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REVIEW PAPER

Ubiquitylation in plants: signaling hub for the integration of environmental signals

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Abstract

A fundamental question in biology is how organisms integrate the plethora of environmental cues that they perceive to trigger a co-ordinated response. The regulation of protein stability, which is largely mediated by the ubiquitin–proteasome system in eukaryotes, plays a pivotal role in these processes. Due to their sessile lifestyle and the need to respond rapidly to a multitude of environmental factors, plants are thought to be especially dependent on proteolysis to regulate cellular processes. In this review, we present the complexity of the ubiquitin system in plants, and discuss the relevance of the proteolytic and non-proteolytic roles of this system in the regulation and co-ordination of plant responses to environmental signals. We also discuss the role of the ubiquitin system as a key regulator of plant signaling pathways. We focus more specifically on the functions of E3 ligases as regulators of the jasmonic acid (JA), salicylic acid (SA), and ethylene hormone signaling pathways that play important roles to mount a co-ordinated response to multiple environmental stresses. We also provide examples of new players in this field that appear to integrate different cues and signaling pathways.

Keywords: Abiotic stress, biotic stress, E3 ubiquitin ligase, environmental cues, ubiquitin code, ubiquitin system.

Introduction

In order to reproduce successfully, plants must be able to perceive, integrate, and respond to combinations of diverse signals from their environment. These signals may indicate mild variations in the plants environment (e.g. time of day), or may be indicative of more severe changes in their conditions such as extreme temperature changes, low oxygen availability, drought, or the presence of invading pathogens. In recent years, a substantial amount of research has made it apparent that the regulation of cellular proteostasis via the ubiquitin system in plants plays a key role in the integration of environmental cues and of downstream signaling pathways, perhaps as a result of their sessile lifestyle (Mazzucotelli *et al.*, 2006; Vierstra, 2009; Sadanandom *et al.*, 2012; Nagels Durand *et al.*, 2016; Serrano *et al.*, 2018). In this review, we discuss how the ubiquitin system and its different components function as an elaborate hub to integrate different signaling pathways that participate in the regulation of plant responses to environmental cues, with an emphasis on biotic stresses.

Overview of the ubiquitin system in plants

The ubiquitin system typically involves the covalent attachment of the 76 amino acid polypeptide ubiquitin to the ε-amino group of a lysine residue of a substrate protein. Ubiquitin is conserved across eukaryotic organisms (Zuin *et al.*, 2014), with the yeast and human ubiquitins differing by only

three amino acids (Ozkaynak *et al.*, 1984). In eukaryotic cells, ubiquitin is redundantly encoded by at least three different loci: as contiguous repeats of ubiquitin units (polyubiquitin) (Sharp and Li, 1987), or as a single ubiquitin moiety translationally fused to another protein such as ribosomal proteins (Finley *et al.*, 1989). In plants (Fig. 1), but not yeast or animals, ubiquitin genes are also expressed as translational head-to-tail fusions with the small ubiquitin-like protein RELATED TO UBIQUITIN (RUB) (Callis *et al.*, 1995). In the model plant *Arabidopsis thaliana* (accession Columbia), 12 genes that express functional ubiquitin have been identified. Five of these genes encode ubiquitin–ribosomal proteins, five encode polyubiquitins, and two encode ubiquitin–RUB fusion proteins (reviewed in Callis, 2014). In order for these fusion proteins to be attached to substrate proteins, ubiquitin must first be processed. This is achieved through the action of a family of hydrolase enzymes termed deubiquitinases (DUBs) (Fig. 1), of which there are ~50 in Arabidopsis (Isono and Nagel, 2014). Free ubiquitin can then be conjugated to a substrate protein via the successive activity of three groups of enzymes: an E1, or ubiquitin-activating enzyme (UBA); an E2 ubiquitin-conjugating enzyme (UBC); and an E3 ubiquitin ligase. E1 enzymes 'activate' ubiquitin by first catalyzing the adenylation of ubiquitin's C-terminus and then forming a thioester bond between the E1 active site cysteine and the ubiquitin C-terminal carboxylate group (Haas *et al.*, 1982). Activated ubiquitin can then

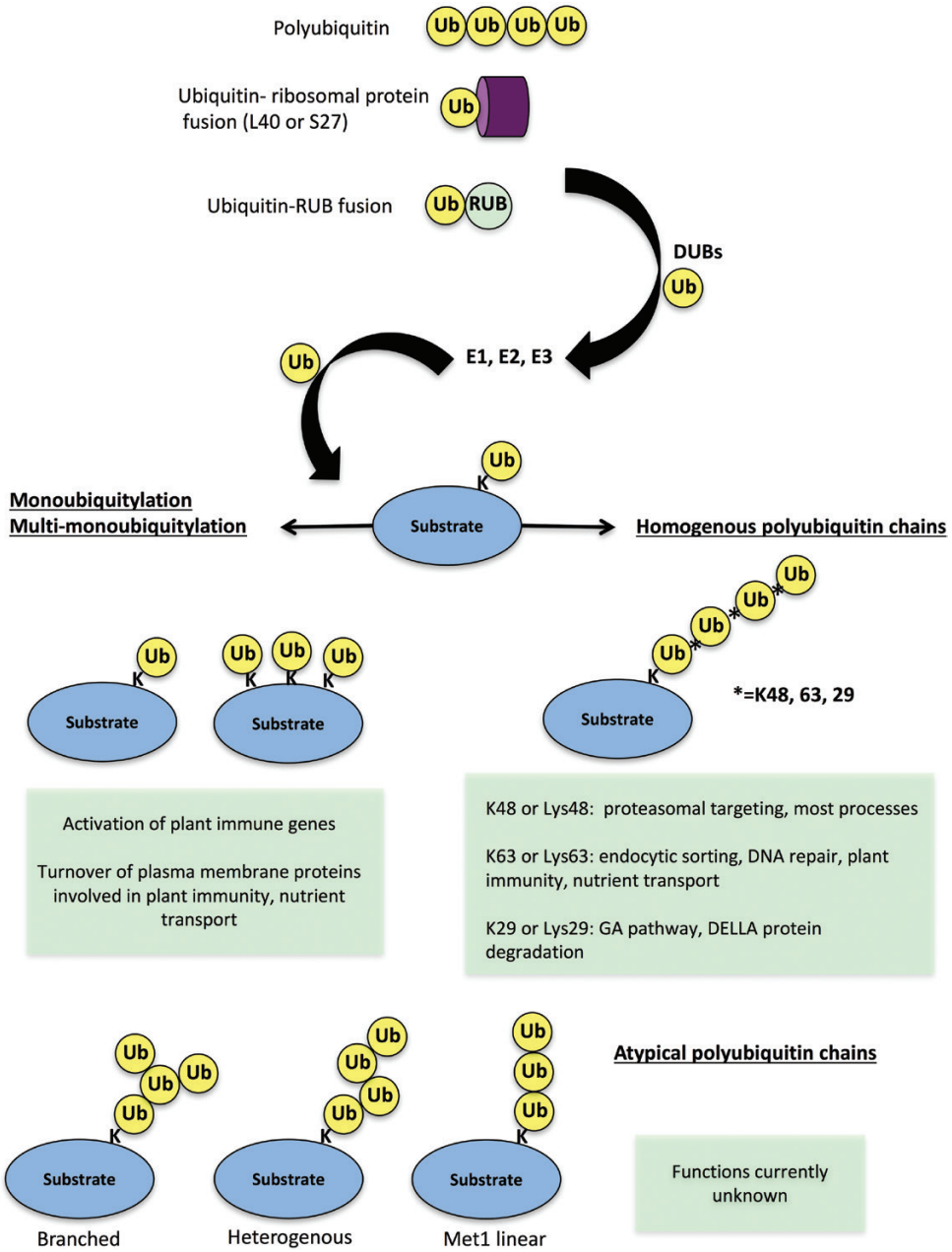

Fig. 1. The ubiquitin code in plants. Ubiquitin genes encoding protein fusions are processed by deubiquitinating enzymes (DUBs) to produce free ubiquitin (Ub). Ubiquitin can then be conjugated to substrates through the action of E1, E2, and E3 enzymes. The conjugated ubiquitin may be further ubiquitylated in a number of different ways, resulting in differential substrate fate. Different types of ubiquitin chains have been shown to play a role in varying environmental responses.

be transferred to a cysteine residue of an E2 enzyme (again through a thioester bond) before the ubiquitin is covalently attached to the substrate protein, typically through the activity of a substrate-specific E3 ligase together with an E2 enzyme. Many higher plants encode more than one enzyme to carry out each of these functions. In Arabidopsis two enzymes have been identified that carry out E1 function, UBA1 and UBA2 (Hatfield *et al.*, 1997), while 37 E2 enzymes are predicted to be encoded in the genome (Kraft *et al.*, 2005).

