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Ubiquitination of pattern recognition receptors in plant innate immunity

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Abstract

Lacking an adaptive immune system, plants largely rely on plasma membrane-resident pattern recognition receptors (PRRs) to sense pathogen invasion. Activation of PRRs leads to the profound immune responses that coordinately contribute to the restriction of pathogen multiplication. Protein posttranslational modifications dynamically shape the intensity and duration of the signaling pathways. In this review, we discuss the specific regulation of PRR activation and signaling by protein ubiquitination, endocytosis and degradation, with a particular focus on bacterial flagellin receptor FLS2 (flagellin sensing 2) in *Arabidopsis*.

Keywords

Ubiquitination; pattern recognition receptors (PRRs); plant innate immunity; endocytosis; protein degradation

INTRODUCTION

Plants and animals are exposed to an environment full of microorganisms and have to contend with the risk of infections. The first line of immune signaling is activated via sensing of the conserved signatures among different microbial species, which are termed as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), by plasma membrane (PM)-resident pattern recognition receptors (PRRs) (Boller & Felix, 2009, Schwessinger & Ronald, 2012). Recent evidence also indicates that PRRs detect endogenous molecules derived from damaged cells, termed as damage-associated molecular patterns (DAMPs) (Yamaguchi & Huffaker, 2011, Albert, 2013). Plant PRRs are often members of receptor-like kinases (RLKs) and receptor-like proteins (RLPs), which mediate PAMP- or

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MAMP-triggered immunity (PTI or MTI) contributing to host resistance against a broad spectrum of microbial infections (Antolin-Llovera *et al*., 2012, Monaghan & Zipfel, 2012). To overcome PTI, adapted pathogens have acquired virulence mechanisms, including the delivery of a cocktail of effectors by bacterial type III secretion system into the host cells (Lindeberg *et al*., 2006). Various effectors have been shown to target components of the immune system and interfere with PTI or host physiological responses, which is termed as effector-triggered susceptibility (ETS) (Jones & Dangl, 2006). To confine pathogens, plants have further evolved disease resistance (R) proteins that directly or indirectly recognize effectors to elicit effector-triggered immunity (ETI) (Chisholm *et al*., 2006, Jones & Dangl, 2006, DeYoung & Innes, 2006). The dynamic co-evolution of plant-microbe interactions is depicted as a zigzag model (Jones & Dangl, 2006, Bent & Mackey, 2007, Dodds & Rathjen, 2010). Accumulating evidence also supports the likely continuum and intimate crosstalks between PTI and ETI (Thomma *et al*., 2011).

Proper activation of PRRs ensures rapid defense responses to fend off potential infections. However, the excessive activation of defense responses can be detrimental even fatal to hosts. For instance, uncontrolled cytokine production in animals often leads to autoimmune or immune-mediated inflammatory diseases, such as rheumatoid arthritis and Crohn's disease (O'Shea *et al*., 2002). In plants, various lesion-mimic or dwarf mutants have been indicated to be associated with elevated or constitutive activation of defense responses (Lorrain *et al*., 2003, Lenk & Thordal-Christensen, 2009). Thus, the activated immune responses must be kept in check to avoid defense from running amok.

Emerging evidence suggests that endocytosis and degradation of receptors serve as one of common mechanisms to modulate signaling outputs (Altenbach & Robatzek, 2007, Sorkin & von Zastrow, 2009). In metazoans, upon growth factor ligand activation, receptor tyrosine kinases (RTKs) undergo endocytosis and subsequent intracellular degradation of both ligands and receptors (Lemmon & Schlessinger, 2010). The ligand-induced internalization and intracellular trafficking of PRRs have been reported in both plant and animal innate immunity (Robatzek *et al*., 2006, Kagan *et al*., 2008). One of the major mechanisms to trigger receptor endocytosis and lysosomal targeting is ubiquitination of the cytosolic domain of membrane receptors (Strous & Gent, 2002, Raiborg *et al*., 2003).

Ubiquitination is a protein post-translational modification in which various numbers of ubiquitin moieties are covalently attached to the substrates (Kerscher *et al*., 2006). Ubiquitination process consists of a stepwise reaction catalyzed by a series of enzymes including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3) (Smalle & Vierstra, 2004). The substrate specificity is largely determined by E3 ligases, which are broadly classified into four groups: HECT (homologous to E6-AP C terminus), RING finger type, U-box and Cullin–RING ligases (CRLs) (Vierstra, 2009). The consequences of ubiquitination vary with the modes of ubiquitination (monoubiquitination vs. polyubiquitination), type of ubiquitin chain linkages and the length of the ubiquitin chain (Komander & Rape, 2012, Tanno & Komada, 2013). An increasing body of evidence suggests the importance of ubiquitination in fine-tuning two major types of plant immune responses mediated by cell surface PRRs and intracellular R proteins (Dielen *et al*., 2010, Cheng & Li, 2012, Marino *et al*., 2012). Here, we focus on the recent advances

on ubiquitination-mediated PRR degradation and endocytosis, and their roles in fine-tuning plant PTI responses.

