (Doelling et al., 2002; Hanaoka et al., 2002). Moreover, two ATG8 isoforms lack C-terminal extensions and thus act without ATG4 intervention. The ATG12-ATG5 conjugate appears to be constitutively synthesized at high levels (Thompson et al., 2005; Phillips et al., 2008). Conversely, the levels of the ATG8-PE adduct are more dynamic, being present at low levels in nonstressed plants and then accumulating to high levels in senescing leaves and in plants deprived of nitrogen or fixed carbon (Chung et al., 2009, 2010; Suttangkakul et al., 2011). Following starvation, numerous ATG8-decorated autophagic vesicles can be seen in the vacuole, implying that cytoplasmic recycling by this route is substantial (Yoshimoto et al., 2004; Thompson et al., 2005).

MUBS

MUBs were added to the UBL collection based on structural studies that discovered an obvious β -grasp fold in an Arabidopsis member (Downes et al., 2006). However, MUBs uniquely differed from the other UBLs by long N- and C-terminal loops and by ending not in a protruding Gly but in an invariant CAAX motif (Fig. 1). CAAX motifs are essential signatures for prenylation, a membrane-anchoring modification in which the Cys becomes conjugated with 15- or 20-carbon-long farnesyl or geranylgeranyl moieties by two distinct families of heterodimeric prenyl transferases, PFT and PGGT, respectively. The prenylated protein is proteolytically trimmed by a CAAX protease to expose the Cys, and its carboxyl group is subsequently methylated by a prenyl Cys methyltransferase (Crowell and Huizinga, 2009; Fig. 2). Sequence database searches subsequently found MUBs in other plant species, animals, and filamentous fungi but not in any unicellular fungi or prokaryotes (Downes et al., 2006). In vitro assays, inhibitor studies, and biochemical analyses of prenyl transferase mutants confirmed that most, if not all, MUBs are substrates for prenylation and in some cases also become palmitoylated. At least in Arabidopsis, the MUBs then become specifically anchored to the plasma membrane (Downes et al., 2006).

The function(s) of MUBs are not yet clear, as the presence of gene families has complicated genetic analyses. Of likely relevance are the observations that *Arabidopsis* MUBs selectively bind a subfamily of Ub E2s that then dock these E2s to the plasma membrane in planta (Dowil et al., 2011). Consequently, MUBs like RUB, SUMO, and ATG8/12 (Fig. 2) might intersect with the Ub system, in this case by recruiting specific components of the Ub conjugation machinery to the plasma membrane, presumably to promote the ubiquitylation of similarly localized targets.

URM1

The emerging picture of URM1 is that it represents an evolutionary bridge between the ancient roles of UBLs in prokaryotic sulfur chemistry (see below) and the presumably more advanced roles of Ub and UBLs as protein modifiers (Fig. 3A). Unlike several others in the Ub/UBL family, URM1 is synthesized in its mature form with an exposed di-Gly sequence protruding from its β -grasp fold (Fig. 1). Studies with yeast and mammalian URM1 showed that it is activated by a novel E1 (UBA4) that comprises a fusion between the canonical E1 that adenylates the C terminus of Ub/UBLs and a rhodanese domain that directs sulfur transfer (Furukawa et al., 2000; Leidel et al., 2009; Van der Veen et al., 2011). By a two-Cys reaction cascade much like those employed by the *Escherichia coli* ThiS and MoaD sulfur transferases used for thiamine and molybdenum cofactor (MoCo) synthesis, respectively, URM1 is first adenylated, then forms an acyl disulfide intermediate with UBA4, before final release to generate an activated URM1 bearing a C-terminal Gly thiocarboxylate (Figs. 2 and 3A). This URM1 thiocarboxylate then participates in two sets of modifications. One likely more ancestral reaction donates the sulfur moiety to the wobble uridine in several transfer RNAs (tRNAs; Leidel et al., 2009). The second involves attachment of the thiocarboxylated URM1 to protein targets through an isopeptide linkage with free Lys residues concomitant with the release of the sulfur moiety (Goehring et al., 2003; Van der Veen et al., 2011). Whether E2, E3, or deurmylating activities also participate in the URM1 pathway is not yet known.

tRNA thiolation by URM1 is proposed to stabilize codon-anticodon interactions on ribosomes to improve translation efficiency (Leidel et al., 2009). Why proteins would be urmylated is not yet clear. Proteomic studies have identified only a handful of targets, including in yeast the peroxiredoxin Ahp1 and, in human cell lines, other components of the tRNA thiolation pathway, the cellular apoptosis susceptibility factor involved in nuclear transport, and several DUBs, thus providing another link between Ub and UBLs (Goehring et al., 2003; Van der Veen et al., 2011). Intriguingly, protein urmylation dramatically rises upon exposing yeast and mammalian cells to oxidative stress, and urmylation mutants are more sensitive to nutrient starvation, oxidants, and heat shock (Furukawa et al., 2000;

