Ubiquitin signalling: controlling the message of surface immune receptors

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Summary
Microbial attack is first detected by immune receptors located at the plasma membrane. Their activation triggers a plethora of signalling cascades that culminate in the immune response. Ubiquitin and ubiquitin-like protein modifiers play key roles in controlling signalling amplitude and intensity, as well as in buffering proteome imbalances caused by pathogen attack. Here I highlight some of the important advances in the field, which are starting to reveal an intertwined and complex signalling circuitry, which regulates cellular dynamics and protein degradation to maintain homeostasis.

I. Introduction
Immune receptors located at the plasma membrane (PM) can perceive conserved molecular motifs termed pathogen-associated molecular patterns (PAMPs), activating PAMP-triggered immunity (PTI). A plethora of subsequently activated signalling cascades leads to changes in enzyme activities and culminates in significant remodelling of the proteome by ramping up of protein biosynthesis and secretion, as well as protein degradation (Box 1). Pathogens, on their part, have evolved effectors, which sabotage immune responses and divert the host’s resources to allow colonization. For these reasons, maintenance of cellular homeostasis during an infection and the concomitant immune response pose a major challenge for plants.

Recent studies have further cemented the key role of ubiquitination in signal dampening, and exciting new studies revealed a role in signal activation, as well as new links have emerged connecting ubiquitination to the delivery of new receptors to the PM. For this Insight article, I will focus on the role of ubiquitin and ubiquitin-like signalling (Box 2) during PTI.

II. Endocytosis – lower the volume
As a means to dampen immune signalling, activated immune receptors are internalized by clathrin-mediated endocytosis, and transported to the vacuole for degradation (Box 2) (Spallek et al., 2013; Ortiz-Morea et al., 2016). Interestingly, in the resting state, several E3s are complexed with kinases, underlining a direct crosstalk between phosphorylations and ubiquitination (Fig. 1a). This is the case for the Arabidopsis PLANT U-BOX (PUB) E3 ligases PUB12 and PUB13, which constitutively interact with BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), but...
associate with FLAGELLIN-SENSING 2 (FLS2) after flg22 treatment (Lu et al., 2011; Liao et al., 2017) (Fig. 1a,b). Both E3s seem to play a more general role negatively regulating signalling of additional receptors, including LYSIN MOTIF RECEPTOR KINASE 5 (LYK5) (Liao et al., 2017). Similarly, SPOTTED LEAF11 (SPL11), the rice orthologue of PUB13, contributes to the destabilization of monocot-specific SPL11 CELL-DEATH SUPPRESSOR 2 (SDS2), an S-domain receptor-like kinase that positively regulates cell death and immunity (Fan et al., 2018). Indeed, signal dampening by ubiquitin-triggered degradation is not restricted to receptors, and includes the NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD), which is ubiquitinated by the PBL13-INTERACTING RING DOMAIN E3 LIGASE (PIRE) to mediate its degradation (Lee et al., 2020).

Evidence supporting a conserved role of ubiquitination in the endocytosis of integral PM proteins in plants is accumulating (Leitner et al., 2012; Martins et al., 2015). However, ubiquitination also plays a role in the ensuing stages on route to the vacuole, particularly at the endosomal sorting complex required for transport (ESCRT) (Mouliner-Anzola et al., 2020). PUB13 was reported to localize to the trans-Golgi network (TGN) (Antignani et al., 2015), which acts as a sorting station for secretory and endocyted cargo, opening up the possibility that receptors targeted by PUB13 were modified after endocytosis. However, Zhou et al. (2018) showed that PUB12 and PUB13 contribute to the endocytosis of BRI1, and to its degradation, placing E3 function at the PM, and suggesting that the same may hold true for FLS2 (Fig. 1c).

Box 1 Routes to degradation.

In addition to being responsible for the basal turnover of proteins, degradation pathways are integral to safeguarding cellular homeostasis during stresses, which result in increased protein or organelle malfunction. However, they also regulate cellular processes by removing enzymes and regulatory proteins, thus, for example, activating or dampening signalling.