PLANT PRR UBIQUITINATION AND SIGNALING

The sessile plants appear to encode much expanded members of gene families potentially involved in the ubiquitination process with about 1415 putative E3 in *Arabidopsis* (Mazzucotelli *et al*., 2006). Interestingly, many of these genes exhibited transcriptional changes during various biotic and abiotic stress responses (Salinas-Mondragon *et al*., 1999, Ramonell *et al*., 2005).

Ubiquitination in FLS2 signaling

Arabidopsis FLS2, a leucine-rich repeat receptor-like kinase (LRR-RLK), functions as a PRR for bacterial flagellin or its active peptide derivative flg22 (Gomez-Gomez & Boller, 2000). Upon flg22 perception, FLS2 instantaneously complexes with another LRR-RLK BAK1 (brassinosteroid insensitive 1-associated kinase 1) (Chinchilla *et al*., 2007, Heese *et al*., 2007, Schulze *et al*., 2010). BAK1 could directly phosphorylate BIK1 (Botrytis-induced kinase 1), a PM-localized receptor-like cytoplasmic kinase (RLCK), to transduce flagellin signaling. BIK1 forms a complex with FLS2/BAK1 and is released from FLS2/BAK1 complex upon flg22 perception (Lu *et al*., 2010, Zhang *et al*., 2010). Activation of MAP kinases (MAPKs) and calcium-dependent protein kinases (CDPKs) functions independently or synergistically downstream of FLS2/BAK1 receptor complex to activate the expression of flg22-responsive genes (Asai *et al*., 2002, Boudsocq *et al*., 2010). In addition, flg22 perception leads to Ca^{2+} ion fluxes, production of reactive oxygen species (ROS) and ethylene, deposition of callose and stomatal closure to prevent pathogen entry (Fig. 1). The reader is directed to many excellent and comprehensive reviews that cover the flg22 and other MAMP perception and signaling events (Dodds & Rathjen, 2010, Boller & Felix, 2009, Schwessinger & Ronald, 2012, Nicaise *et al*., 2009). Plant innate immune signaling appears also under the tight control of negative regulation. A protein phosphatase functions as a negative regulator of FLS2 signaling by interacting with FLS2 (Gomez-Gomez *et al*., 2001). *Arabidopsis* MAPK phosphatase 1, MKP1, negatively regulates flg22 and other MAMP responses and plant immunity likely through dephosphorylation of MAPKs (Anderson *et al*., 2011). FLS2 is ubiquitinated by two closely related plant U-box E3 ubiquitin ligases, PUB12 and PUB13, and subjected for degradation (Lu *et al*., 2011). PUB12 and PUB13 interact with BAK1, and are recruited to FLS2 upon flg22 perception. BAK1 directly phosphorylates PUB12 and PUB13, and is required for FLS2 and PUB12/13 association. PUB12/13 can directly polyubiquitinate FLS2, but not BAK1 or BIK1, suggesting the specificity of substrate ubiquitination of receptor complex (Fig. 1) (Lu et al., 2011).

Protein phosphorylation and ubiquitination are two intertwined posttranslational modifications playing essential roles in diverse intracellular signal transduction pathways and physiological responses (Hunter, 2007). Multiple connections between phosphorylation and ubiquitination, which act either positively or negatively in both directions, have been established. For instance, ligand-induced trans-autophosphorylation of mammalian RTKs could lead to ubiquitination of receptor kinases for degradation (Lu & Hunter, 2009). The

mechanism of activating FLS2 ubiquitination appears unique and distinct from RTK signaling. PUB12 and PUB13 phosphorylation by BAK1 did not enhance its ubiquitination ability on FLS2 (Lu et al., 2011). Instead, phosphorylation seems to be required for flg22-induced FLS2-PUB12/13 association as a kinase inhibitor dramatically suppressed this association. Identification and characterization of PUB12 and PUB13 phosphorylation sites by BAK1 will facilitate the further elucidation of the detailed mechanisms of BAK1-mediated PUB12/13 phosphorylation on FLS2 ubiquitination. PUB22, PUB23 and PUB24, another subgroup of *Arabidopsis* U-box E3 ligases, function redundantly and negatively regulate flagellin-mediated signaling (Trujillo *et al*., 2008). Interestingly, PUB22 interacts with and ubiquitinates Exo70B2, a subunit of the exocyst complex that mediates vesicle tethering during exocytosis (Stegmann *et al*., 2012). Besides its role in the secretion of toxic compounds and cell wall reinforcement, exocytosis in vesicle trafficking contributes to maintain membrane integrity and remodeling in response to environmental cues (Ding *et al*., 2011). Exo70B2 is required for full activation of multiple MAMP-triggered responses and resistance against different pathogen infections. Perception of flg22 stabilizes PUB22 and promotes PUB22-mediated ubiquitination and degradation of Exo70B2 via the 26S proteasome, thereby attenuating flg22-mediated signaling (Fig. 1) (Stegmann et al., 2012). It is possible that Exo70B2-associated exocyst complex contributes to the recycling of certain important components in PTI signaling.

