Journal of Experimental Botany, Vol. 69, No. 3 pp. 371–384, 2018
doi:10.1093/jxb/erx411 Advance Access publication 10 December 2017

REVIEW PAPER

News from the PUB: plant U-box type E3 ubiquitin ligases

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Received 3 August 2017; Editorial decision 25 October 2017; Accepted 25 October 2017

Editor: Christine Raines, University of Essex, UK

Abstract

Plant U-box type E3 ubiquitin ligases (PUBs) are well known for their functions in a variety of stress responses, including immune responses and the adaptation to abiotic stresses. First linked to pollen self-incompatibility, their repertoire of roles has grown to encompass also the regulation of developmental processes. Notably, new studies provide clues to their mode of action, underline the existence of conserved PUB–kinase modules, and suggest new links to G-protein signalling, placing PUBs at the crossroads of major signalling hubs. The frequent association with membranes, by interacting and/or targeting membrane proteins, as well as through a recently reported direct interaction with phospholipids, indicates a general function in the control of vesicle transport and their cargoes. This review aims to give an overview of the most significant advances in the field, while also trying to identify common themes of PUB function.

Keywords: Abiotic stress, development, E2 ubiquitin-conjugating enzyme, E3 ubiquitin ligase, G-proteins, immunity, kinases, protein degradation, PUB, U-box, ubiquitination, vesicle traffic.

Introduction

The attachment of ubiquitin, a 76 amino acid long protein with a remarkable degree of conservation, known as ubiquitination (ubiquitylation), is involved in all aspects of cellular physiology (Fig. 1). Ubiquitination is mediated by an enzymatic cascade, where the last step is performed by E3 ubiquitin ligases, which among other functions mediate substrate specificity (Fig. 1). The U-box mediates interaction with the ubiquitin-conjugating enzyme (E2), is the signature domain for plant U-box type E3 ligases (PUBs) (Fig. 1) (Koegl *et al.*, 1999), and is found in combination with a variation of domains including armadillo (ARM) repeats, the Ser/Thr kinase domain, WD40 repeats, the tetratricopeptide (TPR) domain, or peptidyl-prolyl isomerase (Azevedo *et al.*, 2001; Yee and Goring, 2009).

Ubiquitination can impart different fates to the modified substrate (Fig. 1). The type of ubiquitination is largely endowed by the interacting E2 that is responsible for the catalysis of ubiquitin transfer onto a substrate. Depending on the E2–E3 pair, substrates can be mono- or multi-monoubiquitinated (Fig. 1). Several rounds of ubiquitination can also generate a polyubiquitin chain. Importantly, ubiquitins within the chain can be attached to each other via one of seven lysines, or the N-terminal methionine of ubiquitin (Fig. 1). With the exception of Met1 chains and Lys27, all other linkages have been detected in plants (Maor *et al.*, 2007; Kim *et al.*, 2013).

The different linkage types constitute the ‘ubiquitin code’, which is translated by ubiquitin receptors that recognize specific attributes of chain topologies into cellular responses (Fig. 1) (Kulathu and Komander, 2012). Ubiquitin receptors sort modified proteins into different pathways, such as degradation via the proteasome for Lys48-linked polyubiquitinated proteins, or transport of endocytosed proteins into the vacuole via the endosomal sorting complexes required for transport (ESCRT) in the case of Lys63-linked polyubiquitin chains (Fig. 1) (Vierstra, 2009; Isono *et al.*, 2010).

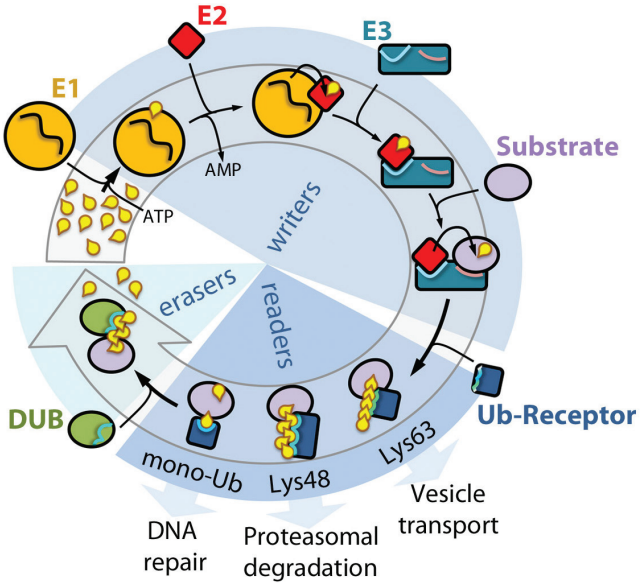

Fig. 1. The ubiquitin modification cycle. Ubiquitination of a substrate protein involves a sequential cascade of enzymatic activities, which can be subdivided into 'writers', 'readers', and 'erasers'. Writers: in the initial step, the ubiquitin-activating enzyme (E1) forms a thioester linkage with ubiquitin with consumption of ATP. Next, ubiquitin is passed on to a ubiquitin-conjugating enzyme (E2) in a transesterification reaction. The E2–ubiquitin conjugate associates with the ubiquitin ligase (E3), which facilitates the transfer of ubiquitin from the E2 onto an available lysine residue on the substrate. Pairing of the E3 with a specific E2 determines the type of ubiquitination. Substrates can be mono- or poly-monoubiquitinated, or ubiquitins can be attached to one another via one of its seven lysines or Met1. Notably, E3s frequently autoubiquitinate (not shown). Readers: ubiquitination is recognized by ubiquitin receptors, which are able to discriminate between the different types of attachment and mediate specific downstream responses. Erasers: ubiquitin moieties are effectively cleaved of by deubiquitinating (DUB) enzymes, which display catalytic preferences for specific types of ubiquitination.

Genetic analyses have revealed the participation of PUBs in a plethora of processes, including cell proliferation during development, adaptation to drought, and immune reactions during pathogen attack. A total of 64 genes are predicted to encode a U-box in the Arabidopsis genome (Azevedo *et al.*, 2001; Andersen *et al.*, 2004; Mudgil *et al.*, 2004; Wiborg *et al.*, 2008), although with some changes. *PUB62* (At3g49065) annotation was amended to PUB32 (AT3G49060). In addition, a homologue of PUB63 (AT5G05230) is now included, namely *PUB62* (AT2G40640), replacing the former *PUB62* (https://www.arabidopsis.org/browse/genefamily/plantubox.jsp). Moreover, 77 have been annotated in the rice genome (Zeng *et al.*, 2008), and 125 were reported for soybean (Wang *et al.*, 2016).

In contrast, the human genome encodes only eight U-box type E3s (Li *et al.*, 2008). The function of plant counterparts of conserved E3s such as CHIP, E4B, or Prp19 is most likely to be maintained. However, a plant-specific subgroup has expanded dramatically, which is characterized by an N-terminal U-box combined with C-terminal ARM repeats. They account for the largest subgroup (64%), and may have adopted new functions in the control of plant-specific processes (Yee and Goring, 2009).

Molecular features

The U-box: a highly conserved E2 docking module

The U-box is a highly conserved E2-interacting module and the defining domain of PUBs (Fig. 2A). Interaction between the U-box and E2 is mediated by hydrophobic surfaces (Fig. 2A) (Zhang *et al.*, 2005). E2s mediate the catalytic step during ubiquitination and are largely responsible for the type of ubiquitination (Ye and Rape, 2009). Therefore, one of the critical questions that still remains unanswered is which E2s do PUBs (or most E3s actually) pair with *in vivo* to carry out their functions? E3s can interact with several E2s (Christensen *et al.*, 2007), and are therefore potentially able to generate different types of ubiquitin chains that mediate distinct outcomes for the modified targets (Fig. 1).