Strikingly, ~1500 genes coding for components of E3 ligases have been identified so far in Arabidopsis, in agreement with the idea that these enzymes provide the bulk of the substrate specificity for the system (Lee and Kim, 2011). The abundance of E3 ligase components encoded by the Arabidopsis genome is also indicative of the particular importance of the ubiquitin system in plants in comparison with other eukaryotes, such as humans or yeast, which encode ~600 and 100 E3 ubiquitin ligases, respectively (Li *et al.*, 2008; Finley *et al.*, 2012). The E3 ligases identified in plants can be divided into three groups, depending on the domains that mediate interaction with the E2 enzyme: (i) HECT (Homology to E6-AP C-Terminus) domain E3 ligases; (ii) RING (Really Interesting New Gene) domain ligases; or (iii) RING finger-like U-box domain ubiquitin ligases (reviewed in Chen and Hellmann, 2013). HECT domain-containing E3 ligases are relatively large (>100 kDa) proteins that form a thioester bond between a conserved cysteine residue located in the HECT domain and a ubiquitin moiety before transfer of the ubiquitin to the substrate (Scheffner *et al.*, 1995; Schwarz *et al.*, 1998). In Arabidopsis seven HECT-containing ubiquitin–protein ligases (UPLs) have been identified, UPL1–UPL7, which can be further divided into four subfamilies (Downes *et al.*, 2003; Marín, 2013). The RING finger domain is a conserved protein–protein interaction domain of 40–60 amino acids that can interact with an E2 UBC (Freemont *et al.*, 1991; Lorick *et al.*, 1999; Deshaies and Joazeiro, 2009). These E3 ligases can be monomeric, whereby the RING finger E3 ligases can interact with the substrate and the E2 without additional binding partners, or can be multimeric and act as part of an E3 ligase complex. Multimeric RING-finger E3 ligases include CULLIN-RING ligase (CRL) complexes (Hua and Vierstra, 2011), which are discussed in more detail below. The U-box domain is made up of a sequence of ~70 amino acid residues. It has a similar fold to RING finger domains but lacks conserved cysteine and histidine residues (Ohi *et al.*, 2003). E3 ligases containing RING-finger domain, and plant U-box (PUB) domains have been implicated in a vast amount of processes in plants, including stress responses (reviewed in Trujillo, 2018).

After the activity of the E1, E2, and E3 ligases, a fourth enzyme, known as an E4 ligase, may also be involved in the elongation of polyubiquitin chains (Koegl *et al.*, 1999). To date, one E4 ligase called MUTANT, SNC1-ENHANCING3 (MUSE3), homologous to the yeast E4 ligase UBIQUITIN FUSION DEGRADATION PROTEIN2 (UFD2), has been identified in Arabidopsis (Huang *et al.*, 2014). One identified function of this E4 ligase is to catalyze the elongation of polyubiquitin chains targeting plant nucleotide-binding leucine-rich repeat (NLR) immune receptors for degradation (Huang *et al.*, 2014).

Conjugation of a chain of four or more ubiquitins to a substrate protein, particularly using the Lys48 residue of ubiquitin, may direct the substrate to the 26S proteasome for proteolysis (Thrower *et al.*, 2000). This large (~2.5 MDa) ATP-dependent multisubunit protease complex contains various sites that facilitate the unfolding and release of free ubiquitin, and subsequent degradation of substrates into peptides (Yang *et al.*, 2004) (also reviewed in Bedford *et al.*, 2010). Ubiquitylation of a substrate is a dynamic process, and ubiquitin can be removed through the hydrolytic activity of a DUB enzyme, adding another layer of regulation to the system. DUBs have now been implicated in the regulation of a variety of cellular processes in plants (reviewed in Isono and Nagel, 2014; March and Farrona, 2017). Two DUBs that play a role in stress response in Arabidopsis are the ubiquitin proteases (UBPs) UBP12 and UBP13. Arabidopsis lines in which *UBP12* and *UBP13* are silenced by transgenic hairpin RNA interference (hpRNAi) are impaired in Lys48 deubiquitylation activity and exhibit increased resistance to the virulent bacterium *Pseudomonas syringae* pathovar tomato DC3000, suggesting that they act as negative regulators of the immune system (Ewan *et al.*, 2011). Recently these UBPs were also shown to play a role in jasmonic acid (JA) signaling, as Jeong *et al.* (2017) observed that UBP12 and UBP13 can remove ubiquitin from a polyubiquitylated species of the master regulator of JA signaling, MYC2, *in vitro* and that overexpression of *UBP12* and *UBP13* stabilized MYC2, probably through their deubiquitylation activity. A substantial amount of research has been carried out demonstrating the importance of the proteolytic function of the ubiquitin system but, in recent years, the significance of the non-proteolytic roles of ubiquitylation has also emerged as an important factor for the regulation of plant signaling pathways.

The ubiquitin code in plants

To function as a signaling hub, the ubiquitin system must be able to integrate and convey large amounts of specific information. One way this can be achieved is through the attachment of different types of ubiquitin chains onto a substrate (Fig. 1) and the use of the surface area of ubiquitin itself to elaborate on the substrate's fate (Komander and Rape, 2012). The most basic form of ubiquitin conjugation is monoubiquitylation, with multi-monoubiquitylation occurring if multiple lysine residues of a substrate protein are conjugated to a single ubiquitin. This modification can target substrates to the proteasome in eukaryotes (Braten *et al.*, 2016), but also acts as a non-proteolytic signal that has been implicated in (i) the activation of various eukaryotic substrates that mainly include transcription factors and histones (Kodadek *et al.*, 2006; Salghetti *et al.*, 2001); and (ii) the regulation of a variety of biological processes in plants (Feng and Shen, 2014), including the endocytic trafficking and vacuolar turnover of plasma membrane proteins (Barberon *et al.*, 2011; Kasai *et al.*, 2011; Martins *et al.*, 2015). In plants, the monoubiquitylation of histone H2B by the E3 ligases HISTONE MONOUBIQUITYLATION1 and 2 (HUB1 and HUB2) has been implicated in some aspects of plant immunity. In Arabidopsis, loss of function *hub1* mutants are more susceptible to necrotrophic fungal pathogens

(Dhawan *et al.*, 2009), and the tomato orthologs of Arabidopsis HUB1/2 have been shown to contribute to disease resistance against the necrotrophic fungus *Botrytis cinerea* (Zhang *et al.*, 2015). Another set of experiments conducted by Zou *et al.* (2014) found that monoubiquitylation of Arabidopsis H2B at the locus of the resistance (R) gene *SUPPRESSOR OF NPR1-1, CONSTITUTIVE1* (*SNC1*), encoding an NLR immune receptor involved in the salicylic acid (SA) defense response pathway, was enhanced upon infection by virulent *P. syringae* DC3000 along with activation of *SNC1*, suggesting a direct link between H2B monoubiquitylation and the activation of plant immune response genes.