Ubiquitination in XA21 and Cf9 signaling

XA21 is an LRR-RLK PRR from rice (*Oryza sativa*) that confers resistance to specific races of *Xanthomonas oryzae* pv. *oryzae (Xoo)* (Song *et al*., 1995). In the absence of pathogen infection, XA21 interacts with XB24 (XA21 binding protein 24), an ATPase, which keeps XA21 in an inactive state (Chen *et al*., 2010b). Upon pathogen infection, XB24 is disassociated from XA21 likely through perception of certain MAMP from *Xoo*, which is proposed to activate XA21 signaling (Chen et al., 2010b). An E3 ubiquitin ligase, XB3, was identified as an interacting protein of XA21 and serves as a kinase substrate of XA21 (Wang *et al*., 2006). XB3 contains an ankyrin repeat domain mediating its interaction with XA21 and a RING finger motif carrying auto-ubiquitination activity. Unlike PUB12/13, XB3 positively regulates XA21 signaling and silencing *XB3* increases rice susceptibility to *Xoo* infection. In addition, XB3 is required for XA21 protein abundance (Wang et al., 2006). However, it remains unknown whether XB3 can directly ubiquitinate XA21 or ubiquitinate other components in XA21 signaling. In addition, attenuation of XA21 signaling is in part achieved by dephosphorylation of XA21 by the protein phosphatase 2C XB15 (Park *et al*., 2008).

Tomato Cf-9 confers resistance to races of the leaf mold fungus *Cladosporium fulvum* expressing the corresponding avirulence gene *Avr9* (Jones *et al*., 1994). Although classified as a plant R gene, *Cf-9* encodes a PM-resident LRR-RLP. Some members of LRR-RLPs have been shown or proposed to function as MAMP receptors (Thomma et al., 2011, Bar & Avni, 2009). Among the Avr9/Cf-9 Rapidly Elicited (ACRE) genes in tomato, there are at least three genes encoding E3 ubiquitin ligases, and two of them, *ACRE276* and *ACRE74*, encode U-box E3 ubiquitin ligases. Silencing of tomato *ACRE276* leads to breakdown of Cf-9-specified resistance against *C. fulvum* leaf mold. Both ACRE276 and ACRE74 are

positive regulators of cell death and disease resistance (Gonzalez-Lamothe *et al*., 2006, Yang *et al*., 2006). ACRE189, also inferred as ACIF1 (Avr9/Cf-9-induced F-box 1), is an F-box protein with an LRR domain. Silencing of tobacco *ACRE189* suppressed the hypersensitive responses (HR) triggered by various elicitors including Avr9, Avr4, AvrPto and the P50 helicase of *tobacco mosaic virus* (TMV) (van den Burg *et al*., 2008). It is likely that these ACRE proteins may function downstream of Cf-9 receptor. Identification of their substrates and activation mechanisms will shed light on their biochemical and physiological involvement in modulating defense responses.

Ubiquitination of PRRs and other host proteins by pathogen effectors

To launch a successful infection, adapted pathogens deploy various virulence strategies to interfere with plant immune responses. Interestingly, certain bacterial type III effectors possess E3 ligase activity and directly ubiquitinate PRRs or other host proteins in the suppression of plant immunity. AvrPtoB was originally identified as an avirulence protein from *Pseudomonas syringae* pv. *tamato* that recognizes tomato protein kinase Pto to trigger ETI responses (Kim *et al*., 2002). In *Arabidopsis*, AvrPtoB suppresses flg22 and many other MAMP responses by targeting BAK1 as one of the virulence mechanisms (He *et al*., 2006, Shan *et al*., 2008, Cheng *et al*., 2011, Zhou *et al*., 2013). AvrPtoB is a modular protein with a carboxy-terminal domain that is an E3 ubiquitin ligase (Janjusevic *et al*., 2006). It has been shown that AvrPtoB is able to ubiquitinate several PRRs, including FLS2 and CERK1, a receptor for fungal chitin (Goehre *et al*., 2008, Gimenez-Ibanez *et al*., 2009). AvrPtoB preferentially ubiquitinates the kinase domain of FLS2 *in vitro*, and promotes FLS2 degradation *in vivo* (Gohre *et al*., 2008). The E3 ligase activity of AvrPtoB is required for its full virulence in *P. syringae* pv. *tamato* and its suppression of host programmed cell death defenses (Janjusevic et al., 2006, Gohre et al., 2008). Similarly, AvrPtoB ubiquitinates the kinase domain of CERK1 *in vitro*, and directs CERK1 degradation *in vivo* (Gimenez-Ibanez et al., 2009). Interestingly, CERK1, a PRR of fungal chitin that mediates plant resistance to fungal pathogens, was found to be an important determinant of plant immunity to bacterial infection, which provides a rationale for CERK1 as a target of bacterial effector AvrPtoB (Gimenez-Ibanez et al., 2009). Indeed, CERK1 was required for peptidoglycan (PGN)-mediated responses and immunity to bacterial infections and was proposed to be a part of plant PGN receptor complex (Willmann *et al*., 2011).