Various studies have addressed this aspect *in vitro* by testing the autoubiquitination activity of specific E3s in combination with various E2s (Kraft *et al.*, 2005; Wiborg *et al.*, 2008; Ramadan *et al.*, 2015). A recent study surveyed the pairing of 17 rice PUBs against 40 E2s by yeast two-hybrid (Y2H) assay using the U-box and the UBC domains only (Bae and Kim, 2014). Tested U-boxes, including that of SPL11, showed a clear preference for class VI E2s. These are the orthologues of the human UBE2D (UbcH5) family that mediates Lys48-linked polyubiquitination and proteasomal degradation (Ye and Rape, 2009). Tested U-box domains also showed a preference for type VII UBCs, which includes homologues of the human UBE2W that is involved in ubiquitin chain initiation and N-terminal ubiquitination (Scaglione *et al.*, 2011, 2013). However, pairing of U-boxes with type VIII UBCs, which are the cognate E2s of the APC/C complex (Ye and Rape, 2009), was also detected. Interaction with type VIII UBCs may thus be indicative of the caveats for such approaches. Even though these initial surveys are key, it still remains a challenge to identify physiological E2–E3 pairs and thereby unveil the true biochemical activities and functions carried out by specific E2–E3 pairs, as well as to demonstrate their biological relevance.

In addition, the U-box can mediate dimerization through its rear side, the surface opposite to the E2-docking region (Fig. 2A). Structural analysis of the *Saccharomyces cerevisiae* Prp19 (Vander Kooi *et al.*, 2006) and human CHIP (Zhang *et al.*, 2005) revealed that the dimer is formed by a hydrophobic patch and a hydrogen bond. Sequence alignment indicated that amino acids mediating the dimerization were conserved for some PUBs, including CHIP and PUB22 (Furlan *et al.*, 2017). Moreover, *in vivo* dimerization via the U-box was demonstrated for PUB22 and PUB24 (Furlan *et al.*, 2017). Structural and biochemical analyses showed that the Arabidopsis PUB14 may also dimerize in a similar fashion to the human CHIP (Andersen *et al.*, 2004).

The U-box is found in combinations with various additional domains, of which ARM repeats are the most common (41 out of 64 PUBs) (Andersen *et al.*, 2004; Mudgil *et al.*, 2004). The common domain arrangement of an N-terminal U-box followed by four and up to 13 predicted ARM repeats is exclusively found in plants. ARM repeats are characterized

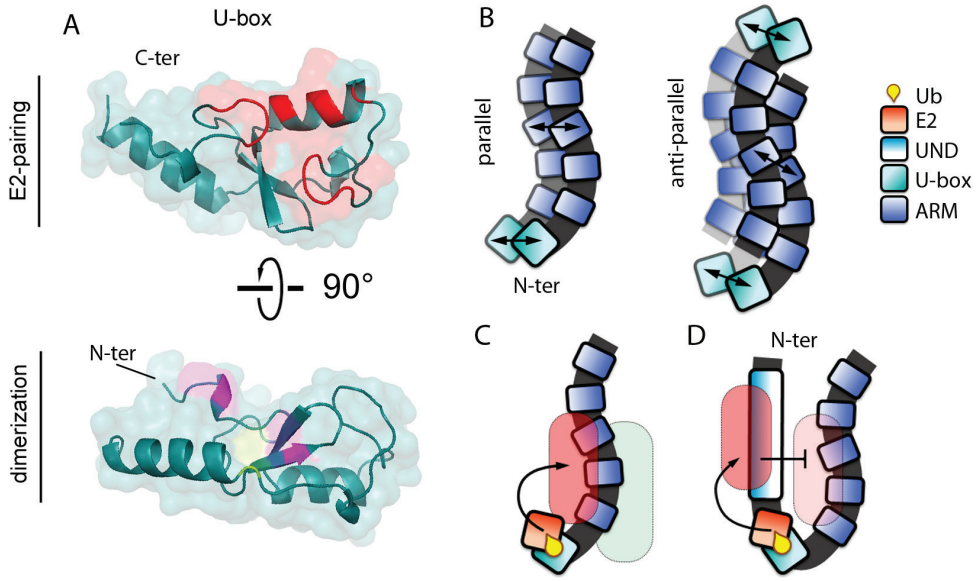

Fig. 2. PUB domains and hypothetical conformations. (A) Structural model of the PUB22 U-box. Shown are the surfaces mediating the interaction with E2. Highlighted in red are the residues predicted to mediate the interaction. The opposite side contains conserved hydrophobic amino acids (in magenta) and H-bond-forming asparagine (yellow), through which dimerization is mediated. Model adapted from Furlan *et al.* (2017). (B) PUB ligases are able to dimerize via a U-box (PUB22) and ARM repeats (PUB10). Shown are potential parallel (left) and anti-parallel arrangements (right) which would result in the formation of dimers or tetramers, respectively. (C) Cartoon of a PUB E3 ligase interacting with an E2–ubiquitin conjugate. Highlighted in red are protein interaction surfaces which would potentially render the interactor available to the E2–ubiquitin complex for ubiquitination, and highlighted in green are interaction surfaces that would not result in ubiquitination. (D) Cartoon of a PUB E3 ligase that possesses a UND. Highlighted in red are potential UND-mediated interaction surfaces, and highlighted in pink are interaction sites on the ARM repeats that could hypothetically be masked by the UND. Conformational changes may expose this surface and allow interaction with additional proteins.

by three α-helices which fold together in tandem as a superhelix (Huber *et al.*, 1997). The human ARM repeat-containing protein β-catenin mediates the interaction with a battery of proteins (Tewari *et al.*, 2010). In the case of PUBs, the ARM repeats have been shown mostly to mediate the interaction with substrates, indicating that interaction renders substrates available for ubiquitination. However, interaction with the ARM repeats does not necessarily result in the ubiquitination of the interactors if not accessible to the E2–ubiquitin conjugate (Fig. 2C). This appears to be the case for some PUB-interacting kinases (Samuel *et al.*, 2008; Mbengue *et al.*, 2010; Furlan *et al.*, 2017).

However, *in vitro* interaction assays using the ARM repeats of PUB10 suggest they can also play a role in dimerization (Jung *et al.*, 2015). These results open up the possibility that PUB proteins are also able to form higher order oligomers with different conformations (Fig. 2B). Dimer formation could be mediated by interactions through both the U-box and the ARM repeats in a parallel arrangement. Alternatively, a dimer formed by interaction through ARMs in an antiparallel fashion would be able to interact with another dimer via the U-box to form a tetramer (Fig. 2B). Such an interaction constellation could potentially control target binding and its subsequent ubiquitination. Indeed, oligomer formation was shown to regulate E3 ubiquitination activity in plants (Furlan *et al.*, 2017) and in other systems (Deshaies and Joazeiro, 2009).

However, the U-box protein Ufd2 from yeast, which is conserved across species including human and plants, displays an elongated structure consisting of several irregular ARM-like repeats with two additional helical hairpins protruding from it and a U-box domain attached to its C-terminus (Tu *et al.*, 2007). This indicates that the combination of ARM repeats and a U-box is evolutionarily ancient, and has experienced a reorientation and evolved into a large protein family in plants.