After ubiquitin has been conjugated to a lysine residue of a substrate protein, the ubiquitin moiety itself can also be ubiquitylated on one of its seven lysine residues (Lys6/11/27/29/33/48/63) or on its N-terminal methionine (Met1), resulting in polyubiquitin chains. These chains can be homogenous, whereby ubiquitin is attached to the same position on each consecutive ubiquitin molecule, or can be mixed with ubiquitin being conjugated at different sites throughout the chain. Furthermore, a specific ubiquitin molecule in a chain may also be conjugated to two ubiquitins through two different lysine residues of the same ubiquitin molecule, resulting in branched chains (Komander and Rape, 2012). Proteomic analysis of the Arabidopsis ubiquitylome conducted by Kim *et al.* (2013) identified six of the seven homogenous lysine chains attached to substrates, with the chain types found in an order of abundance of Lys48>Lys63>Lys11 followed by lower levels of Lys33>Lys6>Lys29. The most abundant type of chain found to be attached in this study, Lys48, has been well characterized as a proteolytic signal for the conjugated substrate via the activity of the 26S proteasome and has been implicated in almost all aspects of plant signaling (reviewed in Sadanandom *et al.*, 2012; Walsh and Sadanandom, 2014), some of which are discussed in more detail below.

Ubiquitin chains linked through Lys63 residues are also abundant in plants, though substrates conjugated to Lys63 chains have not been as extensively characterized. Although this modification can target substrates to the proteasome (Saeki *et al.*, 2009), it appears to have many proteasome-independent roles in eukaryotes, including acting as a signal for lysosome- and vacuolar-dependent degradation pathways (Welchman *et al.*, 2005; Kirkin *et al.*, 2009). In plants, Lys63 chain conjugation has been reported to be involved in a number of processes (Tomanov *et al.*, 2014; Johnson and Vert, 2016; Romero-Barrios and Vert, 2018). These include endocytic sorting (Martins *et al.*, 2015), DNA repair (Wen *et al.*, 2008; Pan and Schmidt, 2014), and plant immunity (Mural *et al.*, 2013). This type of substrate modification also plays a role in plant nutritional deficiency responses such as iron starvation (Li and Schmidt, 2010). A recently conducted proteomic screen in Arabidopsis using sensor-based affinity purification of Lys63 chain-bound proteins identified a number of other nutrient transporters, including the phosphate transporter PHOSPHATE TRANSPORTER1 (PHT1) (Johnson and Vert, 2016). Previous studies have reported that one way in which PHT1 is regulated is through vacuolar degradation (Bayle *et al.*, 2011; Cardona-Lopez *et al.*, 2015). The correct endocytic processing of PHT1 is dependent on the activity of an adaptor protein APOPTOSIS-LINKED GENE 2-INTERACTING PROTEIN X (ALIX), that can interact with members of the endosomal sorting complexes required for transport III (ESCRT-III) and contains a domain that can bind Lys63 polyubiquitin chains *in vitro* (Cardona-Lopez *et al.*, 2015; Kalinowska *et al.*, 2015).

Another polyubiquitin chain type that has been implicated in plant signaling is through ubiquitin Lys29. Using a cell-free-based assay system, Wang *et al.* (2009) found Lys29 to be the major site of ubiquitin chain formation in the targeting of DELLA proteins for proteasomal degradation. DELLA proteins act as central repressors of the gibberellic acid (GA) pathway which plays a role in a number of plant environmental responses including light, temperature, and water responses (Gupta and Chakrabarty, 2013). Other atypical linkages such as Lys6, Lys11, and Lys33 have not been well characterized in plants, although these linkages have been found to play a role in other eukaryotes such as in mammalian mitophagy (Durcan *et al.*, 2014), regulation of cell cycle by the anaphase-promoting complex (APC/C) (Durcan *et al.*, 2014), and protein trafficking (Callis, 2014). To date, chains that are linked through Lys27 or Met1 have not been identified in plant extracts (Kim *et al.*, 2013; Callis, 2014).

In sum, rapid progress is currently being made to uncover the functions and protein substrates for ubiquitin chains involving lysine residues other than Lys48. Many of these new functions highlight the essential and diverse roles of the ubiquitin system in the regulation of plant signaling pathways.

CRLs: master regulators of plant hormone signaling pathways

In the past two decades, phytohormone signaling pathways, as well as their crosstalk, have been established as essential features of a co-ordinated response to combined environmental cues and stresses. The ubiquitin system, and in particular CRLs, act as central regulators of many phytohormone signaling pathways. The role of CRLs as signaling hubs largely depends on their ability to target for degradation transcriptional repressors or activators that act as master regulators of the gene regulatory networks downstream of phytohormone signaling.

CRLs are modular E3 ligases

In the Arabidopsis genome, >6% of genes are predicted to encode components of the ubiquitin–proteasome system (Vierstra, 2009), most of which are E3 ligase components. In Arabidopsis, as well as in other plants, CRLs are among the largest and most diverse families of E3 ligases. CRLs are multisubunit E3 ligases composed of a scaffold subunit (Cullin or CUL) that brings together the substrate recognition component of CRLs and the RING-domain subunit RBX, that interacts with the E2. For the purpose of this review, we will focus on CUL1-based ligases [CRL1; also known as SKP1/CUL1/F-box protein (SCF) ligases] and CUL3-based ligases (CRL3), because of their predominant and recurrent roles in the control of multiple hormone signaling pathways.

CRL1 ligases are composed of four different subunits. Three of them (RBX, CUL1, and SKP1 homologs) form the core of

the enzyme, while a fourth subunit (an F-box domain protein or FBP), interacts with substrate proteins and thus mediates the specificity of the complex (Deshaies, 1999). The Arabidopsis genome encodes almost 700 FBPs (Gagne *et al.*, 2002), implying a very large number of CRL1 complexes with different substrate specificities. CRL1 ligases are well known for their roles in regulating phytohormone signaling pathways, including those of auxin, GA, JA, and ethylene (Gray *et al.*, 1999; Xu *et al.*, 2002; Guo and Ecker, 2003; Potuschak *et al.*, 2003; Chini *et al.*, 2007; Fu *et al.*, 2004; Thines *et al.*, 2007; Qiao *et al.*, 2009). CRL3 ligases also rely on an RBX subunit to mediate the interaction with an E2 enzyme, but substrate recognition is typically mediated by a Bric-a-Brac, Tramtrack and Broad Complex/Pox virus and Zinc Finger (BTB/POZ) domain protein, of which there are ~80 encoded in the Arabidopsis genome (Gingerich *et al.*, 2005; Choi *et al.*, 2014). Other CRLs also play important roles in hormone signaling and will be briefly discussed below. These include CUL4-based E3 ligases (CRL4) which encompass a DAMAGED DNA BINDING PROTEIN1 (DDB1) adaptor protein and DWD-domain proteins (Choi *et al.*, 2014).

CRLs are key regulators of hormone signaling pathways

As mentioned above, CRLs are essential to control the activity of transcription factors that act as master regulators of different phytohormone signaling pathways. In this review, we will focus on the SA, JA, and ethylene signaling pathways (Fig. 2). We refer the reader to the following excellent reviews for details of the role of the ubiquitin system in the regulation of other hormone signaling pathways (Sadanandom *et al.*, 2012; Shabek and Zheng, 2014; Stone, 2014; Larrieu and Vernoux, 2015; Lavy and Estelle, 2016; Nagels Durand *et al.*, 2016; Yang *et al.*, 2017).