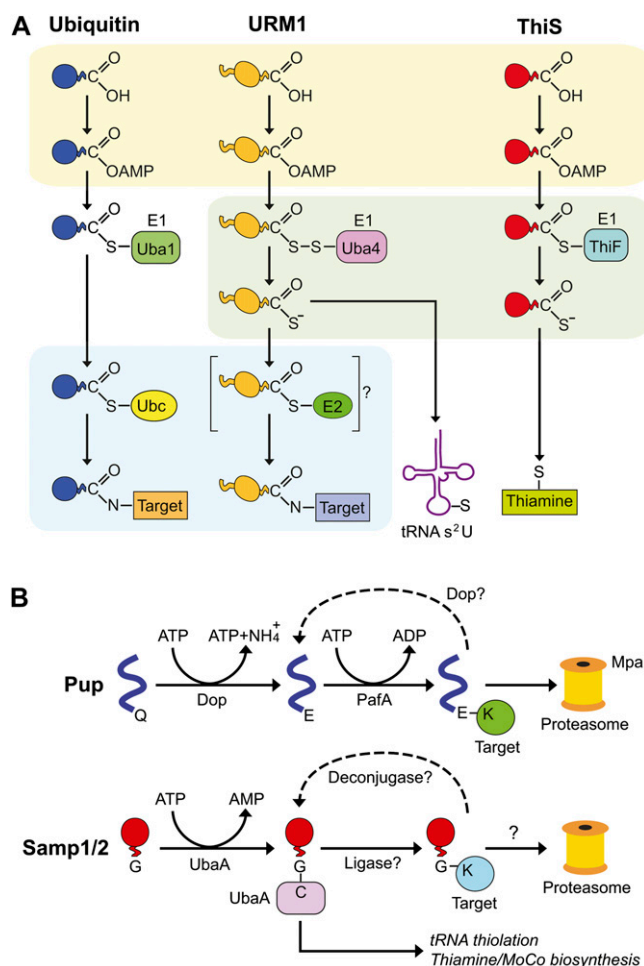


Figure 3. Evolution of the eukaryotic Ub/UBL modification pathways from prokaryotic reaction schemes. A, The reaction mechanisms for *E. coli* ThiS involved in sulfur transfer chemistry, yeast UFM1 involved in tRNA thiolation and protein modification, and Ub involved in protein modification. B, Schematics for the prokaryotic conjugation pathways involving *H. volcanii* Samp1/2 and *M. tuberculosis* Pup. A was adapted from Petroski et al. (2011).

Goehring et al., 2003; Leidel et al., 2009; Van der Veen et al., 2011).

At present, little is known about urmylation in plants, and no protein targets have been reported. *Arabidopsis* contains two active genes encoding URM1, with 37% to 39% identity to their yeast counterpart. Nakai et al. (2012) recently showed that one *Arabidopsis* isoform but the not the other participates in cytosolic tRNA thiolation and can functionally replace yeast URM1. In a search for *Arabidopsis* mutants resistant to sirtinol, a small molecular activator of auxin-inducible genes and a promoter of auxin-related developmental phenotypes, an ortholog of the UBA4 E1 (called SIR1) was unexpectedly discovered (Zhao et al., 2003). Subsequent sirtinol screens identified additional loci involved in sulfur transfer and MoCo synthesis, suggesting that the sirtinol resistance of UBA4/SIR1 loss-of-function plants is caused by compromised

MoCo assembly (Dai et al., 2005); however, additional effects on protein urmylation cannot be ruled out.

UFM1

UFM1 closely follows the Ub enzymatic paradigm, but knowledge of its corresponding targets and associated functions in any eukaryote is lacking. Even with only 14% amino acid sequence identity to Ub, UFM1 adopts a remarkably similar β -grasp fold (Fig. 1), and like several other UBLs, it is synthesized as a precursor (seven residues longer for Arabidopsis UFM1) that requires processing by the UFSP2 protease to expose the functioning C-terminal Gly (Komatsu et al., 2004). Ufmylation as deduced in mammalian cells involves an E1 (UBA5), a single E2 (UFC1), and only one known E3 (UFL1), which will conjugate UFM1 to targets both in vivo and in vitro (Fig. 2; Komatsu et al., 2004; Tatsumi et al., 2010). Although driving the same biochemical reactions, both UFC1 and UFL1 have little sequence in common with the E2s and E3s from other Ub/UBL conjugation systems, implying that they work differently. Biochemical studies with mammalian cells suggest a limited number of UFM1 substrates; currently, the only bona fide target is the endoplasmic reticulum-resident protein UFBP1, which is modified at one or more Lys residues via isopeptide linkages (Tatsumi et al., 2010; Lemaire et al., 2011).