A peculiarity of a subgroup of 17 PUBs is the presence of a so-called U-box N-terminal domain (UND), which is ~200–250 amino acids in length. Semi *in vitro* ubiquitination assays suggest that UND does not affect interaction with E2s (Seo *et al.*, 2016). Until recently the function of the UND remained cryptic, but two studies may help us understand its purpose.

First, PUB13 required its U-box and UND to interact with RabA4b in a Y2H assay, suggesting that it is involved in protein–protein interactions (Antignani *et al.*, 2015). A second study showed that PUB18 required the presence of its UND to interact with the exocyst complex subunit Exo70B1 *in vivo*, and is physically associated with it, as shown by *in vitro* pull-downs. Indeed, the UND alone was able to interact with Exo70B1 in a Y2H assay (Seo *et al.*, 2016). Accordingly, a chimeric PUB22 fused to the PUB18 UND enabled interaction with Exo70B1, while at the same time suppressing Exo70B2 association, which is mediated via its ARM repeats. Remarkably though, a truncated variant of PUB18 lacking the UND was able to interact with Exo70B2, even though pairwise amino acid sequence identity of PUB22 and PUB18 ARM repeats is only 19.4%. These results suggest that besides mediating interaction with Exo70B1, PUB18 UND suppresses Exo70B2 interaction, potentially by folding back onto the interaction surface (Fig. 2D). These observations

open up the possibility that PUB18 may also target Exo70B2 upon release of negative regulation by its UND.

The Arabidopsis CHIP is highly similar to animal CHIPs, with three tetratricopeptide repeats (TPRs) at the N-terminal side, and a U-box domain at the C-terminal side. Like its human homologue, Arabidopsis CHIP interacts with molecular chaperones such as Hsc70 through its TPRs, and therefore is likely to modulate the switch from chaperone-assisted protein folding to proteasomal degradation (Lee *et al.*, 2009).

An additional highly conserved U-box E3 ligase is Prp19. The human Prp19 is an essential component of the Prp19 complex required for correct function of the spliceosome (Song *et al.*, 2010). In Arabidopsis, there are two homologues, Prp19A (MAC3A, PUB59) and Prp19B (MAC3B, PUB60), both of which contain a C-terminal WD40 domain. The Prp19 complex in humans modifies Prp3, a component of the U4 small nuclear riboprotein (snRNP), with non-proteolytic Lys63-linked ubiquitin chains dependent on the WD40 domain (Cho *et al.*, 2008).

PUB–kinase modules and activity regulation

One of the hallmarks of PUBs, which initially drew the attention of several labs, was their transcriptional induction in response to a wide array of stresses (Yee and Goring, 2009). More recent work, however, has started to peel off the various layers of PUB regulation. One of the most recurrent themes for PUBs is the interaction with kinase domains, especially receptor kinases, which in several cases also results in the phosphorylation of PUBs (Fig. 3). These kinase–PUB modules may be representative of feed-back loops by directly controlling proteolysis of cellular components and, hence, the processes in which they participate (Samuel *et al.*, 2008; Mbengue *et al.*, 2010; Furlan *et al.*, 2017). The first of such interactions to be reported was between ARC1 and the S-locus receptor SRK (Gu *et al.*, 1998), which results in the phosphorylation of ARC1 *in vitro* (Samuel *et al.*, 2008). A Y2H survey using various family members of the S-domain receptor kinases against different PUBs identified several potential interaction partners, suggesting that they represent conserved modules (Samuel *et al.*, 2008). Indeed, subsequent studies have uncovered additional PUB–kinase interactions in Arabidopsis (Lu *et al.*, 2011; Furlan *et al.*, 2017; Liao *et al.*, 2017), *Nicotiana benthamiana* (Kim *et al.*, 2003), *Medicago truncatula* (Mbengue *et al.*, 2010), as well as in rice (Ding *et al.*, 2009; Wang *et al.*, 2015). From the reported PUB–kinase modules, PUB13 is the only one reported to mediate the ubiquitination of the interacting kinase (Lu *et al.*, 2011;

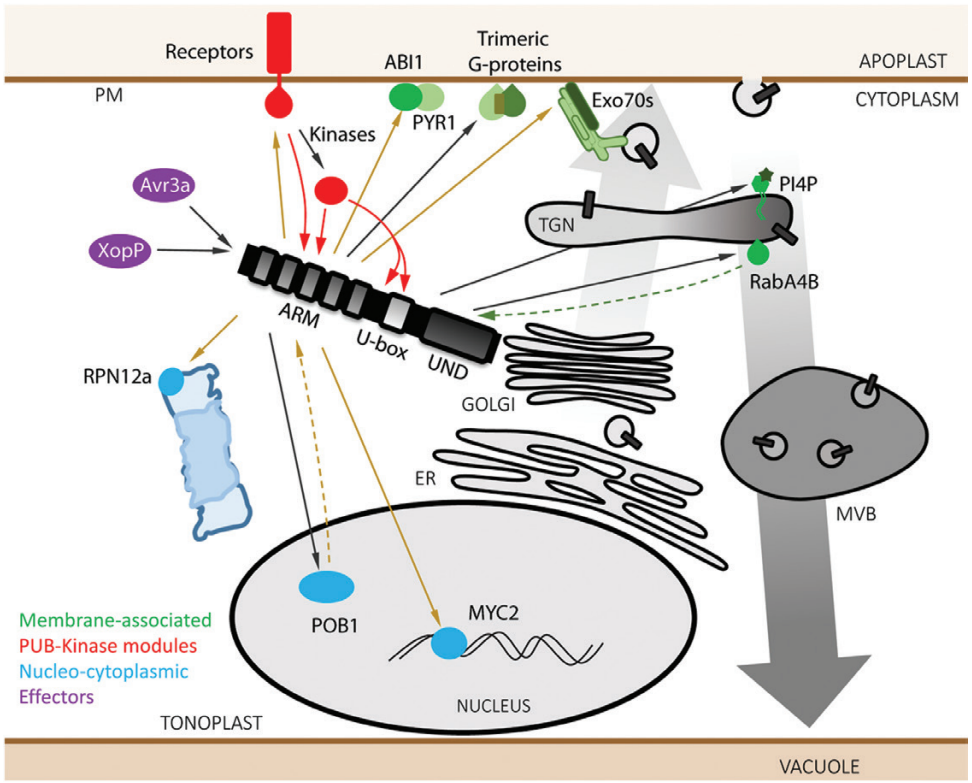

Fig. 3. Proteins and processes targeted by PUBs. Overview of a selection of PUB-interacting proteins and their subcellular localization. A PUB E3 cartoon with the domains UND, U-box, and five ARM repeats is shown, as a representative for all (including those without an UND). PUBs interact with several plasma membrane (PM)-located receptor-like kinases (RLKs) via soluble kinases, or phosphatases in the case of ABI1 (shown in red). Connection between PUB ligases and vesicle traffic, by interacting with proteins associated with membranes such as Exo70 subunits of the exocyst complex, PM-located proteins such as the ABA receptor PYR1, trimeric G-proteins, and the GTPase RabA4B, or direct interaction with phosphatidylinositol 4-phosphate (PI4P; shown in green). Protein levels of nuclear-localized PUBs can be regulated by POB1 and, in addition, transcription factors, such as Myc2, are targeted by PUBs (shown in blue). Ubiquitination of subunit RPN12a may regulate proteasomal activity (shown in blue). Bacterial (XopP) and oomycete effectors (Avr3a) target PUBs and manipulate their activity (shown in pink). TGN, *trans*-Golgi network; ER, endoplasmic reticulum; MVBs, multivesicular bodies. Black arrows denote interaction, red phosphorylation, and yellow ubiquitination. Dashed arrows represent a potential interaction.