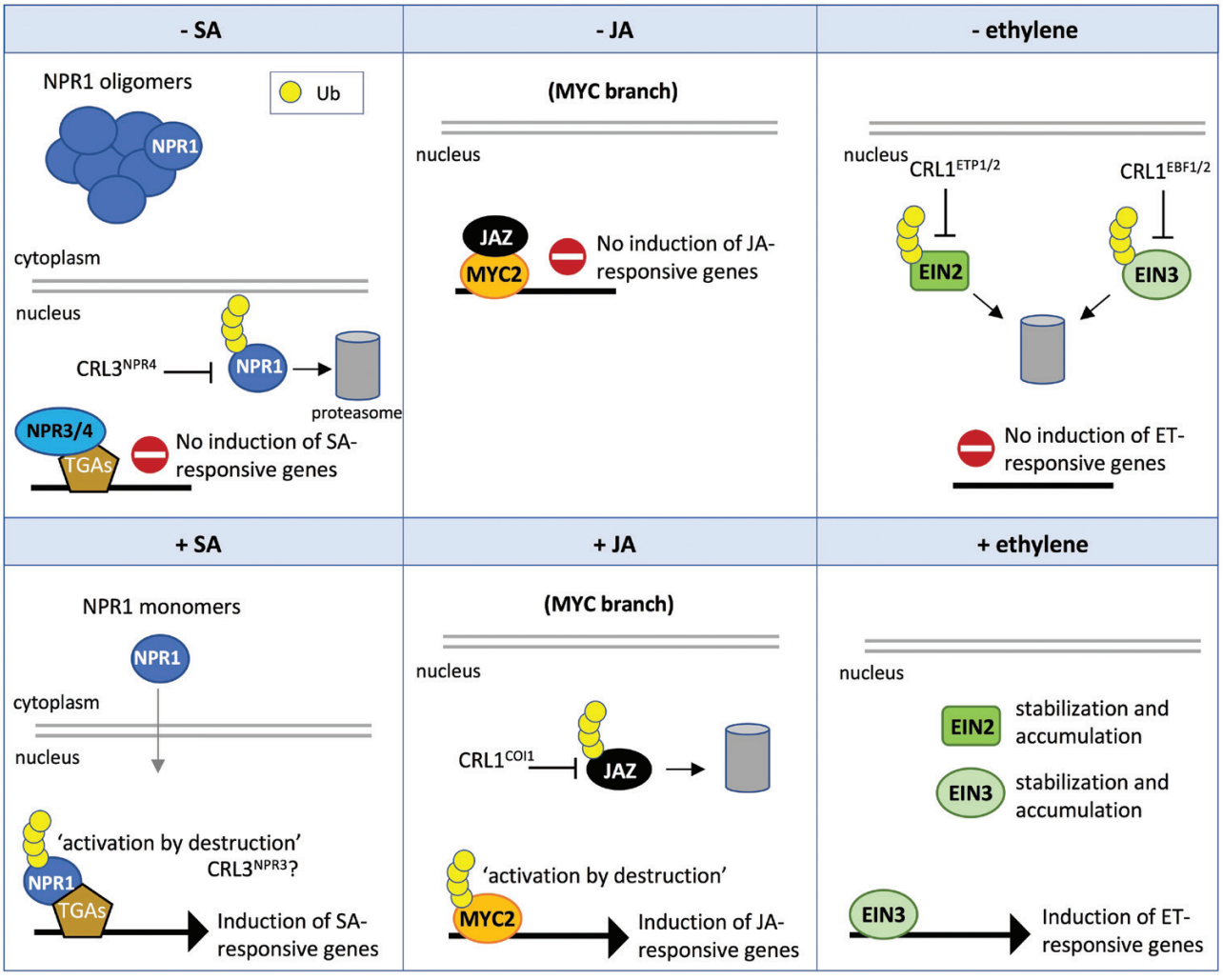

Fig. 2. Brief overview of the SA, JA, and ethylene signaling pathways. SA triggers the dissociation of NPR1 oligomers in the cytoplasm and the translocation of NPR1 to the nucleus, where it associates with other transcription factors (e.g. TGA transcription factors) and acts as a transcriptional co-activator. NPR1 levels in the nucleus are controlled by $CRL3^{NPR3}$ and $CRL3^{NPR4}$, depending on SA levels. NPR3/4 were also recently shown to act as transcriptional co-repressors of SA-responsive genes in the absence of SA. The negative effect of NPR3/4 depends on their interaction with TGA transcription factors. In the absence of JA, MYC2 interacts with the JAZ repressors, which recruit other proteins, resulting in repression of JA response genes. Binding of JA-Ile by $CRL1^{COI1}$ promotes the interaction between COI1 and JAZ, and results in the degradation of the JAZ repressors and the activation of MYC2. In the absence of ethylene (abbreviated ET), EIN2 and EIN3 are targeted for degradation by CRL1 ligases. In contrast, in the presence of ethylene, EIN2 and EIN3 are stabilized, thus facilitating the activation of ethylene response genes. Ubiquitin is depicted in yellow.

SA signaling

SA plays key roles in plant immunity and is typically associated with plant defense against biotrophic pathogens (that feed on living tissue). SA signaling is important for the regulation of programmed cell death and for the onset of systemic acquired resistance, which leads to a longer broad-spectrum immunity (reviewed in Furniss and Spoel, 2015; Withers and Dong, 2016; Dempsey and Klessig, 2017). The BTB domain protein NONEXPRESSOR OF PR GENES1 (NPR1) acts as a transcriptional co-activator of SA response genes in conjunction with other transcriptional regulators, such as TGA transcription factors (Zhang *et al.*, 1999; Després *et al.*, 2000; Zhou *et al.*, 2000; Boyle *et al.*, 2009) (Fig. 2). While NPR1 has the domains required to interact with CUL3, it does not appear to interact directly with this cullin (Dieterle *et al.*, 2005; Spoel *et al.*, 2009; Fu *et al.*, 2012). Instead, NPR1 stability is tightly regulated by CRL3s that comprise the BTB domain proteins NPR3 and NPR4, both of which are paralogs of NPR1 (Fu *et al.*, 2012). Importantly, the untimely activation of the SA signaling pathway in the absence of SA is prevented through the degradation of NPR1 by the $CRL3^{NPR4}$ ligase, as well as by oligomerization of NPR1 in the cytosol, which prevents its translocation into the nucleus (Fu *et al.*, 2012). A recent study by Ding *et al.* (2018) also shows that, in the absence of SA, NPR3 and NPR4 act as transcriptional co-repressors of SA response genes. This negative effect of NPR3/4 depends on their interaction with TGA transcription factors, presumably on the promoter of target genes.

NPR1, NPR3, and NPR4 have been shown to bind SA, suggesting that these BTB domain proteins may also act as SA receptors (Maier *et al.*, 2011; Fu *et al.*, 2012; Wu *et al.*, 2012; Ding *et al.*, 2018), although this is still a matter of debate (Kuai *et al.*, 2015; Dempsey and Klessig, 2017). In the case of NPR1, biochemical evidence suggests that SA binding might trigger conformational changes of NPR1 that release its transactivation domain from the autoinhibitory effect of its BTB domain (Wu *et al.*, 2012). It has also been proposed that binding of SA to NPR3 and NPR4 might regulate their interaction with their substrate NPR1 (Fu *et al.*, 2012). For example, SA promotes interaction of NPR1 with NPR3, resulting in the $CRL3^{NPR3}$-mediated degradation of NPR1 (Fu *et al.*, 2012). Interestingly, NPR1 turnover is important for the activation of SA response genes, indicating that NPR1 is probably regulated through an 'activation by destruction' mechanism, whereby following the initiation of transcription, a transcriptional activator becomes inactive (also termed 'spent') and is targeted for degradation to allow an active, or 'fresh', transcription factor to bind the promoter elements of target genes to trigger additional rounds of transcription (Lipford *et al.*, 2005; Spoel *et al.*, 2009; Fu *et al.*, 2012; Geng *et al.*, 2012). In addition to the regulation of NPR1, binding of SA to NPR3 and NPR4 appears to inhibit the NPR3/4-mediated repression of SA response genes (Ding *et al.*, 2018). However, it remains unclear if the formation of an active $CRL3^{NPR3/4}$ complex is required (Ding *et al.*, 2018).