The two main routes for protein degradation in plants are the proteasome and the lytic vacuole. Each one is specialized in the degradation of specific types of proteins. The proteasome is a large nucleo-cytosolic complex, which mainly recognizes soluble proteins tagged with Lys48-linked ubiquitin chains. It can also degrade nonsoluble proteins (e.g. aggregates), but only with the assistance of additional cellular machineries. The lytic vacuole is catered for by mainly two pathways, the endocytic pathway and autophagy. The endocytic pathway consists of distinct membrane compartments, mediating the internalization of plasma membrane (PM) proteins (e.g. receptors, channel proteins, NADPH oxidases, etc.), and their transport by vesicular traffic. Internalized proteins can be recycled to the PM or delivered to the vacuole for degradation. Autophagy comprises different pathways of which macro-autophagy is best described in plants. It is characterized by double membrane compartments that deliver a wide variety of substrates to the vacuole, including defective or aggregated proteins, and large cargoes such as damaged organelles. A common denominator for these pathways is ubiquitination of substrates/cargoes to be degraded.

Box 2 Ubiquitin modification cycle.

Ubiquitin, which plays a central role in coordinating many aspects of protein degradation, is a small 76-amino-acid protein modifier. Its attachment involves a sequential cascade of enzymatic activities. In the initial step, the ubiquitin-activating enzyme (E1) forms a thioester bond with ubiquitin under 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 through a reaction termed aminolysis. Ubiquitin can be attached to the E3 (autoubiquitination), a substrate, as well as to ubiquitin itself to build ubiquitin polymers. Ubiquitin can be linked to one another via one of its seven Lys residues, or Met1, giving rise to structurally and functionally distinct polymers (Yau & Rape, 2016). E2s largely determine the linkage between ubiquitin moieties and, thus, the ubiquitin signal. This signal can have different consequences for the modified protein, ranging from degradation by the proteasome, to changes in activity, to relocation. The differently linked ubiquitin chains adopt different topologies. This structural information is decoded by ubiquitin receptors, to mediate specific downstream responses. Ubiquitination is highly dynamic, because moieties are effectively cleaved off by deubiquitinating (DUB) enzymes, which display catalytic preferences for specific types of ubiquitin chains. It is notable that similar sets of machineries are also responsible for the attachment of ubiquitin-like proteins, such as SMALL UBIQUITIN-LIKE MODIFIER (SUMO), and AUTOPHAGY-RELATED 8 (ATG8), that are required for macro-autophagy.
Fig. 1 Ubiquitination during the immune response in three acts. Ubiquitin and SMALL UBIQUITIN-LIKE MODIFIER (SUMO) attachment during the immune response follow a chronological order, which has not yet been well defined. The schematic proposes a sequence of events based on indirect observations such as the timing of kinase activation (seconds to minutes) vs the endocytosis of immune receptors (c. 45 min). (a) First act – resting state. E3 ubiquitin ligases are not active towards their targets and associate with the nonactivated signalling complex that includes different kinases, and can include the heterotrimeric G protein complex (HTGC) composed of of XLG2/XLG3 (Gα), AGB1 (Gβ), and AGG1/AGG2 (Gγ). PUB22 is in its autoubiquitination mode and mediates its own degradation by the proteasome. The exocyst with its Exo70B2 subunit, mediates the tethering of secretory vesicles (SV), which deliver low amounts of FLS2 and other cargo. The SUMO E2 SCE is active in the nucleus and suppresses transcription of immune-related genes, but does not mediate SUMOylation of the nonactivated FLS2. The NADPH oxidase RBOHD is inactive. (b) Second act – early events (0–20 min). Ligand binding induces the heterodimerization of FLS2 and the EF-Tu receptor (EFR) with the coreceptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), activating intracellular kinase domains. BAK1 phosphorylates PUB12/13, CPK28 phosphorylates PUB25/26 and PUB4 is also phosphorylated potentially by EFR or BOTRYTIS-INDUCED KINASE 1 (BIK1). PUB22 is phosphorylated by MPK3, inhibiting autoubiquitination and stabilizing it. In addition, PUB22 switches the E2 to potentially mediate a different type of ubiquitin chain. SCE1 SUMOylates FLS2 and RHA3A/B monoubiquitinates BIK1, together allowing BIK1 dissociation. RHA3A/B monoubiquitination of BIK1 is also required for its endocytosis, potentially through clathrin-mediated endocytosis (CME). Subsequently, BIK1 (now cytosolic?) is degraded by the proteasome after being ubiquitinated by PUB25/26. Nuclear SCE1 is nitrosylated, inhibiting SUMOylation. (c) Third act (but not last!) – following events (>20 min). Ubiquitination of plasma membrane (PM) proteins, including FLS2 and RBOHD, initiates endocytosis by CME. FLS2 is deSUMOylated by DESY. Internalized cargo progresses through the endomembrane system, maturing into multivesicular bodies (MVBs) and is delivered to the vacuole for final degradation, terminating signalling. Exo70B2 is ubiquitinated by the active PUB22, contributing to its recruitment into autophagosomes (APSs), and delivery to the vacuole by macro-autophagy, thus reducing the secretion of naive receptors and other signalling components, avoiding excessive signalling. Owing to the inhibition of SCE1, SUMO is potentially removed by a SUMO-specific protease, relieving repressor activity and releasing transcription. The type of chain with which substrates are modified remains unknown. Notably, chain-building activity of E2–E3 pairs on a specific substrate have not been demonstrated. Therefore, depicted chain types are only the most likely ones and other chain types are possible. Ubiquitin in dashed lines has not been demonstrated. Proteasome structure was obtained from Protein Data Bank entry 4CR2.
responses to chitin by the receptor CERK1 (Desaki et al., 2019). More recently, PUB4 was proposed to play a dual role in BIK1 stability by promoting the degradation of nonactivated BIK1, while preserving activated BIK1 from degradation (Derkacheva et al., 2020) (Fig. 1b).