Liao *et al.*, 2017). PUB13 was shown to ubiquitinate both FLS2 and LYK5 to mediate their degradation, potentially via the endocytic degradatory pathway (Liao *et al.*, 2017; Lu *et al.*, 2011).

The fact that most reported PUB–kinase interactions result only in the phosphorylation of the E3 may reflect the challenges posed by the analysis of ubiquitination. However, it may instead indicate that the modification between PUB–kinase modules is not in all cases reciprocal. For instance, PUB22 and *M. truncatula* PUB1 are phosphorylated by MPK3 and LYK3, respectively, but are unable to ubiquitinate them under the tested conditions (Mbengue *et al.*, 2010; Furlan *et al.*, 2017). The geometry of the PUB–kinase interaction may allow the kinase to phosphorylate the E3 while remaining inaccessible to the E2–ubiquitin conjugate docked on the U-box. Indeed, phosphorylation by MPK3 of PUB22 occurs on Thr62 located on the rear side of the U-box, indicating that MPK3 docks on a surface distant from the E2–ubiquitin conjugate (Furlan *et al.*, 2017). This nicely illustrates that interaction with an E3 ligase does not automatically imply that the interactor also serves as a substrate, a point which needs to be kept in mind.

Another potential outcome of PUB modification by kinases is subcellular relocalization. Transient co-expression of *Brassica napus* SRK_{910} with ARC1 in tobacco BY-2 cells resulted in relocalization of ARC1 from the cytosol and nucleus to endoplasmic reticulum-associated bodies (Stone *et al.*, 2003). Moreover, co-expression of Arabidopsis PUB13 with the cytoplasmic kinase MLPK, but not with the S-domain receptor kinase ARK1, resulted in its relocalization to as yet unidentified compartments (Samuel *et al.*, 2008). In addition, transient co-expression of PUB9 and ARK1 resulted in a striking relocalization to the plasma membrane (PM), which was dependent on ARK1's kinase activity and could also be triggered by abscisic acid (ABA) treatment (Samuel *et al.*, 2008).

Nevertheless, the mechanism by which phosphorylation impinges on PUB function was hitherto unknown. Analysis of PUB22 phosphorylation uncovered a mechanism that controls its modes of ubiquitination activity. PUB22 displays autoubiquitination, which *in vivo* mediates its degradation (Stegmann *et al.*, 2012). It was later shown to be phosphorylated by MPK3 at residues Thr62, located on the rear side of the U-box domain, and Thr88, which is found between the U-box and the first ARM repeat, a region predicted to be disordered (Furlan *et al.*, 2017). Both phosphorylation events contribute additively to the stabilization of PUB22 in response to the perception of pathogen-associated molecular patterns (PAMPs), which are conserved molecular motifs detected by PM-located receptors. While the mechanism by which Thr88 mediates PUB22 stabilization remains unclear, phosphorylation of Thr62 inhibited homodimerization, which is required for autoubiquitination *in trans* (meaning that one PUB22 molecule modifies the other), and thus allows PUB22 to accumulate and carry out its function.

Notably, autoubiquitination is inherent to most E3s *in vitro*, and may be responsible for their high instability *in vivo*. This may serve as a safety interlock mechanism, which allows them to function only when needed (Lee *et al.*, 2014). The back of the U-box and the related RING domain commonly mediate dimerization of E3 ligases (Deshaies and Joazeiro, 2009). The ability of PUB22 to heterodimerize *in vitro* may indicate that PUBs are linked via an interdependent network that controls their stability.

The effector proteins Avr3a and XopP from *Phytophthora infestans* and *Xanthomonas oryzae*, respectively, contribute to pathogen virulence. Both Avr3a and XopP inhibited autoubiquitination of CMPG1 and OsPUB44 *in vitro* and correlated with their accumulation *in vivo*, indicating that autoubiquitination may occur *in vivo* (Bos *et al.*, 2010; Ishikawa *et al.*, 2014). XopP specifically interacted with OsPUB44 via the unique residues Leu86 and His94. Both are located at the C-terminal end of the U-box, and predicted to be on an α-helix distal and opposite to the E2-interacting surface. It is therefore conceivable that stabilization of OsPUB44 by XopP is achieved by inhibition of dimerization in a fashion reminiscent to PUB22 regulation (Furlan *et al.*, 2017). However, dimer formation can also be required for activity as shown for the human RING E3s BIRC7 and RNF4 (Dou *et al.*, 2012; Plechanovová *et al.*, 2012).

In line with OsPUB44 function in immunity, incubation of the rice PUB15 with the kinase domain of the blast resistance transmembrane B-lectin-type receptor-like kinase (RLK) induced autoubiquitination, leading to the idea that phosphorylation may trigger its activity (Wang *et al.*, 2015).

Following the principle of inactivation by destruction of PUB22, E3 degradation can also be controlled by additional factors, as in the case of *N. benthamiana* PUB17 (ACRE276). PUB17 interacts with the BTB-BACK domain protein POB1, which results in PUB17's degradation in the nucleus (Fig. 3) (Orosa *et al.*, 2017). BTB domains are present in proteins such as NPR1, NPR3, and NPR4, and may mediate the interaction with Cullin3, a subunit of Cullin-RING-Ligases (CRLs) (Fu and Dong, 2013). This opens up the possibility that CRLs control PUB17 stability by ubiquitination.

Finally, ubiquitination can be reversed by ubiquitin-specific proteases called deubiquitinases (DUBs; Fig. 1). PUB10 ubiquitinates MYC2 *in vitro* and mediates its degradation *in vivo* (Fig. 3). MYC2 degradation is counteracted by the deubiquitinases USP12 and USP13, nicely illustrating an additional layer of how ubiquitination can be regulated (Jeong *et al.*, 2017).

Additional intersection points with cellular signalling

A second emerging theme is the interaction of PUBs with different types of G-proteins or proteins involved in their regulation (Fig. 3) (Kobayashi *et al.*, 2012; Hu *et al.*, 2013; Liu *et al.*, 2015; Wang *et al.*, 2017). G-proteins act as molecular switches that are involved in transmitting signals from a variety of stimuli.

A first report indicated that PUB20 interacts with the β-subunit AGB1 of a heterotrimeric G-protein, and that the interaction is mediated by the ARM repeats (Kobayashi *et al.*, 2012). PUB20 and PUB21 are rapidly transcriptionally induced by flg22, similarly to the closely related PUB22–PUB24 triplet (Navarro *et al.*, 2004; Trujillo *et al.*, 2008).

However, the function of PUB20 remains to be elucidated. Also TUD1, a PUB in rice, genetically interacted with a Gα subunit, known as D1/RGA1, to regulate brassinosteroid-mediated growth (Hu *et al.*, 2013). The Arabidopsis genome additionally encodes three EXTRA-LARGE G PROTEINs (XLGs) which, as canonical Gα subunits, contain a C-terminal Gα-like domain with GTP-hydrolysing activity (Wang *et al.*, 2017). All three XLGs encoded in the Arabidopsis genome interacted with PUB2 and PUB4, although the constellation of these interactions still requires further investigation (Wang *et al.*, 2017).

SPL11 interacts via its ARM repeats with SPIN6, a Rho-GAP, to mediate its degradation (Liu *et al.*, 2015). In addition, PUB13, the closest homologue of SPL11 in Arabidopsis, interacted with the small GTPase RAB4A (Antignani *et al.*, 2015).