JA signaling

JA is involved in the regulation of plant defenses against pathogens (typically necrotrophic pathogens, which feed on dead tissue) and herbivorous insects, as well as to different abiotic stresses (Wasternack and Hause, 2013; Kazan, 2015; Goossens *et al.*, 2016). Upon JA perception, the JAZ transcriptional repressors are recognized (bound) by the FBP CORONATINE INSENSITIVE1 (COI1) as part of the E3 ligase $CRL1^{COI1}$ (Fig. 2). Subsequent ubiquitylation and proteasomal degradation of the JAZ repressors result in the activation of JA response genes (Chini *et al.*, 2007; Thines *et al.*, 2007). Importantly, the COI1 subunit acts both as the substrate adaptor for the JAZ repressor proteins and as the JA-Ile receptor, so that interaction with the JAZ proteins is strongly enhanced following binding of JA-Ile to COI1 (Katsir *et al.*, 2008; Melotto *et al.*, 2008; Fonseca *et al.*, 2009; Sheard *et al.*, 2010). However, interaction of certain JAZ proteins, such as JAZ12, with the E3 ligase KEEP ON GOING (KEG), can instead protect JAZ12 from its $CRL1^{COI1}$-mediated ubiquitylation (Pauwels *et al.*, 2015), thus highlighting how JAZ protein interaction with E3 ligases other than $CRL1^{COI1}$ can in fact lead to their stabilization. More recently, An *et al.* (2017) have shown that, at basal JA levels, COI1 also interacts with MED25, a subunit of the Mediator complex, on the promoter of MYC2 target genes. It has been proposed that binding of JA-Ile to COI1 not only promotes interaction of COI1 with the JAZ repressors (resulting in their ubiquitylation and degradation), but also triggers enhanced interaction of MED25 with MYC2 (Chen *et al.*, 2012), resulting in the recruitment of polymerase II and the activation of MYC2 target genes (An *et al.*, 2017). Furthermore, in agreement with the essential roles of COI1 both as a receptor and as a regulator of JA signaling, the levels of this FBP are tightly regulated *in planta*. COI1 interaction with ASK1 and the functionally redundant ASK2 promotes the stabilization of the COI1 protein (Yan *et al.*, 2013; Zhou *et al.*, 2013). There is hence a balance between the levels of COI1 and its incorporation into $CRL1^{COI1}$ complexes (Yan *et al.*, 2013). Interestingly though, degradation of COI1 appeared to be independent of the $CRL1^{COI1}$ ligase, suggesting that it might be targeted for degradation by another, still unknown, E3 ligase (Yan *et al.*, 2013). In addition to $CRL1^{COI1}$, other E3 ligases participate in the regulation of JA signaling. These include KEG (Pauwels *et al.*, 2015), as well as RING DOMAIN LIGASE3 (RGLG3) and RGLG4, which modulate JA signaling in response to wounding and *P. syringae* DC3000 infection (Zhang *et al.*, 2012).

JA responses are typically divided into two branches, depending on the identity of the transcription factors involved in the regulation of the respective JA-responsive genes. The MYC branch of JA signaling (Fig. 2) relies on the activity of the functionally redundant basic helix–loop–helix (bHLH) transcription factors MYC2, MYC3, MYC4 (Fernández-Calvo *et al.*, 2011), as well as MYC5 during stamen development (Qi *et al.*, 2015), in conjunction with other transcription factors (Chini *et al.*, 2016). This branch is typically associated with response to wounding and defenses against herbivorous insects (see Goossens *et al.*, 2017 for a comprehensive review). In the absence of JA, the activity of the MYC2/3/4 transcription

factors is repressed through their interaction with the JAZ repressor proteins (Goossens *et al.*, 2015; F. Zhang *et al.*, 2015). Interestingly, the degradation of MYC2 seems to be important to maintain the activation of JA-responsive genes, possibly through an 'activation by destruction' mechanism akin to that for NPR1 in SA signaling (Zhai *et al.*, 2013). However, the E3 ligase responsible for the degradation of MYC2 as part of this 'activation by destruction' mechanism remains to be identified. The U-box E3 ligase PUB10 has also been shown to play a role in MYC2 degradation, although it is likely that other E3 ligases also target MYC2 (Jung *et al.*, 2015). Interestingly, as mentioned earlier, the effects of PUB10, and potentially of other E3 ligases, may be counteracted by the activity of the deubiquitylating enzymes UBP12 and UBP13 (Jeong *et al.*, 2017).

In contrast, the ETHYLENE RESPONSE FACTOR (ERF) branch of JA signaling (Fig. 3), which is typically involved in plant defense against necrotrophic pathogens, is largely dependent on the activity of the ERF transcription factors ERF1 and OCTADECANOID-RESPONSIVE ARABIDOPSIS APETALA2/ETHYLENE RESPONSE FACTOR DOMAIN PROTEIN59 (ORA59) (Berrocal-Lobo *et al.*, 2002; Lorenzo *et al.*, 2003; Pré *et al.*, 2008). Importantly, the regulation of JA-responsive genes of the ERF branch also depends on ethylene signaling (Lorenzo *et al.*, 2003; Pré *et al.*, 2008), so that this branch integrates signals from both the JA and ethylene pathways. As discussed below, the complex regulation of MYC2, ERF1, and ORA59 stability is key to the crosstalk between these two hormonal pathways.

Ethylene signaling

The gaseous hormone ethylene is often viewed as a modulator of different hormone signaling pathways (Broekgaarden *et al.*, 2015) and has been shown to regulate plant responses to both biotic (Broekgaarden *et al.*, 2015) and abiotic stresses [e.g. flooding (Sasidharan and Voesenek, 2015) and high salinity (Peng *et al.*, 2014); see also (Lin *et al.*, 2009)]). Ethylene is also well known for its functions in the regulation of physiological and developmental processes, including germination, senescence, and fruit maturation (see Dubois *et al.*, 2018 for a comprehensive review). Two important regulators of ethylene signaling are under the control of CRL1s (Fig. 2). The protein ETHYLENE INSENSITIVE2 (EIN2) plays a central role in this process (reviewed in Ju and Chang, 2015) and is tightly regulated through different mechanisms involving phosphorylation (Qiao *et al.*, 2009, 2012; Ju *et al.*, 2012), proteolytic cleavage (Cooper, 2013; Qiao *et al.*, 2012, 2013) and ubiquitin-dependent protein degradation. Specifically, EIN2 interacts through its C-terminal domain with the FBPs EIN2 TARGETING PROTEIN1 (ETP1) and ETP2 (Qiao *et al.*, 2009). These two FBPs and associated CRL1 complexes are required to maintain low levels of EIN2 in the absence of ethylene (Qiao *et al.*, 2009), thus preventing the unnecessary activation of the ethylene response. In contrast, ethylene production results in increased levels of EIN2, which triggers the activation of ethylene response genes (Qiao *et al.*, 2009) through the activity of the transcription factor EIN3 and related EIN3-LIKE (EIL) proteins (Chao *et al.*, 1997; Solano *et al.*, 1998; Chang *et al.*, 2013). In the absence of ethylene, EIN3 and EIL1 are targeted for degradation by CRL1s comprising the FBPs EIN3 BINDING F-BOX PROTEIN1 (EBF1) or EBF2, thus limiting induction of the ethylene response pathway in the absence of signal (Guo and Ecker, 2003; Potuschak *et al.*, 2003; Binder *et al.*, 2007). Importantly, EIN3/EIL1 are stabilized in the presence of ethylene and this correlates with the proteasomal degradation of EBF1/2 in an EIN2-dependent manner (An *et al.*, 2010). In addition, in the presence of ethylene, EIN2 prevents translation of *EBF1/2* mRNA, thus further contributing to a rapid accumulation of EIN3 (Li *et al.*, 2015), which is essential for a timely response to ethylene. Importantly, EIN3 also up-regulates the transcription of *EBF2*, suggesting that $CRL1^{EBF2}$ ligases may play a role in preventing a prolonged activation of ethylene response (Potuschak *et al.*, 2003; Konishi and Yanagisawa, 2008). In sum, several FBPs and CRL1s are key to controlling the outputs of ethylene signaling. The complex regulation of EBF1/2 activity highlights the importance of their role in controlling EIN3 levels.