BIK1 levels are negatively regulated by the calcium-dependent kinase CPK28 (Monaghan et al., 2009) and positively regulated by the heterotrimeric G protein complexes, composed of XLG2/ XLG3 (Gα), AGB1 (Gβ) and AGG1/AGG2 (Gγ) (Liang et al., 2016). Interestingly, both G protein complexes and CPK28 regulation of BIK1 meet at the related E3s PUB25 and PUB26 (Wang et al., 2018a) (Fig. 1a,b). Both E3s favour nonactivated BIK1 as a substrate, indicated by increased interaction and ubiquitination of the nonactivated form (Wang et al., 2018a). In addition, PUB22 and PUB26 were also shown in the same study to regulate the levels of BIK1. One possible scenario is that after its release from FLS2, BIK1 is dephosphorylated (Couto et al., 2016), becoming available to the active PUB25/26, thus restricting the pool of nonactive BIK1s available for reactivation and continued signalling. Some parallels are observed in rice where the CPK28 homologue OsCPK4 regulates the stability of the BIK1 homologue OsRLCK176 (Wang et al., 2018b).

Although most research has been aimed at the BIK1 fraction anchored to the PM, it remains unclear how BIK1 localization is regulated, and if/how nuclear levels of BIK1 are regulated by ubiquitination (Lal et al., 2018). However, because BIK1’s degradation appears to be mainly driven by the proteasome (Box 2), it suggests that PM association is reversible in order to be accessed by the proteasome (Fig. 1b).

III. Secretion – pump up the volume

Perception of pathogen attack activates a reprogramming of the cell, which includes a marked increase in the secretory pathway activity to deliver naïve receptors to the PM, among other things (Wang et al., 2005; Tateda et al., 2014). The interplay between secretion and endocytosis/degradation determines the amount of receptors available at the PM.

The exocyst is an octameric complex that mediates the initial recognition between the secretory vesicle and the PM (Saeed et al., 2019). The Exo70B2 subunit and the related Exo70B1 are required for full activation of responses triggered by PM located receptors (Stegmann et al., 2012). Accordingly, FLS2 was recently shown to require Exo70B2 and Exo70B1 for its delivery to the PM (Wang et al., 2020). Triggered by FLS2 activation, Exo70B2 is ubiquitinated by the E3 PUB22, leading to its degradation (Stegmann et al., 2012). Of note, Exo70B2 is phosphorylated by MPK3, directly linking it to immune signalling. Its phosphorylation diverts it to the vacuole by autophagy for degradation, suggesting a link between ubiquitination and autophagy at this stage (Brillada et al., 2020). Thus, PUB22 may contribute to control the delivery of newly synthesized signalling components such as FLS2.