With the exception of SPIN6, the outcome of the interaction between PUBs and G-proteins remains open. Two main scenarios are possible. First, the G-proteins serve as E3 substrates and are ubiquitinated, potentially leading to their degradation. Indeed, several examples from the mammalian field also support such a scenario (Chen *et al.*, 2009; Torrino *et al.*, 2011; Oberoi *et al.*, 2012). Alternatively, G-proteins could be effectors of PUBs. Most identified substrates of PUB ligases are targeted via the ARM repeats. However, interaction between PUB13 and RAB4A requires both the UND and U-box domains (Fig. 3) (Antignani *et al.*, 2015). The fact that RAB4A only interacts with PUB13 in its GTP-bound state opens up the possibility that PUB13 is modulated by RAB4A, instead of RAB4A being a ubiquitination substrate.

Cellular sites of PUB action

In addition to the recent insight into the regulation of PUB activity, the cellular sites at which they function are slowly being unravelled and include mainly the nucleus and membranes.

Nicotiana benthamiana PUB17 interaction with POB1 takes place in the nucleus (Orosa *et al.*, 2017), and it was shown that nuclear localization is required for Cf4-triggered cell death (Fig. 1) (He *et al.*, 2015). PUB10 targets MYC2 for degradation, suggesting that it may act in the nucleus (Jung *et al.*, 2015).

There is a growing body of evidence which links PUBs to processes related to vesicle transport (Fig. 3). Transient expression of PUBs suggests that some are associated with different endomembrane compartments (Stone *et al.*, 2003; Samuel *et al.*, 2008). In addition, a majority of the identified PUB substrates and interacting proteins are inherent membrane proteins, associated with membranes, or regulators of membrane transport (Samuel *et al.*, 2009; Mbengue *et al.*, 2010; Lu *et al.*, 2011; Stegmann *et al.*, 2012; Wang *et al.*, 2015; Seo *et al.*, 2016; Liao *et al.*, 2017).

PUB13 is one of the best studied E3s, and was recently shown to reside in both the Golgi and the *trans*-Golgi network (TGN). In plants, both endocytic and secretory pathways transit via the TGN (Viotti *et al.*, 2010), therefore suggesting that PUB13 participates in the sorting of endocytic cargo. However, Golgi localization may also indicate that it is involved in protein secretion.

PUB13 can target the phosphatase 2C (PP2C) ABI1, which, together with its cognate substrates, the ABA receptors PYR/PYLs, can localize to the PM (Demir *et al.*, 2013; Rodriguez *et al.*, 2014). ABI1 is also able to associate directly with ROP11 (Li *et al.*, 2012) and RopGEF1 (Li *et al.*, 2016). Interestingly, RopGEF1 is degraded via the endocytic pathway in response to ABA, which is likely to result in ROP11 deactivation and downstream activation of the ABA pathway via ABI1 inactivation (Li *et al.*, 2016). These observations highlight how FLS2 and ABI1 regulation, both cognate targets of PUB13, as well as potentially of its nearest homologue PUB12, is tightly linked to the endocytic degradatory pathway.

Another E3 shown to associate with membranes by biochemical fractionation is PUB4 (Wang *et al.*, 2017). Mutants of *PUB4* are less sensitive to CLV3, a peptide hormone perceived by the CLV1 receptor in conjunction with RLKs and receptor-like proteins (Kinoshita *et al.*, 2015*b*). Similar to the immune receptor FLS2, CLV1 is endocytosed and transported into the vacuole for degradation (Nimchuk *et al.*, 2011). The reported interactions of PUBs with kinase domains suggest the potential association between PUB4 and one of the RLKs involved in the CLV3 pathway. However, interaction could not be detected with CLV1, SOL2, RPK2, ACR4, or BAM1 in a Y2H assay (Kinoshita *et al.*, 2015*b*).

Interestingly, PUB13 interacts directly with phosphatidylinositol 4-phosphate (PI4P) via its ARM repeats (Antignani *et al.*, 2015). Phosphoinositides play a critical role in regulating membrane dynamics and vesicular transport, and specific phosphoinositide species display a unique distribution within the endomembrane compartments, acting as organelle-specific trafficking markers. This opens up the exciting possibility that PUBs directly interact with membranes, instead of indirectly associating with them via their targets. Also SAUL1 (PUB44) associates with the PM in an ARM repeat-dependent manner, which is potentially conserved in homologues from other species (Drechsel *et al.*, 2011; Vogelmann *et al.*, 2014). Structural analyses show that the superhelix formed by ARM repeats contain a long, positively charged groove (Valenta *et al.*, 2012). If conserved in PUBs, a similar feature may mediate the interaction with the negatively charged phospholipids (Antignani *et al.*, 2015).

Moreover, PUBs may also control vesicle transport by directly targeting its components. These include in *B. napus* Exo70A1 by ARC1 during the pollen self-incompatibility response (Samuel *et al.*, 2009), and Exo70B2 by PUB22 and potentially the closely related PUB23 and PUB24 (Stegmann *et al.*, 2012). Similarly, PUB18 targets Exo70B2's closest homologue Exo70B1, and was shown to associate with membranes in biochemical fractionation experiments (Seo *et al.*, 2016).

Together, these studies indicate that PUB function may be closely linked to the regulation of the transport of vesicles and membrane proteins.

The many engagements of PUBs

Development

Recent publications have uncovered new and exciting functions for PUBs in development. For instance, *pub4* mutants showed higher levels of cell proliferation in both shoot and root apical meristems. The root apical meristem of *pub4* seedlings displayed reduced inhibition of root cell proliferation and columella stem cell maintenance by CLV3 (Kinoshita *et al.*, 2015*b*). CLV3, a member of the CLE peptide family characterized by a 14 amino acid sequence motif, termed the CLE-box, which is secreted from stem cells and perceived in underlying cells by the CLV1 receptor kinase, plays a key role. Although previously shown to be involved in maintenance of the root meristem, CLV1 is best known for its function in stem cell maintenance of the shoot apical meristem (SAM; Somssich *et al.*, 2016). Interestingly, *PUB4* additionally contributes to SAM development. Crossing the *pub4* allele into *clv1*, *clv2*, and *clv3* resulted in a synergistic increase of SAM size. Accordingly, the number of carpels, which is known to reflect the relative size of the flower meristem and has been used as an indicator for the *clv* phenotype, was also increased (Kinoshita *et al.*, 2015*a*). In contrast to the phenotypes reported for the female organs, mutants of *pub4* and the related *pub2* displayed a reduced number of stamens and incomplete degeneration of the pollen tapetal cells (Wang *et al.*, 2013; Wang *et al.*, 2017).

Previous studies have demonstrated that meristem size is determined by the interactions between the two plant hormones: auxin as a promoter of cell division and cytokinin as a regulator for cell differentiation (Chandler and Werr, 2015). When grown in the presence of the artificial cytokinin benzylaminopurine, inhibition of seedling growth was reduced in *pub4*, and *pub2 pub4* plants were almost insensitive (Wang *et al.*, 2017). Treatment with *trans*-zeatin also resulted in small differences in SAM development in *pub4* plants compared with the wild type (Kinoshita *et al.*, 2015*b*).

Both cytokinin and CLV3 pathways are initiated by receptor kinases located at the PM (Larrieu and Vernoux, 2015). Phenotypes in *pub4* mutants may hence suggest that substrate(s) of PUB4 are components of general regulatory processes involved in both peptide and hormone signalling.