Another interesting feature of the ethylene signaling pathway is the co-operation between CRL1- and CRL3-type ubiquitin ligases. Indeed, CRL3s comprising the BTB domain proteins ETHYLENE OVERPRODUCER1 (ETO1), ETO1-LIKE1 (EOL1), and EOL2 appear to mediate the degradation of enzymes involved in ethylene biosynthesis (Wang *et al.*, 2004; Yoshida *et al.*, 2005; Christians *et al.*, 2009).

Hormonal crosstalk and the integration of environmental signals by the ubiquitin system

In this section of the review, we will consider the role that E3 ligases play in regulating the crosstalk between different hormone pathways, with a focus on the crosstalk between JA and other signaling pathways.

JA and ethylene crosstalk

JA and ethylene can act synergistically to regulate genes of the ERF branch (see 'JA signaling' above). The integration of the JA and ethylene pathways is thought to be mediated via (i) the JA-dependent stabilization of ERF transcription factors such as ORA59 (He *et al.*, 2017), which are important for the regulation of ERF branch JA response genes; (ii) the stabilization of EIN3/EIL1 by ethylene; and (iii) the $CRL1^{COI1}$-dependent degradation of the JAZ transcriptional repressors which have been shown to interact with and repress EIN3/EIL1 (Zhu *et al.*, 2011) (Fig. 3). Hence, the degradation of the JAZ proteins triggered by JA-Ile combined with the stabilization of ERF and EIN3/EIL1 provide a suitable framework to explain the synergistic interaction between the JA and ethylene signaling pathways, which is relevant in the context of plant defenses against necrotrophic pathogens (Berrocal-Lobo *et al.*, 2002; reviewed in Pieterse *et al.*, 2012). It is also noteworthy that the expression of ERF1 is under the control of EIN3 (Solano *et al.*, 1998). In addition, the stability of ERF1 is tightly regulated by the E2 conjugating enzyme UBC18 (Cheng *et al.*, 2017). The latter appears to target ERF1 for degradation in the

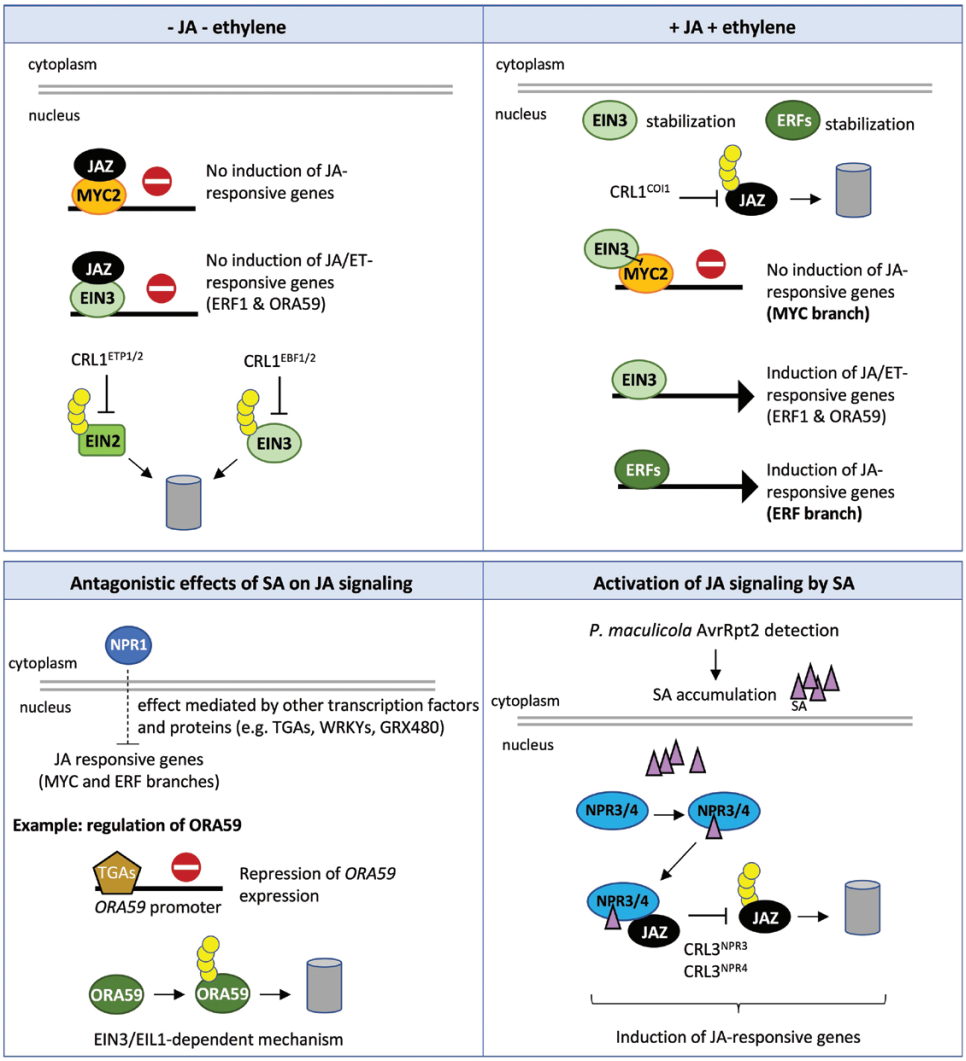

Fig. 3. Schematic representation of the crosstalk between the JA, ethylene, and SA signaling pathways. In the absence of ethylene and JA, the JAZ proteins interact with EIN3 and MYC2, thus preventing the activation of JA (from both the MYC and the ERF branch) and ethylene response genes. In the presence of JA and ethylene, EIN3 is stabilized and the degradation of the JAZ repressors results in the expression of *ERF1* and *ORA59* in an EIN3-dependent manner. These two ERF transcription factors (abbreviated ERFs) then up-regulate JA response genes of the ERF branch. EIN3 also interacts with MYC2 and represses its activity, thus antagonizing the MYC branch. SA has an antagonistic effect on JA signaling, which is mediated through different mechanisms involving cytosolic NPR1, as well as other proteins such as TGA and WRKY transcription factors or the glutaredoxin GRX480 (Ndamukong *et al.*, 2007). In particular, *ORA59* expression is repressed by TGA transcription factors, and the ORA59 protein is also targeted for degradation through a mechanism that requires EIN3/EIL1. However, SA can also trigger the activation of JA-responsive genes in the context of *P. maculicola* AvrRpt2-triggered ETI, through the NPR3- and NPR4-dependent degradation of the JAZ repressors in the presence of SA. Ubiquitin is depicted in yellow.

dark (Cheng *et al.*, 2017), which plays an important role in the regulation of hypocotyl elongation in an ethylene-dependent manner (Zhong *et al.*, 2012).

The JA and ethylene pathways can also act antagonistically through the mutual repression of MYC2/3/4 and EIN3/EIL1 (Song *et al.*, 2014; Zhang *et al.*, 2014) (Fig. 3). Indeed, the MYC2 and EIN3 proteins have been shown to interact and repress each other (Song *et al.*, 2014; Zhang *et al.*, 2014). In addition, MYC2 can directly induce the expression of EBF1, which can target EIN3 for degradation (Zhang *et al.*, 2014). Hence protein–protein interactions and the regulation of $CRL1^{EBF1}$ activity by MYC2 both contribute to the antagonistic effects of JA and ethylene, and also provide some explanation for the antagonistic effects of the ERF and MYC branches of JA signaling in mediating responses to necrotrophic pathogens and herbivorous insects or wounding, respectively.