As a modular protein, the N-terminal domain ($AvrPtoB_{1-387}$) of AvrPtoB lacking E3 ligase activity elicits a Pto-independent plant immunity, termed as Rsb (resistance suppressed by AvrPtoB C terminus), in tomato varieties lacking Pto and in *Nicotiana benthamiana* (Abramovitch *et al*., 2003). Fen, a homolog of Pto, interacts with $AvrPtoB_{1-387}$ and mediates the Rsb phenotype in tomato, but does not recognize full length AvrPtoB (Rosebrock *et al*., 2007). Interestingly, the C-terminal E3 ligase domain of AvrPtoB specifically ubiquitinates Fen, but not Pto or other Pto homologs, and promotes its degradation, thereby leading to the lack recognition of Fen by AvrPtoB (Rosebrock et al., 2007). Then, how does Pto activate immunity without being ubiquitinated by AvrPtoB? An elegant study shows that Pto phosphorylates AvrPtoB at Thr450 (T450) accompanied with the inactivation of AvrPtoB E3 ligase activity (Ntoukakis *et al*., 2009). Importantly, $AvrPtoB^{T450D}$, a phospho-mimic mutant, lost the E3 ligase activity and was able to trigger Fen-mediated Rsb phenotype, just

like AvrPtoB$_{1-387}$ or its E3 ligase mutants. AvrPtoB ubiquitinates Fen at Lys164 (K164), an invariant residue in protein kinase that often mediates phosphotransfer during phosphorylation reaction. The study suggests a model in which AvrPtoB polyubiquitinates Fen within the catalytic cleft of the kinase for proteasome-mediated degradation, whereas Pto avoids degradation by phosphorylating and inhibiting AvrPtoB E3 ligase activity (Ntoukakis et al., 2009). The research provides an example of intertwined relationship between protein phosphorylation and ubiquitination regulating plant immune responses.

CONSEQUENCE OF PRR UBIQUITINATION

Different types of ubiquitin chains generated by E3 ubiquitin ligases provide versatility of target proteins to distinct fates. One major consequence of protein ubiquitination is the subsequent targeting substrates to 26S proteasome for degradation, which can be experimentally inhibited by various proteasome inhibitors, such as MG132. In addition, ubiquitination can modulate the activity or localization of a target protein. For integral membrane proteins, ubiquitination often serves as one of major triggers for protein internalization through endocytic pathways to early endosome (EE) for signaling activation or further to late endosome (LE)/multivesicular body (MVB) and finally fuse with vacuoles/lysosomes for degradation (Vierstra, 2009, Haglund & Dikic, 2012, Komander & Rape, 2012). Evidence also indicates that 26S proteasome-mediated protein degradation pathway may be associated with endocytic pathways, and it is possible that they are not two completely independent pathways in mediating protein degradation (van Kerkhof *et al*., 2000, Abas & Wisniewska, 2006, Clague & Urbe, 2010).

PRR endocytosis

In mammals, LPS and its receptor TLR4 are endocytosed, trafficked to EE/sorting endosome, and then LE/lysosome for degradation. Endosomal trafficking of the LPS receptor complex is essential for signal termination (Husebye *et al*., 2006). It has been shown that FLS2 translocates into intracellular vesicles upon flg22 perception, followed by degradation (Robatzek et al., 2006). Recently, evidence suggests that internalized FLS2 proteins enter endocytic pathways with two distinct trafficking routes depending on its activation status (Beck *et al*., 2012). In the absence of flg22, non-activated FLS2 constitutively recycles between PM and EE independent of its signaling partner BAK1 (Fig. 2). This endocytic recycling is likely to regulate the abundance of receptors at PM and maintain a constant pool of signaling receptors (Beck et al., 2012). Upon flg22 perception, FLS2 enters a distinct endocytic trafficking pathway in which it transiently localizes at trans-Golgi network (TGN)/EE in the early stage, and transports to intermediate compartments with features between the TGN/EE and LE/MVB, followed by sorting into LE/MVB and vacuole for degradation (Fig. 2) (Beck et al., 2012, Choi *et al*., 2013). In *Arabidopsis* seedlings, FLS2-GFP fluorescence signal disappeared from PM after approximately 20–40 minutes upon flg22 treatment and subsequently fluorescence-labeled vesicles appeared in cytoplasm. These vesicles likely arise from a Wortmannin-sensitive endocytic process and can be blocked by actin inhibitors (Robatzek et al., 2006). In plants, endocytosis is mainly mediated by vesicle coat protein clathrin, called clathrin-mediated endocytosis (CME) (Chen *et al*., 2011). Recently, plant defense hormone salicylic acid (SA)