Additional PUBs have been implicated in the regulation of growth in response to different stimuli. Jasmonic acid (JA) inhibits root growth, and *pub10* plants were more sensitive to this effect (Jung *et al.*, 2015). JA-mediated inhibition was dependent on the transcription factor MYC2. Involvement of PUBs in hormone signalling was also reported for rice, where mutants of the PUB-encoding *TUD1* displayed stunted growth and insensitivity to brassinolide (Hu *et al.*, 2013).

The S-type receptor ARK2 interacts with PUB9 in a Y2H study (Samuel *et al.*, 2008). Single mutants of both *ARK2* and *PUB9* grew normally, but root growth and lateral root formation were reduced under phosphate-starved conditions in the *ark2-1*/*pub9-1* double mutants (Deb *et al.*, 2014). These phenotypes correlated with altered auxin responses.

Flowering time has been reported to be accelerated in *pub12*/*pub13* double mutants, which may reflect the elevated salicylic acid (SA) levels in these plants (W. Li *et al.*, 2012; Antignani *et al.*, 2015; Zhou *et al.*, 2015). In contrast, mutation of the nearest homologue in rice, *spl11*, resulted in delayed flowering under long-day conditions (Vega-Sánchez *et al.*, 2008).

The first PUB protein to be functionally characterized in plants was the *B. napus* ARC1 (Arm-Repeat Containing) (Stone *et al.*, 1999, 2003). ARC1 is required for pollen self-incompatibility in two different Brassicaceae species, *B. napus* and *Arabidopsis lyrata*. It is frequently deleted in self-compatible species, including *Arabidopsis thaliana*, and silencing of ARC1 in *A. lyrata* partially breaks self-incompatibility, allowing fertilization (Stone *et al.*, 1999; Indriolo *et al.*, 2012). Self-incompatibility signalling is activated with the landing of a self-pollen grain on a stigmatic papilla, and the pollen S-locus cysteine-rich/S-locus protein 11 (SCR/SP11) ligand is perceived by the allele-specific S receptor kinase (SRK) (for a detailed review, see Doucet *et al.*, 2016). Notably the reconstitution of this signalling circuit in *A. thaliana* resulted in a robust pollen self-incompatibility, indicating that ARC1 plays a key role in this response (Indriolo *et al.*, 2014). ARC1 is proposed to impede self-pollination by mediating the degradation of Exo70A1, a subunit of the exocyst mediating early tethering of vesicles during exocytosis, and thus down-regulating the secretory pathway and the delivery of components required for pollen germination (Fig. 1) (Samuel *et al.*, 2009; Indriolo *et al.*, 2014; Safavian *et al.*, 2015). In addition, the glyoxalase GLO1, which detoxifies methylglyoxal, a by-product of glycolysis, is also targeted by ARC1 (Sankaranarayanan *et al.*, 2015). Self-pollination was suggested to result in increased levels of methylglyoxal and of methylglyoxal-modified proteins, including GLO1. Data indicate that modified GLO1 was more effectively targeted for degradation by ARC1 in order to reject self-pollen (Sankaranarayanan *et al.*, 2015).

Abiotic stress

Plants are extremely resilient to a bewildering range of environmental conditions, including temperatures, light intensities, and water availability. In order to maintain cellular homeostasis, proteolytic systems are engaged at different levels, and include the degradation of non-functional proteins or entire organelles, such as damaged chloroplasts.

Light stress

During photosynthesis, high light conditions can result in the excessive excitation of chlorophyll, leading to its triplet state and the production of highly reactive singlet oxygen ($^1O_2$) (Dietz *et al.*, 2016). *Ferrochelatase 2* (*FC2*) mutants accumulate high levels of protoporphyrin, resulting in higher singlet oxygen production, leading to stronger chloroplast damage and degradation, when etiolated seedlings were transferred to an 8 h light regime (Woodson *et al.*, 2015). In order to identify the components involved in the clearing

of damaged chloroplasts, the authors performed a suppressor screen in which *PUB4* was identified. In contrast to *fc2* plants, greening of seedlings was restored in *pub4*/*fc2* double mutants. Enhanced ubiquitination detected in chloroplast fractions of *fc2* seedlings was additionally reduced in *pub4*/*fc2*. These observations led to the idea that PUB4 contributes to the clearing of chloroplasts by ubiquitination of as yet undetermined chloroplast proteins. Accordingly, *pub4* plants were more sensitive to high light (Woodson *et al.*, 2015). It remains open how removal of a PUB4-dependent quality control mechanism suppresses phototoxicity in *fc2* seedlings. Cytokinin controls chloroplast function and structure, and it increases chlorophyll biosynthesis (Cortleven and Schmülling, 2015). It will be interesting to know whether the *pub4* cytokinin phenotype (see above) is also linked to its effects on chloroplast homeostasis.

Reactive oxygen species (ROS), such as singlet oxygen, activate chloroplast retrograde signalling relaying information from the chloroplast to the nucleus to regulate nuclear genes involved in chloroplast function and stress adaptation (Van Aken and Van Breusegem, 2015). Nuclear-encoded chloroplast proteins are translated in the cytoplasm and transported into the chloroplast via N-terminal transit sequences recognized by specialized import complexes on the inner and outer envelope membranes. CHIP is a chaperone-dependent U-box E3 shown to mediate the degradation of ClpP4, the proteolytic subunit of the Clp chloroplast protease, and FtsH1, a subunit of the chloroplast FtsH protease (Shen *et al.*, 2007*a*, *b*). Plants overexpressing CHIP display lesions and enhanced ROS accumulation, which correlates with reduced levels of FtsH1 and ClpP4, leading to the assumption that it provides cytosolic control over the amount of FtsH1 and ClpP4 precursors available for chloroplast import.

CHIP also plays a more general role in the surveillance of chloroplast precursor proteins. It interacts with the chaperone protein Hsc70-4, which recognizes specific sequence motifs in transit peptides and thereby probably facilitates the ubiquitination of substrates and their degradation via the proteasome (Lee *et al.*, 2009).

The interaction of CHIP with chaperones facilitates the targeting of misfolded or damaged proteins, which may result from abiotic stresses, such as elevated temperatures. Plants with mutations in *CHIP* were more sensitive to heat shock, which resulted in the accumulation of insoluble protein aggregates (Zhou *et al.*, 2014). Mutant plants accumulated a number of proteins from the light-harvesting complex after heat stress. In contrast, mutants of the autophagic receptor *NBR1* accumulated a different set of proteins, which led to the suggestion that CHIP and NBR1 mediate distinct but complementary anti-proteotoxic pathways (Zhou *et al.*, 2014).

Drought stress

Cellular signalling in response to drought or osmotic stress by the ABA pathway is intimately involved in ubiquitination and proteolytic systems (Yu *et al.*, 2016). Several studies have underlined the role of several PUBs in drought stress and ABA signalling. As mentioned previously, PUB12 and PUB13 were reported to target ABI1, in response to ABA (Kong *et al.*, 2015). Notably, ABI1 ubiquitination by PUB12 and PUB13 required the presence of ABA and its receptor PYR1. Similarly, the ubiquitination of the A subunit of PP2A by CHIP was suggested to participate in ABA signalling (Luo *et al.*, 2006). In addition, plants lacking SAUL1 were impaired in ABA-induced inhibition of germination, suggesting that like PUB12/PUB13, they also are less sensitive to ABA (Salt *et al.*, 2011). Furthermore, *pub46 pub48* plants were found to be more sensitive to drought (Adler *et al.*, 2017).