Comparable ufmylation pathways, including the UFBP1 target, can be readily found encoded in the genomes of other metazoans and in plants such as Arabidopsis but not in yeast or *Schizosaccharomyces pombe*, suggesting a unique role for this UBL in multicellular organisms. *Uba5*^{-/-} mice are embryonic lethal, possibly caused by defective erythroid development (Tatsumi et al., 2011), and cultured human cells silenced for *Ufm1* and *Ufl1* show accelerated apoptosis (Lemaire et al., 2011), possibly due to heightened endoplasmic reticulum stress. Little is currently known about the phenotypic functions of the UFM1 pathway in plants.

HUB1

Despite being nearly identical to Ub structurally (Fig. 1), the HUB1 proteins are the most unconventional members of the UBL family. They invariably terminate in a di-Tyr sequence followed by a non-conserved amino acid, all of which remains linked to the active polypeptide (Dittmar et al., 2002). Rather than becoming covalently attached to other proteins, studies using yeast and mammalian cells show that HUB1 likely acts noncovalently, and accordingly no E1s, E2s, E3s, or processing enzymes have been identified for HUB1 in any eukaryote (Fig. 2). Genetic analyses of yeast HUB1 revealed roles in polarized growth, cell cycle progression, and the mitochondrial unfolded protein response, which might be collectively manifested by defective mRNA splicing (Dittmar et al., 2002; Mishra et al., 2011). In particular, HUB1 appears

essential for cleaving noncanonical 5' splice sites through interactions with the spliceosomal tri-small ribonucleoprotein particle, which in Arabidopsis likely involves binding to the Prp38 subunit (Mishra et al., 2011). Whereas $\Delta hub1$ mutants in yeast are viable, consistent with its strong avoidance of alternative splicing, *S. pombe* $\Delta hub1$ mutants are lethal (Wilkinson et al., 2004; Mishra et al., 2011). Arabidopsis contains two loci that encode HUB1 proteins with 95% amino acid sequence identity; one (At5g42300) shows strong expression, whereas the other (At3g45180) is expressed poorly, if at all, and may be a pseudogene (A. Lomax and R.D. Vierstra, unpublished data). Whether HUB1 is essential to Arabidopsis or other plants is not yet known.

UBL DOMAIN FUSIONS

In line with the versatile roles of β -grasp domains in promoting protein-protein interactions or encouraging protein folding, numerous proteins have been found that contain a Ub/UBL domain translationally linked to other domains, thus skirting the need for subsequent covalent attachment. In the Arabidopsis genome alone, over 50 loci are predicted to encode proteins with sequence homology to Ub, and where examined, these domains assume an obvious β -grasp fold (e.g. At2g23250 [Fig. 1]). Two essential ribosomal proteins are expressed only as Ub fusions (Arabidopsis *UBQ1/2* and *UBQ3/4* loci) but then are rapidly processed cotranslationally to release the ribosomal proteins and Ub in free, functional forms (Callis et al., 1990). In yeast, this cosynthesis dramatically elevates expression, presumably by exploiting the efficient folding of Ub to encourage folding of the more recalcitrant ribosomal subunit sequences downstream (Finley et al., 1989).

Examples of stable UBL fusions in Arabidopsis include the UBL-UBA family (RAD23 [HHR23A in humans], DSK2, DDI1, NUB1, and UBL1 [Farmer et al., 2010]), the Ub HECT E3 UPL5, the proteasome-associated deubiquitylating enzyme UBP6, and the RNA splicing factor 3A, as well as a number of uncharacterized proteins (e.g. At2g23250). To prevent inadvertent release by deconjugases, these UBL domains lack the C-terminal Gly. Members of the UBL-UBA family are uniquely organized in having an N-terminal UBL domain followed by one or more UBA domains that recognize Ub (Farmer et al., 2010; Fatimababy et al., 2010). By simultaneously binding ubiquitylated proteins via the UBA domain and the proteasomal receptors RPN1, RPN10, or RPN13 through the UBL domain, these proteins chaperone Ub targets to the 26S complex for turnover (Fu et al., 2010).