has been reported to affect CME of several PM-resident proteins, but not FLS2 (Du *et al*., 2013). These results suggest that various pathways may involve in the endocytosis of plant PM proteins.

The mechanisms of activation of FLS2 internalization and differential cargo sorting of endocytosis remain largely unknown. Phosphorylation and ubiquitination have been shown to play essential roles to activate the endocytosis and cargo sorting for various proteins (Goh *et al*., 2010). Kinase inhibitor K252a completely abolished flg22-mediated FLS2 endocytosis. A mutation in a potential phosphorylation site, $FLS2^{T867V}$, also compromised its endocytosis (Robatzek et al., 2006). Consistent with the potential transphosphorylation between BAK1 and FLS2, BAK1 is required for ligand-induced FLS2 endocytosis. It remains elusive whether phosphorylation serves as a trigger for FLS2 internalization. The involvement of ubiquitination in endocytosis could occur either in endocytosis initiation or cargo sorting step. For mammalian PM-resident RTKs, TLRs and G-protein-coupled receptors (GPCRs), ubiquitination appears not required for efficient endocytosis initiation as prevention of receptor ubiquitination, in many cases, has been shown to have little effect on the endocytosis (Hislop & von Zastrow, 2011, Haglund & Dikic, 2012, Clague *et al*., 2012). On the other hand, ubiquitination is an important signal to direct sorting of receptors into the MVB for lysosomal degradation (Tanno & Komada, 2013). Mutation of the ubiquitination sites often blocks the degradation of internalized mammalian receptors (MacGurn *et al*., 2012, Haglund & Dikic, 2012). *Arabidopsis* BOR1 (Requires High Boron 1) is mono- or di-ubiquitinated upon boron application. Boron-induced ubiquitination of BOR1 is not required for endocytosis from PM but is crucial for further sorting to MVB and subsequent degradation in vacuoles (Kasai *et al*., 2011). In another case, *Arabidopsis* IRT1 (iron-regulated transporter 1) is found in TGN/EE and likely undergoes endocytosis and subsequent degradation in vacuoles (Barberon *et al*., 2011). IRT1 is monoubiquitinated via unknown E3 ubiquitin ligase(s) on several cytosol-exposed residues *in vivo*. Mutations of two putative mono-ubiquitination sites stabilize IRT1 at PM and leads to extreme lethality by metal overload (Barberon et al., 2011). Recently, a RING-type E3 ubiquitin ligase IDF1 (IRT1 degradation factors 1) has been found to be required for IRT1 protein stability (Shin *et al*., 2013). It remains unknown whether IDF1 or other E3 ubiquitin ligases mediate the IRT1 endocytosis process.

FLS2 possesses a PEST-like motif at its C-terminus, which is often associated with monoubiquitination in yeasts and mammals (Roth & Davis, 2000). Mutation in PEST motif impairs FLS2 endocytosis, suggesting that modification on PEST motif, likely through monoubiquitination, may be involved in initiation of ligand-induced FLS2 endocytosis (Robatzek et al., 2006). It remains an open question whether PUB12 and PUB13 are involved in FLS2 internalization. Evidence suggests that PUB12 and PUB13-mediated FLS2 ubiquitination and flg22-induced FLS2 internalization are likely uncoupled. FLS2 PEST motif and kinase inactive mutants are compromised in FLS2 endocytosis, but do not affect FLS2 ubiquitination by PUB12 and PUB13 *in vitro* (Lu et al., 2011). This finding is not surprising since FLS2 ubiquitination by PUB12/13 is mainly polyubiquitination, which often leads to different substrate fates from mono-ubiquitination. The intracellular juxtamembrane domain of FLS2 is required for PUB12/13-mediated ubiquitination (D.L. & L.S.

unpublished data). This is consistent with the role of juxtamembrane domain of receptor kinases in creating docking sites for recruiting components in fine-tuning the signaling output (Lemmon & Schlessinger, 2010). The likely uncoupling of ligand-induced FLS2 endocytosis and degradation suggests distinct ubiquitination mechanisms operating these two linked processes. It is possible that a distinct E3 ligase mediates initiation of FLS2 endocytosis through PEST domain.