Similar to *pub12*/*pub13* mutants, *pub18*/*pub19* plants were less sensitive to salt- and ABA-dependent inhibition of germination (Bergler and Hoth, 2011; Kong *et al.*, 2015). In contrast, in a subsequent study, *pub18*/*pub19* plants were more resilient to drought stress and displayed enhanced stomatal closure in response to ABA (Seo *et al.*, 2012). A potential reason for this apparent discrepancy may be the result of additional functions of PUB18/PUB19, at different stages of development. In seedlings, PUB18 was shown to target Exo70B1 for degradation and, accordingly, *exo70B1* plants were less responsive to ABA (Seo *et al.*, 2016). Notably, *pub22 pub23* mutants displayed a comparable phenotype that was ABA independent, suggesting that they contribute to drought responses via an alternative pathway (Cho *et al.*, 2008; Seo *et al.*, 2012). PUB22 and PUB23 interact and ubiquitinate RPN12 and RPN6 without affecting their levels (Cho *et al.*, 2008, 2015). Both RPN12 and RPN6 are regulatory particles of the proteasome, located in the base and the lid, respectively. The function of RPN12 is to recruit the RPN10 ubiquitin receptor (Riedinger *et al.*, 2010), while RPN6 is proposed to act as a molecular clamp holding the regulatory and the core subcomplexes together (Pathare *et al.*, 2012). In transgenic lines overexpressing PUB22 and PUB23, RPN12 associated with smaller molecular size complexes than in the wild type. Significantly, drought stress resulted in the association of RPN12 with complexes of a similar size. These results suggest that PUB22 and PUB23 ubiquitination may regulate proteasome composition, and hence imply a general role in protein homeostasis. In line with a similar function to PUB22 and PUB23 in drought, *pub9* mutants were more sensitive to ABA-dependent inhibition of germination (Samuel *et al.*, 2008).

The function of PUBs in drought is also conserved across species. Heterologous overexpression of the soybean *PUB8* in Arabidopsis showed decreased drought tolerance and inhibited ABA-mediated stomatal closure (Wang *et al.*, 2016). In rice, *PUB70* mutants were less sensitive to ABA, while overexpression resulted in the opposite effect (Tang *et al.*, 2016). PUB70 promoted the degradation of the transcription factor bZIP46 by using the MODD protein as an adaptor.

Immunity

Many PUBs have been implicated in the regulation of the immune response and cell death. One prominent role is the dampening of signalling triggered by PM-located receptors. Mutants of the related *PUB22*, *PUB23*, and *PUB24*, as well as *PUB12* and *PUB13*, display enhanced immune responses and

resistance against pathogens, indicating that they are involved in the dampening of responses (Trujillo *et al.*, 2008; Jacobs *et al.*, 2011; Lu *et al.*, 2011; Chen *et al.*, 2014). Negative regulation is inherent to all signalling networks (Ferrell, 2013), and is therefore critical to maintain cellular homeostasis.

Availability of receptors, and thus signal output potential, is controlled through vesicle transport by both the secretory pathway, which delivers newly synthesized proteins to the PM, and the endocytic degradatory pathways that transport activated or defective receptors to the vacuole for degradation. The function of ubiquitination as a sorting signal during vesicle trafficking is relatively well described (Isono *et al.*, 2010; Furlan *et al.*, 2012; Leitner *et al.*, 2012; Martins *et al.*, 2015). However, ubiquitination also plays a role in the regulation of the trafficking machinery. PUB22 was shown to mediate the degradation of the Exo70B2 subunit of the exocyst after activation of the immune receptor FLS2 (Stegmann *et al.*, 2012). Accordingly, *exo70B2* mutants show impaired activation of immunity and enhanced susceptibility (Pecenková *et al.*, 2011; Stegmann *et al.*, 2012). Although few cargoes of the exocyst are known in plants, pharmacological approaches suggest that it contributes to recycling of PM proteins (Drdová *et al.*, 2013). Similar processes are apparently in place to control pollen self-incompatibility, as mentioned earlier (Doucet *et al.*, 2016).

The closely related PUB12 and PUB13 are recruited to the activated FLS2 signalling complex through the co-receptor BAK1, to ubiquitinate FLS2 and contribute to its degradation (Fig. 1) (Lu *et al.*, 2011; Zhou *et al.*, 2015). Upon activation, FLS2 is internalized and transported to the vacuole for degradation via the endocytic degradatory pathway (Beck *et al.*, 2012; Spallek *et al.*, 2013; Mbengue *et al.*, 2016). However, it remains unknown at which step of its internalization FLS2 is ubiquitinated. The reported localization of PUB13 to the Golgi and the TGN (Antignani *et al.*, 2015) opened up the possibility that FLS2 is ubiquitinated at the TGN, which in plants participates in both secretion and endocytic traffic (Viotti *et al.*, 2010). However, PUB12 and PUB13 were shown to interact constitutively with BAK1 (Lu *et al.*, 2011). BAK1 is mostly located at the PM (Bücherl *et al.*, 2013), resulting in the prediction that a subpopulation of PUB12 or PUB13 should also be PM associated.

PUB13 also interacts with the chitooligosaccharide receptor LYK5, and mediates its ubiquitination *in vitro* (Liao *et al.*, 2017). Early responses to chitooctaose were increased in *pub13*, but were similar to the wild type in *pub12*. This specificity was absent for FLS2-mediated signalling, in which both PUBs contributed equally to its regulation (Lu *et al.*, 2011). Interestingly, long-term responses to chitooctaose, such as gene expression and callose deposition, were not changed in *pub13*.

Inactivation of the Arabidopsis *PUB13* also results in spontaneous cell death, enhanced levels of the defence hormone SA, and early flowering (Li *et al.*, 2012; Antignani *et al.*, 2015; Zhou *et al.*, 2015). Notably, mutation of *FLS2*, which is targeted by PUB12/13 via BAK1 (Lu *et al.*, 2011), was epistatic to *pub13*-caused cell death. This suggests that phenotypes are a consequence of FLS2 or BAK1 deregulation, while also indicating that other interactors, such as LYK5 and ABI1, are most probably not involved (Kong *et al.*, 2015; Liao *et al.*, 2017). Cell death was also SA dependent, since inactivation of key pathway components, *PAD4* and *SID2*, also suppressed the phenotype (Li *et al.*, 2012).

The spontaneous cell death phenotype of *pub13* plants is reminiscent of the *spl11* mutant, its closest sequence homologue in rice (Li *et al.*, 2012). SPL11 interacts with and ubiquitinates SPL11-interacting protein 6 (SPIN6), a Rho GTPase-activating protein. Although results indicate that SPL11 mediates the degradation of SPIN6, knock-out plants displayed phenotypes reminiscent of *spl11*, suggesting that it also played a negative regulatory role in cell death and innate immunity in rice (Liu *et al.*, 2015).

A second group of PUBs is involved in the activation of cell death responses. The founding member is the *N. benthamiana* CMPG1, which is required to trigger cell death by the tomato receptor-like protein Cf-9 and the fungal elicitor Avr9 (González-Lamothe *et al.*, 2006), and constitutes the closest homologue of the Arabidopsis PUB20 and PUB21. A subsequent study using transient silencing of *CMPG1* indicated that it is required for cell death responses triggered by PM-located Cf-9/Avr9, Cf-4/Avr4, Pto/AvrPto, or the oomycete PAMP, cellulose-binding elicitor lectin (CBEL). However, CMPG1 was dispensable for responses mediated by the nucleotide-binding leucine-rich repeat (NLR) proteins R3a, R2, and Rx, which are intracellular immune receptors that reside in the nucleus. Avr3a only impaired cell death triggered by PM-located receptors, suggesting that CMPG1 controls receptor activity at the PM (Gilroy *et al.*, 2011).