EVOLUTION OF THE Ub/UBL SUPERFAMILY

A long-standing question has been the evolutionary origins of these complex Ub/UBL systems. A major breakthrough was the discovery that the prokaryotic pathways directing the acquisition of inorganic sulfur

during thiamine and MoCo biosynthesis use the structurally related β -grasp proteins, ThiS and MoaD (Lake et al., 2001; Wang et al., 2001), which also share common adenylate/thioester intermediates generated by E1-related enzymes, ThiF and MoaE, respectively (Figs. 1 and 3A). It is easy to imagine that the URM1 pathway evolved directly from these sulfur transferase reactions and thus represents the ancestral bridge between sulfur chemistry and protein conjugation (Fig. 3A).

Remarkably, as in eukaryotes, it also appears that prokaryotes further elaborated upon the ThiS/MoaD reaction scheme to generate their own protein-tagging systems, some of which might incorporate E1→E2→E3 conjugation cascades as well as deconjugases to reverse attachment (Hochstrasser, 2009; Nunoura et al., 2011; Burroughs et al., 2012). Where fully understood, conjugation also directs turnover of the modified protein. One recently discovered in Archaea species such as *Haloferax volcanii* revolves around two β -grasp proteins, Samp1 (for small archaeal modifier protein1) and Samp2 (Fig. 1), and their corresponding E1 UbaA (Humbard et al., 2010; Miranda et al., 2011). Samp1 and Samp2 have retained their participation in thiamine/MoCo and tRNA thiolation much like ThiS/MoaD and URM1, respectively (Fig. 3B). And like URM1, they also become isopeptide linked via their di-Gly C terminus to a collection of protein substrates by a streamlined pathway absent of E2 or E3 activities. Sampylation affects numerous targets in *H. volcanii*, including self-modification, to form poly-Samp chains much like eukaryotic Ub and SUMO (Humbard et al., 2010). The consequences of Samp1/2 addition are not yet known; that the levels of Samp1/2 conjugates rise during nitrogen starvation and are possibly turned over by a stripped-down archaeal version of the proteasome imply a role in amino acid recycling during nutrient deprivation (Humbard et al., 2010).

An intriguing variation on the theme is Pup (for prokaryotic Ub-like protein) found in a number of actinobacteria and nitrospira, including *Mycobacterium tuberculosis* (Pearce et al., 2008; Wang et al., 2010). Pup is structurally unrelated to β -grasp proteins and is in fact intrinsically disordered. However, like Ub/UBLs, *M. tuberculosis* Pup becomes conjugated to proteins and even directs their degradation by a primitive eubacterial proteasome consisting of just the CP capped by a ring of the AAA-ATPase protein Mpa, which serves as the Pup receptor (Wang et al., 2010). Surprisingly, the conjugation chemistry is markedly distinct from those used by Ub/UBLs, suggestive of convergent evolution (Fig. 3B). The C-terminal Gln residue of Pup is first deamidated by the Dop deamidase, and then Pup is isopeptide linked via the resulting Glu γ -carboxyl group to accessible target Lys residues using a Gln synthetase-like PafA ligase (Striebel et al., 2009). Interestingly, Dop also has depupylating activity that can reverse Pup addition (Burns et al., 2010). The extensive catalog of Pup substrates from *M. tuberculosis* and *Mycobacterium smegmatis* imply significant proteolytic roles for this modifier in metabolism, respiration, and adaptation (Pearce et al., 2008; Poulsen et al., 2010).

CONCLUSION

Certainly, the expanding collection of Ub/UBL proteins and their plethora of targets place these polypeptide modifiers at the center of many aspects of plant biology. In fact, based on the sheer number of associated components and phenotypic impact, Ub and the UPS are rivaled only by transcription factors and protein kinase cascades as the dominant regulators of plant growth, development, and survival. It is also intriguing that while these Ub/UBLs have substantially diversified with respect to the biological processes they control and their underpinning enzymology, several points of cross talk have evolved that connect some Ub/UBLs to each other, particularly between Ub and RUB, Ub and SUMO, Ub and MUBs, ATG8 and ATG12, and Ub and autophagy mediated by ATG8/12. Given the facts that (1) animals have UBLs (ISG15 and FAT10 [Hochstrasser, 2009]) not yet found in plants, (2) more UBLs will likely emerge in prokaryotes, and (3) our limited structural knowledge of most plant proteins, it is highly possible that more plant UBLs will be forthcoming. Furthermore, using bacterial Pup for illustration, it is also possible that new polypeptide modifiers will emerge that are independent of the hallmark β -grasp fold. Consequently, we should expect that the known functional space encompassed by these polypeptide modifiers in the plant realm will continue to increase.

ACKNOWLEDGMENTS

I thank past and present members of the Vierstra laboratory for helpful discussions and for generating the data described herein from our laboratory. Special thanks goes to Aaron Lomax for unpublished information on various UBLs. I apologize to those whose work was not cited due to space constraints.

Received May 17, 2012; accepted June 9, 2012; published June 12, 2012.

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