IV. Ubiquitin signals – what is the message?

The 37 E2 ubiquitin-conjugating enzymes (UBCs) catalyse ubiquitin attachment, with the assistance of E3s, of which there are more than 1300 in Arabidopsis (Box 1) (Vierstra, 2009). Although previous studies had tested E2-E3 activity in vitro (Kraft et al., 2005), Turek et al. (2018) screened the pairing of all 37 Arabidopsis E2s against the E3 PUB22 and E2 subsets against other E3s in vivo, identifying physiological pairs. PUB22, which dampens signalling mediated by various immune receptors (Trujillo et al., 2008), interacts with a subset of 11 E2s. Hence, not only does one E2 pair with many E3s, but one E3 also interacts with several E2s, indicating that one E3 can mediate the modification of substrates, or itself, with different types of chains. Moreover, interaction of PUB22 with UBC35 (aka UBC13) was induced by flg22 treatment, while interaction with UBC8 was inhibited (Turek et al., 2018). This reveals that pairing is dynamic...
and E3 ligases are endowed with different catalytic properties, depending on the cellular status (Fig. 1b). UBC35 and its close homologue UBC36 are the main sources of the Lys63 chain in Arabidopsis (Romero-Barrios et al., 2020), which play a key role in vesicle trafficking (Leitner et al., 2012; Martins et al., 2015; Moulinier-Anzola et al., 2020). Pairing of PUB22, its closely related homologue PUB24, and PUB4 with UBC35, supports a role in regulating vesicular traffic (Turek et al., 2018; Desaki et al., 2019; Derkacheva et al., 2020). PUB13, however, which mediates endocytosis of BRI1 (Zhou et al., 2018) and most likely that of FLS2 (Lu et al., 2011), interacted with a different subset of E2s. This suggests that it mediates the generation of a different type of chain to regulate receptor internalization or other steps of its trafficking to the vacuole.

The ubc35 ubc36 double mutant displays enhanced responses to flg22, reminiscent of the pub22 pub23 pub24 mutant (Turek et al., 2018). However, ubc35 ubc36 were more susceptible to virulent Pseudomonas syringae pv. tomato (Pt) (Turek et al., 2018), similar to pub4, which was more susceptible to Pt COR– and AhrcC (Derkacheva et al., 2020). These and other observations indicate that Lys63 chains have a manifold function and are likely to pair with a large complement of E3s.

While at early time points Lys63 chains are required for receptor transport to the vacuole, at later stages it may be engaged by selective autophagy to buffer cellular stress caused by the infection. Ubiquitin chains have recently emerged as signals decoded by autophagy receptors that physically link the autophagy machinery to ubiquitin-tagged substrates. In animals, autophagy receptors, including NEIGHBOR OF BRCA1 (NBR1), display a preference for Lys63 chains (Grunati & Dikic, 2018). It remains to be demonstrated which type of ubiquitin chain is preferentially bound by the Arabidopsis NBR1 (Svenning et al., 2011). However, NBR1 helps to dampen HopM1-dependent bacterial virulence, which induces proteophagy (Ustun et al., 2018).

The Arabidopsis E2 PUB8 and related group VI members are commonly used for in vitro ubiquitination assays as a result of their high activity (Kraft et al., 2005). Silencing of the putative Nicotiana benthamiana group VI orthologues inhibits flg22-induced reactive oxygen species burst and results in increased susceptibility to virulent bacteria (Zhou et al., 2017). The same study also showed that the effector AvrPtoB interacted in vivo with the group VI E2s, and were required to degrade the kinase FEN, an AvrPtoB substrate. This suggests that while group VI E2 contribute to the perception and mounting an immune response in the host, AvrPtoB hijacks the same group of E2s to undermine immune responses. Indeed, AvrPtoB autoubiquitination activity was highest with the Arabidopsis group VI E2 PUB8, as determined by a synthetic biology approach (Kowarschik et al., 2018). AvrPtoB was also active and interacted in vivo with the group III UBC3. UBC3 is related to UBC1, shown to mediate monoubiquitination and to affect responses to necrotrophic pathogens (Dhawan et al., 2009). Mass spectrometry analysis of the chain types produced by AvrPtoB with UBC3 and UBC8 suggested a tendency to the generation of Lys33-linked chains in both cases, which are not well characterized (Kowarschik et al., 2018). This hints at the possibility that AvrPto exceptionally determines the type of chain, instead of the E2s.

V. Regulation of ubiquitination – communicating with others

Many studies have underlined the dynamic nature of ubiquitination in response to the activation of immune signalling. We nevertheless still know little about how intracellular signalling, and in particular kinase-driven signalling, integrates into the modulation of ubiquitination. However, a few examples have started to reveal some basic mechanisms.