JA and SA crosstalk

SA and JA are classically considered as mutually antagonistic (reviewed in detail in Pieterse *et al.*, 2012). For example, the expression of two JA-responsive genes, *PDF1.2* and *VSP2*, which are targets of the ERF and MYC branches, respectively, is repressed in the presence of SA. Furthermore, the negative regulation of JA signaling by SA requires the activity of cytosolic NPR1 (Spoel *et al.*, 2003; Leon-Reyes *et al.*, 2009).

Additional studies have shown that the repression of *PDF1.2* upon SA treatment correlated with a reduced accumulation of the ORA59 protein (a positive regulator of the ERF branch), possibly through its ubiquitin-mediated degradation (Van der Does *et al.*, 2013). Interestingly, the degradation of ORA59 upon SA treatment appears to require the EIN3/EIL1 transcriptional regulators, which are essential for the regulation of ethylene-response genes and the ERF branch (He *et al.*, 2017).

However, the SA and JA pathways do not always antagonize each other (Schenk *et al.*, 2000; van Wees *et al.*, 2000; Mur *et al.*, 2006). Liu *et al.* (2016) have recently shown that in the presence of a strain of *P. syringae* pathovar *maculicola* that codes for the effector protease AvrRpt2, the onset of effector-triggered immunity (ETI) involved the induction of JA biosynthesis in an SA-dependent manner. The SA-mediated induction of JA response genes depends on the activity of NPR3 and NPR4, but does not require the activity of the $CRL1^{COI1}$ E3 ligase (Liu *et al.*, 2016). In fact, in this particular context (i.e. RPS2-mediated ETI in response to AvrRpt2 activity), binding of SA to NPR3 and NPR4 promotes the interaction of these two BTB-domain proteins with several JAZ proteins. In the case of JAZ1 specifically, NPR3 and NPR4 can target this repressor of JA response genes for degradation. Hence, it appears that JAZ protein degradation can be mediated by both $CRL1^{COI1}$ and $CRL3^{NPR3/4}$ depending on the physiological context. This example also illustrates how two types of CRLs that are typically associated with antagonistic pathways may converge in function to degrade JAZ proteins.

Regulation of JA signaling by far-red light

Light quality (e.g. shade) plays an important role in how plants allocate resources between growth and JA-mediated defenses against pathogen or herbivores (Moreno *et al.*, 2009; Cerrudo *et al.*, 2012). The regulation of MYC2 stability is key to this light-dependent resource allocation process. Indeed, MYC2, MYC3, and MYC4 are stabilized in the presence of JA, through a mechanism that requires the activity of $CRL1^{COI1}$ (Chico *et al.*, 2014), thus promoting herbivore defenses. In contrast, far-red (FR) light triggers the proteasome-dependent degradation of MYC2/3/4 through a mechanism that necessitates, directly or indirectly, the activity of CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) as part of a CRL4 E3 ligase complex (Zhang *et al.*, 2008). Concomitantly with the degradation of MYC2 in FR light, the JAZ repressors are also stabilized, thus further enhancing the repression of JA signaling. This destabilization of MYC2 and stabilization of JAZ proteins in FR light correlate with an increased susceptibility to necrotrophic pathogens such as the fungus *B. cinerea* (Cerrudo *et al.*, 2012; Chico *et al.*, 2014). Hence, light quality is an important regulator of JA signaling, and the crosstalk between CRL4- and CRL1-mediated protein degradation in the regulation of JA signaling is important in co-ordinating resource allocation between growth and defense. However, while the notion that there is a continuous balance to be found between allocating resources for defense or for growth prevails, there is also mounting evidence that this growth versus defense trade-off is not simply due to a competition for resources, but may in fact be an emerging property of the gene regulatory networks orchestrated by JA signaling (Campos *et al.*, 2016). This interesting paradigm shift stems from the observation that plants mutant for five of the JAZ repressors and for the *phytochrome B* (*phyB*) photoreceptor (mutant denoted *jazQ phyB*) retain the increased insect resistance of the *jazQ* quintuple mutant with few negative effects on growth (Campos *et al.*, 2016).

New players in the field

Over the past few decades, research focused on the expanding field of the plant ubiquitin system revealed novel E3 ligases and pathways that are central to the regulation and co-ordination of plant responses to environmental cues. In this section of the review, we focus on two new players, which are likely to play a more prominent role in the coming years. These two examples were chosen because the substrates and molecular mechanisms have been dissected in some detail, and also because they regulate signaling pathways that are activated by different environmental signals.

KEG: regulation of hormone signaling and plant defenses against pathogens

In recent years, KEG has been shown to play important roles in abscisic acid (ABA) signaling, which is essential in the regulation of plant responses to a wide range of abiotic stresses (e.g. drought and osmotic stress) and biotic interactions (reviewed in Stone, 2014; Lievens *et al.*, 2017; Yang *et al.*, 2017; Jurkiewicz and Batoko, 2018). In addition, KEG contributes to the crosstalk between ABA and JA, as well as the regulation of plant immunity (Fig. 4).

KEG is a RING E3 ligase that has a unique domain architecture, comprising a kinase domain, as well as ankyrin and 'HECT and RCC1-like' (HERC2-like) repeats (Stone *et al.*, 2006). The HERC2-like repeats regulate KEG's subcellular localization to the *trans*-Golgi network/early endosome vesicles, as well as its dimerization (Gu and Innes, 2011). KEG was initially identified as an E3 ligase that targets for degradation the transcription factor ABA INSENSITIVE5 (ABI5), a positive regulator of ABA response genes that plays important roles in ABA-mediated growth arrest following germination (Lopez-Molina *et al.*, 2001). More specifically, KEG targets ABI5 for degradation in the absence of ABA (Fig. 4), presumably to prevent the untimely activity of ABI5 and allow seedling development (Stone *et al.*, 2006; Liu and Stone, 2010, 2013). In response to ABA treatment, KEG's abundance decreases through a mechanism that appears to involve its auto-ubiquitylation (Liu and Stone, 2010) (Fig. 4), thus contributing to ABI5 accumulation and the activation of ABA response genes. Interestingly, in the presence of ABA, ABI5 may be targeted for degradation by CRL4-type E3 ligases, such as those encompassing the DWD proteins ABA-HYPERSENSITIVE DCAF1 (ABD1) and DWD-HYPERSENSITIVE TO ABA1 (DWA1), DWA2, and DWA3. Indeed, mutant plants for the latter accumulate higher levels of ABI5 in the presence of ABA (Lee *et al.*, 2010, 2011; Seo *et al.*, 2014)