In contrast, the *Nicotiana tabacum* ACRE276 (PUB17) was also required for Cf-9/Avr9- and Cf-4/Avr4-triggered cell death in *N. tabacum*, but mutants of the closest homologue in Arabidopsis, *PUB17*, were compromised in NLR RPM1- and RPS4-mediated resistance (Yang *et al.*, 2006; He *et al.*, 2015). Transcriptional responses triggered by flg22 treatment were reduced by ACRE276 silencing (He *et al.*, 2015). Although PUB17 and ACRE276 apparently have a much broader function, silencing did not compromise cell death triggered by the *P. infestans* PAMP INF1, or co-expression of R3a/Avr3a, demonstrating that not all PAMP-triggered and cell death-associated responses require ACRE276 (He *et al.*, 2015).

Genetic and biochemical screens using the constitutive active NLR *suppressor of npr1-1, constitutive1* (*snc1*) have helped to dissect the connection between NLRs and PUBs. These studies have identified a complex which is essential for immunity mediated by various NLRs and include the two highly similar U-box proteins MAC3A (Prp19A) and MAC3B (Prp19B) (Palma *et al.*, 2007; Monaghan *et al.*, 2009). Because Prp19 is required for the activation of the spliceosome in yeast (Chan *et al.*, 2003), changes in splicing may regulate transcriptional activation of defence genes in plants during NLR-activated immunity (Monaghan *et al.*, 2009). Alternatively, it was recently shown that the Prp19 complex is recruited to transcribed genes and that it interacts with RNA polymerase II and the TREX complex, coupling transcription to nuclear mRNA export (Chanarat *et al.*, 2011). It is therefore conceivable that MAC3A and MAC3B are required

for full transcriptional activation during the immune response by contributing to similar processes.

A forward genetic screen in *mos4*/*snc1* mutants uncovered an additional PUB E3 mutant, *snc1-enhancing 3* (*MUSE3*), which is involved in regulating NLR levels. MUSE3 is the plant homologue of UFD2, an evolutionarily conserved protein with ubiquitin chain elongation function in yeast, known as E4 (Johnson *et al.*, 1995). Knocking out the Arabidopsis *UFD2* resulted in increased protein levels of the NLRs SNC1 and RPS2 (Huang *et al.*, 2014). Co-expressing UFD2 together with CPR1, a substrate adaptor for CRL E3s involved in the regulation of NLR levels, enhanced NLR degradation. It was therefore proposed that MUSE3 enhances CPR1 activity by elongating ubiquitin chains, thus acting as an E4 (Tu *et al.*, 2007).

SAUL1 was shown to influence senescence and cell death in an SA-dependent manner, which is characteristic of autoimmune mutants (Vogelmann *et al.*, 2012; Disch *et al.*, 2016). Accordingly, growth at high temperature and humidity, known to inhibit NLR signalling, suppressed the phenotype. This has led to the idea that SAUL1 may be guarded by an NLR protein and that its absence activates cell death. Indeed a recent publication identified SOC3, a Toll interleukin 1 receptor (TIR)-type NLR, to be responsible for the autoimmune phenotype in *saul1* plants (Tong *et al.*, 2017). However, SAUL1 and SOC3 do not appear to interact directly, suggesting that SOC3 activation occurs via an associated protein. It is possible that SOC3 surveils the status of a SAUL1 substrate. Moreover, mutant analysis of *SAUL1* and its nearest homologue *PUB43* in the *soc3* background indicated that they redundantly contribute to PAMP-triggered immunity (Tong *et al.*, 2017).

Guarding of PUBs by NLRs would make sense in light of two studies which showed that they are targeted by effector proteins. Avr3a, an effector of the potato blight pathogen *P. infestans*, interacts with and stabilizes CMPG1 in *N. benthamiana* (Bos *et al.*, 2010). CMPG1 stabilization by Avr3a was proposed to prevent host cell death during the biotrophic phase of infection (Fig. 1). Following a strategy similar to that of Avr3a on CMPG1, the XopP effector of the rice pathogen *Xanthomonas oryzae* pv. *oryzae* directly interacts with the rice PUB44 U-box domain and inhibits autoubiquitination *in vitro* (Ishikawa *et al.*, 2014). Transient overexpression of XopP resulted in the accumulation of OsPUB44. Silencing of OsPUB44, suppresses peptidoglycan (PGN)- and chitin-triggered immunity and *X. oryzae* resistance, and expression of XopP resulted in similar phenotypes, indicating that XopP inhibits OsPUB44 function.

Guarding by NLRs and targeting by pathogen effectors underlines the importance of PUBs in immunity.

New directions of PUB E3 research

One of the most prevalent themes that have surfaced recently is the interaction of PUBs with two types of key signalling components, namely kinases and G-proteins. The existence of PUB–kinase modules is well documented, with both receptor kinases and soluble kinases. Within the identified modules, the phosphorylation of PUBs is common and has been shown to control the E3 activity. Future studies will hopefully provide needed insight into the relationship between PUBs and G-proteins. The interaction with kinases and G-proteins positions PUBs at the crossroads of two major signalling pathways in which they may act as a rheostat of cell signalling and survey protein homeostasis.

It is generally expected that interaction with an E3 results in the ubiquitination of the interacting protein. However, absence of ubiquitination is not surprising since the constellation of the interaction may not necessarily render accessibility to the E2–ubiquitin conjugate. Hence, E3s may themselves serve as substrates for modifications, effectors of G-proteins, or, alternatively, use an interacting protein as a platform to gain access to a larger set of targets indirectly.

Evolutionary questions regarding the formation of the plant-specific combination of an N-terminal U-box and ARM repeats in the C-terminal half also require attention and may guide the elucidation of their biochemical function. A similar arrangement of domains in inverted order is present in the highly conserved Ufd2. In contrast to yeast and higher eukaryotes, inactivation of the Arabidopsis *Ufd2*/*MUSE3* orthologue leads to viable plants, indicating that other E3s, such as PUBs, may have assumed some of Ufd2's ancestral functions.

With an increasing number of PUB substrates being isolated, the need to characterize PUB function beyond genetic terms is becoming ever more significant. The identification of physiological PUB–E2 pairs and their biochemical analyses will be key to unveil their true *in vivo* functions. An important point to bear in mind is to discriminate between auto-ubiquitination and substrate ubiquitination, as some E3s may switch between these two activity modes in response to stimuli, as shown for PUB22.

Finally, to gain a deeper understanding of PUB function, structural analyses will be instrumental to decipher the complex interplay between the domains present in PUBs. The need for structural information is most acute for PUBs composed of an UND, a U-box, and ARM repeats, because they are unique to plants, in contrast to conserved E3s, such as Prp19, Ufd2, or CHIP.

A vibrant scientific community has emerged in the last decade that has begun to unravel the many implications of PUB functions in plants. Their key role in controlling stress response positions them as a key source of new research avenues to understand the ubiquitination process and strategies to improve crop resilience to ever-increasing environmental challenges.

Acknowledgements

I am grateful to Jacqui Monaghan and the Trujillo team for critical reading of the manuscript. Research in the laboratory of MT is supported by grants from the Leibniz Association, the Deutsche Forschungsgemeinschft (DFG), and the Boehringer Ingelheim Foundation.

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