Hitherto, we lacked insight into how E1s and E2s are regulated. However, Skelly et al. (2019) elucidated a mechanism by which SUMOylation and functions together with SNC1 to activate transcriptional responses (Zhu et al., 2010; Niu et al., 2019).

Downstream, PUB E3s have been shown to form signalling modules with different types of kinases, which suggests that their activity may be directly relayed by kinase activation (Trujillo, 2018) (Fig. 1a). PUB22 specifically interacts with MPK3, which phosphorylates PUB22 at two residues, resulting in its quick stabilization (Stegmann et al., 2012; Furlan et al., 2017). Only phosphorylation of Thr62 inhibited autoubiquitination activity, by preventing PUB22 homo- and heterologimerization through its conserved U-box domain (Furlan et al., 2017). However, Thr62 phosphorylation increased substrate-directed ubiquitination to potentially avoid an excessive immune response (Furlan et al., 2017). A similar mechanism was proposed for the E3 TRAF6 that activates the NF-κB pathway in humans. TRAF6 is directly phosphorylated by MST4, preventing its oligomerization via a trimerization interface, and autoubiquitination, avoiding excessive inflammation (Jiao et al., 2015). Notably, autoubiquitination and concomitant degradation are common features of many E3s, suggesting that inhibition of autodestruction may generally serve as a molecular failsafe mechanism.

The mechanism by which the second phosphorylation site Thr88 regulates PUB22 remains unknown. CPK28 phosphorylation on corresponding sites was later reported for PUB25 (Thr95) and PUB26 (Thr94) (Fig. 2) (Wang et al., 2018a). These are located in the stretch linking the U-box and the Armadillo (ARM) repeats, and are conserved among the closely related PUB20–PUB26 (Fig. 2). The PUB25 T95E phosphomimic displayed enhanced BIK1 ubiquitination, but, in contrast to PUB22, it also increased autoubiquitination (Wang et al., 2018a). PUB13 is phosphorylated by BR1 on Thr343 in the linker stretch connecting the U-box with the ARM repeats adjacent to a highly conserved proline, also present on PUB4 (Fig. 2). Thr343 controls interaction of PUB13 with BR1, and kinase activity was required for BR1 ubiquitination (Wang et al., 2018a; Zhou et al., 2018). However, phosphorylation of PUB13 did not affect autoubiquitination (Zhou et al., 2018). Interestingly, theRalstonia solanacearum effector RipAC causes changes in PAMP-induced phosphorylation and reduction of PUB4 levels (Fig. 2). Some of the stretches linking the U-box are predicted to be intrinsically disordered for several PUBs (Fig. 2). Phosphorylation may therefore trigger a transition
into a structured state that allows interaction with substrates or modulates activity.

Beyond phosphorylation, both PUB25/26 and PUB4 constitutively associate with the heterotrimeric G protein complex inhibiting ubiquitination of nonactive BIK1 (Fig. 1a) (Wang et al., 2018a; Derkacheva et al., 2020). However, it remains unclear how the G protein complex is released from FLS2, allowing PUB25/26 and PUB4, which remain attached to XLG2/3 and AGB1, to engage BIK1.

VI. Conclusions and future challenges

New examples have confirmed that ubiquitin signalling is hard-wired to kinase-mediated signalling through E3-kinase complexes (Fig. 1a). Kinase activation relays ubiquitin signalling to control signal output by contributing to receptor-triggered signalling, mediating removal of active receptors, and controlling the delivery of new ones (Fig. 1). However, mechanisms by which phosphorylation translates into activity changes of the ubiquitination cascade are only beginning to be revealed.

Ubiquitin E2s shape ubiquitin signalling by catalysing attachment and the building of different types of chains with distinct functions. It will therefore be essential to include them in the conversation. Most E2s remain poorly characterized, but identifying E2 catalytic properties, and the signals (chain type) they generate, will provide a first step to elucidating their function. The inhibition of the SUMO E2 by nitrosylation has further revealed that additional layers of regulation are in place upstream of E3s. Moreover, E2–E3 pairing is dynamic, changing during the immune response, and adding unsuspected degrees of complexity.

It is important to understand how pathways controlling cellular homeostasis contribute to plant robustness. This insight will help us to improve traits to buffer the impact of pathogen attack and climate change on crop yields.

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Data availability

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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