The rice RLK XA21 is internalized and likely transported via TGN/EE compartment (Chen *et al*., 2010a). Similar to the ligand-independent constitutive endocytosis trafficking of FLS2, XA21 is internalized via BFA-sensitive vesicles in rice protoplasts. It will be of interest to investigate the endocytosis and recycling of XA21 during pathogen infection. Nevertheless, whether XA21-associated E3 ligase XB3 or other E3 ligase-mediated ubiquitination serves as one of triggers for this endocytosis process remains unknown.

The tomato RLP receptor LeEix2 initiates defense responses upon perception of fungal protein EIX (Ethylene-Inducing Xylanase). EIX triggers the internalization of LeEix2 from PM to EE compartments labeled by FYVE (Fab-1, YGL023, Vps27, and EEA1) domain (Bar & Avni, 2009). Inhibition of internalization by chemical treatments resulted in a complete arrest of EIX-induced signaling. Furthermore, some EE compartments undergo a directional movement to a greater distance at an elevated speed upon EIX application. The data suggest that internalization of LeEix2 receptor is required for LeEix2-mediated signaling (Bar & Avni, 2009, Sharfman *et al*., 2011). It remains to be determined whether LeEix2 undergoes ubiquitination upon EIX treatment. LeEix2 endocytosis requires the tyrosine-based motif YXXΦ, a putative internalization signal that binds to clathrin-associated proteins. Mutation of this motif inhibits LeEix2 internalization and abolishes its ability to induce HR in tomato (Ron & Avni, 2004). A similar motif is present in the cytoplasmic C-terminus of tomato RLP Cf-4 and is required for Cf-4 function (Voossen et al., MPMI congress abstracts, 2009). Moreover, analysis of LRR-RLPs from *Arabidopsis* and rice revealed that 9 of the 56 *Arabidopsis* proteins and 20 of the 90 rice proteins contain the YXXΦ motif (Fritz-Laylin *et al*., 2005). In addition, some LRR-RLKs including EFR, XA21 and BAK1 also possess this motif (Geldner & Robatzek, 2008). Although the biological function of this motif is not clear, this suggests the existence of a common mechanism of endocytosis in mediating RLK and RLP signaling.

PRR degradation

In mammals, a RING-type E3 ubiquitin ligase Triad3A ubiquitinates Toll-like receptors TLR4 and TLR9 and promotes their proteolytic degradation (Chuang & Ulevitch, 2004). The extent of Triad3A-dependent TLR9 ubiquitination is increased in the presence of the proteasome inhibitor MG132. Consistently, the degradation of TLR9 is blocked by the treatment with the irreversible proteasome inhibitor lactacystin but not by the lysosomotropic agent NH_4Cl or the lysosomal protease inhibitor E64. Together, the data support that Triad3A-mediated ubiquitination promotes 26S proteasome-mediated TLR degradation. Genetic analysis with overexpression and loss-of-function of Triad3A suggests that it negatively regulates TLR activation and controls the intensity and duration of TLR signaling (Chuang & Ulevitch, 2004). Evidence also exists for the 26S proteasome-mediated

degradation of plant PRRs. The degradation and internalization of FLS2-GFP were substantially compromised by the treatment of MG132 (Robatzek et al., 2006). PUB12/13-mediated ubiquitination and degradation of FLS2 also involve 26S proteasome since MG132 blocks flg22-induced degradation. Similar with Triad3A, PUB12/13 negatively regulate plant PTI signaling as the *pub12/13* mutant exhibits enhanced immune responses to bacterial infection (Lu et al., 2011). The control of duration and intensity of immunity by E3 ubiquitin ligase-mediated degradation is important for plant normal growth and development. Mutation of rice *PUB13* ortholog *SPL11* causes plants with spontaneous cell death, a likely uncontrolled immune response (Zeng *et al*., 2004). The similar phenotype was observed in *Arabidopsis pub13* mutant under excessive light conditions and high humidity (Li *et al*., 2012).

The *Lotus japonicus* SYMRK (Symbiosis RLK) is required for signal transduction in root symbiosis (Stracke *et al*., 2002). SINA4, a SYMRK interacting protein, belongs to SINA (Seven in Absentia) E3 ubiquitin ligase family. Overexpression of SINA4 in both *N. benthamiana* and *L. japonicus* nodulated roots induces SYMRK degradation and re-localization (Den Herder *et al*., 2012). However, it is not clear whether SINA4 mediates SYMRK ubiquitination. It is also possible that SINA4 ubiquitinates other components that regulate SYMRK protein stability.

Ubiquitin-tagged proteins can be degraded via three major pathways: proteasome, lysosome/vacuole, and autophagosome (Clague & Urbe, 2010). The internalized membrane-located receptors are often degraded in the vacuole or the lysosome. For instance, AvrPtoB-mediated CERK1 degradation is likely vacuolar dependent since it is blocked by Bafilomycin A1, a vacuolar-type H+-ATPase inhibitor, not by MG132 (Gimenez-Ibanez et al., 2009). However, a considerable number of studies also indicate that the proteasome inhibitor MG132 could block membrane protein endocytosis and degradation. In addition to the above mentioned FLS2 and TLR9 (Robatzek et al., 2006, Lu et al., 2011, Gohre et al., 2008, Chuang & Ulevitch, 2004), auxin efflux carrier PIN2 and water channel aquaporin PIP2;1 could be stabilized with MG132 treatment (Abas & Wisniewska, 2006, Lee *et al*., 2009). The ligand-induced degradation of mammalian growth hormone receptor (GHR) and EGFR can be blocked by various proteasome specific inhibitors (van Kerkhof et al., 2000, Longva *et al*., 2002). It is likely that the effects of proteasome inhibitors on lysosome degradation of integral membrane proteins might be indirect. These inhibitors, such as MG132, may affect the activity of lysosomal enzymes, or reduce the ubiquitin pool in the cell (Gorbea *et al*., 2010, Melikova *et al*., 2006). Existing evidence from mammalian studies also suggests the direct involvement of the proteasome activity in certain steps of membrane protein endosomal sorting processes. Translocation of the activated EGFR from the outer membrane to inner membrane of MVBs could be blocked by various proteasome inhibitors (Longva et al., 2002). Ligand-induced lysosomal EGFR degradation is preceded by EGFR de-ubiquitination, which requires 26S proteasome activity (Alwan *et al*., 2003). Similarly, the lysosomal degradation of human TrkA (neurotrophic tyrosine kinase receptor type I) also requires proteasome-dependent de-ubiquitination. Ubiquitinated TrkA employs the endosomal-lysosomal pathway for degradation and a proteasome-dependent de-ubiquitination step precedes its delivery to lysosomes (Geetha & Wooten, 2008). In addition,

evidence also suggests the existence of a proteasome pool that associates with endosomes and influences receptor endosomal sorting. Human Ecm29-associated 26S proteasomes are present on flotillin-positive endosomes and Ecm29 functions as an adaptor in the localization of the 26S proteasome on endosomes, ER membrane, and centrosome (Gorbea et al., 2010). Thus, it is possible that the proteasome pathway is involved in an endosomal sorting step of ubiquitinated proteins to lysosomes, thereby providing a mechanism for regulated degradation.

Conclusion and perspective

The PM-resident PRRs serve as an array of surveillance radar antennas, which promptly detect the microbial and danger signals and launch robust defense responses. Precise and efficient activation and attenuation of PRR signaling are crucial for any organism survival. It has become an emerging theme that ubiquitination and endocytosis play important roles in fine-tuning PRR signaling. Despite the fact that a mechanistic understanding is still missing, various components involved in ubiquitination and endocytosis processes, for instance, distinct families of E3 ubiquitin ligases, have been found to be involved in PRR signaling. Certain E3 ubiquitin ligases are able to directly ubiquitinate PRR receptors and mediate their degradation whereas some seem to ubiquitinate the components associated with PRR signaling. However, the connection between ubiquitination and endocytosis has not been established. In addition, it remains largely unknown what are the mechanisms underlying ligand-induced PRR endocytosis activation and whether endocytosis is linked with signaling activation. Ubiquitination as one of the most prevalent post-translational modifications is likely involved in the regulation of various signaling components in plant innate immunity. Genome-wide characterization of ubiquitination dynamics in plant immune signaling will provide a global view of the role of protein ubiquitination in fine-tuning a variety of signaling outputs. Development of versatile and sensitive *in vivo* and *in vitro* ubiquitination assays in combination with label free quantitative proteomics will identify novel components in ubiquitination-mediated plant PRR signaling.

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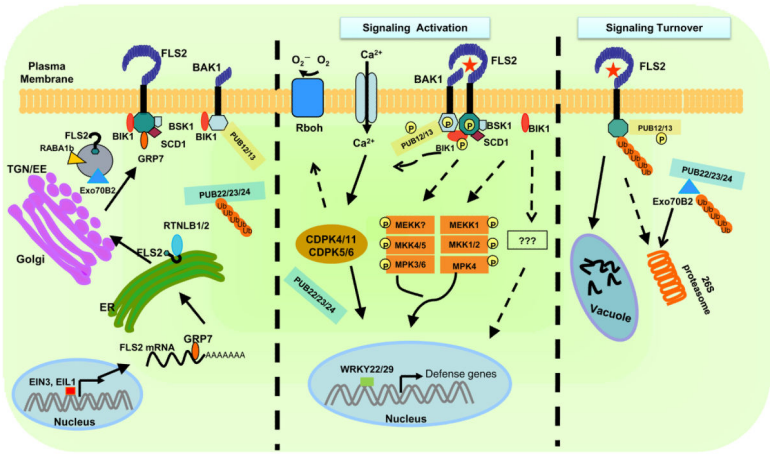

Figure 1. FLS2 signaling pathway in *Arabidopsis*

The transcription, translation and maturation of FLS2, the *Arabidopsis* receptor of bacterial flagellin (flg22), require EIN3 (ethylene insensitive 3)/EIL1 (ethylene insensitive 3-Like 1) (Boutrot *et al*., 2010, Mersmann *et al*., 2010), GRP7 (glycine-rich protein 7) (Nicaise *et al*., 2013), ER-resident reticulon-like proteins RTNLB1/RTNLB2 (Lee *et al*., 2011) and RABA1b (Ras genes from rat brain a1b) (Choi et al., 2013). FLS2 constitutively interacts with BIK1 and BSK1(BR-signaling kinase 1) (Shi *et al*., 2013), two RLCKs that positively regulate FLS2 signaling, and SCD1 (stomatal cytokinesis-defective 1) required for certain FLS2 responses (Korasick *et al*., 2010). BAK1 constitutively interacts with BIK1 and E3 ubiquitin ligases PUB12/13. Binding of flg22 likely causes conformational change of FLS2, which further recruits BAK1 to the complex. The dimerization of FLS2/BAK1 leads to the phosphorylation of FLS2/BAK1/BIK1 complex, and subsequent release of BIK1. Rapid Ca^{2+} influx, oxidative burst mediated by PM-resident NADPH-oxidase Rboh, and activation of MAPK and CDPK cascades collectively activate and amplify the defense gene reprogramming and other defense responses. PUB12/13 are phosphorylated by BAK1 and interact with FLS2 upon flg22 perception, thereby promoting polyubiquitination of FLS2 to tune down the signaling. PUB22/23/24 polyubiquitinate Exo70B, a subunit of the exocyst complex, for 26S proteasome degradation to down-regulate the defense signaling.

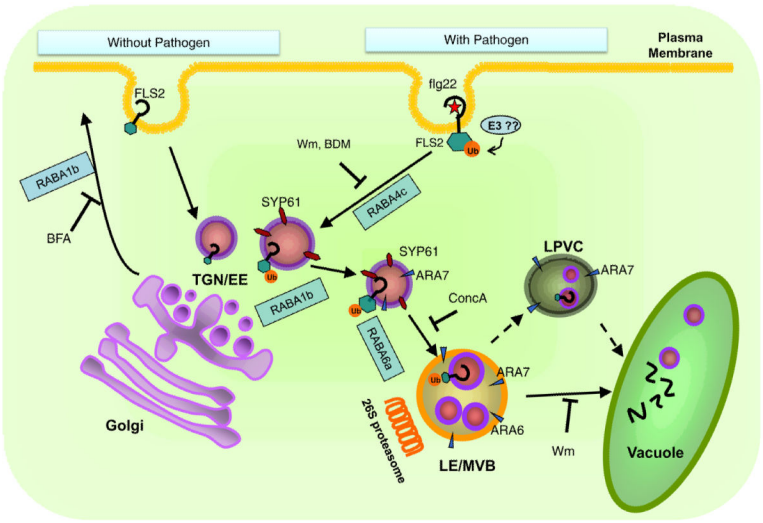

Figure 2. Endocytic pathways involved in FLS2 internalization

The non-activated FLS2 undergoes a constitutive recycling between plasma membrane (PM) and trans-Golgi network (TGN)/early endosome (EE) compartment via a Brefeldin A (BFA)-sensitive endosomal pathway. The flg22-activated FLS2 receptor traffics via a Wortmannin (Wm) - and Concanamycin A (ConcA)-sensitive pathway and is further sorted into vacuole for degradation. Monoubiquitination mediated by PEST motif of FLS2 might be involved in the FLS2 endocytosis initiation or protein sorting steps. The route of activated FLS2 endocytosis includes SYP61-labeled TGN/EE compartment, SYP61- and ARA7-labeled intermediate compartment with properties between TGN/EE and late endosome (LE)/multivesicular body (MVB), ARA6-labeled LE/MVB compartment and finally vacuole for degradation. MVB containing FLS2 may also traffic into late prevacuolar compartment (LPVC) before fusion with vacuole. RABA family protein RABA6a and RABA4c play roles in distinct steps of FLS2 endocytosis, and RABA1b is required for normal morphology of TGN/EE and transport of newly synthesized FLS2 to PM.