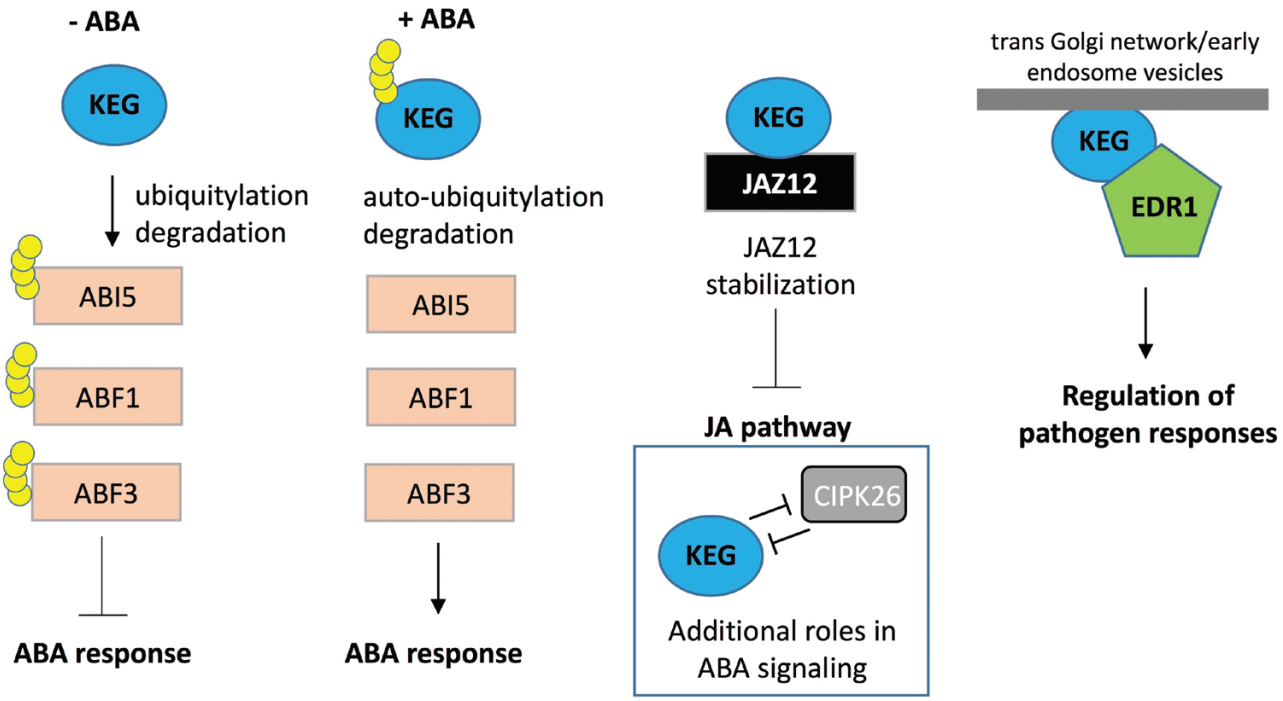

Fig. 4. Overview of KEG's roles in the regulation of hormone signaling and of plant defenses. In the absence of ABA, KEG targets ABI5, ABF1, and ABF3 for degradation, thus inhibiting ABA response. In the presence of ABA, KEG is degraded, possibly as a result of its auto-ubiquitylation. *In vitro*, KEG is also phosphorylated by CIPK26, which could enhance its auto-ubiquitylation activity (inset). In addition, KEG interacts with JAZ12, thus preventing its degradation by $CRL1^{COI1}$ and contributing to the repression of JA response genes. Finally, KEG retains EDR1 at the *trans*-Golgi network/early endosome vesicles (represented in dark gray), which is important for the regulation of plant defenses against pathogens. Ubiquitin is depicted in yellow.

In addition to ABI5, *in vitro* experiments indicate that the ABRE BINDING FACTOR1 (ABF1) and ABF3 transcription factors, which are related to ABI5 and play a role in ABA signaling, could also be targets of KEG, although their degradation is thought also to be mediated by other, still unknown, E3 ligase(s) (Chen *et al.*, 2013). In normal growth conditions, KEG also targets for degradation the CALCINEURIN B-LIKE INTERACTING PROTEIN KINASE26 (CIPK26), which can phosphorylate ABI5 *in vitro* (Lyzenga *et al.*, 2013). Interestingly, CIPK26 can also phosphorylate KEG *in vitro*, which could promote its auto-ubiquitylation and degradation (Lyzenga *et al.*, 2017). Lyzenga *et al.* (2017) therefore propose a model in which KEG and CIPK26 reciprocally regulate each other and positions both CIPK26 and KEG as important regulators of ABA signaling. Furthermore, KEG interacts in the cytoplasm with JAZ12. Surprisingly, though, based on both molecular and biochemical data, KEG does not appear to target JAZ12 for degradation, but instead protects it from $CRL1^{COI1}$-mediated ubiquitylation (Pauwels *et al.*, 2015) (Fig. 4). This study therefore highlights the role of KEG in the crosstalk between the JA and ABA signaling pathways.

In addition to its roles in the regulation of hormone signaling, an allele of KEG (*keg-4*) that harbors a mutation in its HERC2-like repeats was identified in a genetic suppressor screen of the enhanced pathogen resistance of plants mutant for the Ser/Thr protein kinase ENHANCED DISEASE RESISTANCE1 (EDR1) (Frye and Innes, 1998; Wawrzynska *et al.*, 2008; Hiruma and Takano, 2011). KEG was then shown to interact with EDR1 (Wawrzynska *et al.*, 2008; Gu and Innes, 2011). More specifically, interaction of EDR1 with KEG facilitates the localization of EDR1 to the *trans*-Golgi network/early endosome vesicles through a mechanism that requires the HERC2-like repeats of KEG (Fig. 4). Additional experiments established that KEG plays a more general role in protein trafficking (Gu and Innes, 2012). Interestingly, this function probably depends both on its HERC2-like repeats and the presence of the RING domain, although the role of protein ubiquitylation or KEG's substrates in this process has not yet been clearly established.

The N-end rule pathway: sensor and integrator of environmental cues

In recent years, the ubiquitin-dependent N-end rule pathway has emerged as an important signaling hub that senses and modulates plant responses to several environmental cues and stresses of both abiotic and biotic origins (Fig. 5). The N-end rule pathway targets proteins for degradation based on the identity of a protein's N-terminal residue, or some of its modifications (e.g. N-terminal acetylation or cysteine oxidation). This protein degradation pathway is present in all eukaryotes examined so far, and is widely studied in mammals and in yeast (for comprehensive reviews on the topic, see Varshavsky, 2011; Tasaki *et al.*, 2012; Lee *et al.*, 2016), but its relevance in plants has only been uncovered in more recent years (reviewed in Gibbs *et al.*, 2016; Dissmeyer *et al.*, 2017, 2018). Besides its roles in plant development and in the regulation of physiological processes (Graciet *et al.*, 2009; Holman *et al.*, 2009; Dong *et al.*, 2017; Zhang *et al.*, 2018), several recent studies position the N-end rule as a key pathway for the regulation of diverse signaling pathways.

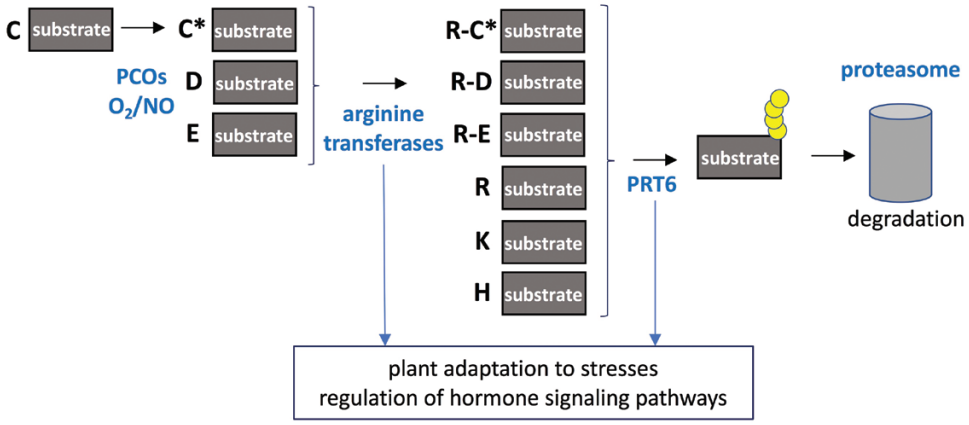

Fig. 5. Simplified overview of the N-end rule pathway and its functions in the integration of signaling pathways. The N-end rule pathway of protein degradation targets proteins for degradation based on the identity of their N-terminal residues (indicated here using single-letter abbreviations for amino acids). Recognition of N-end rule substrates by E3 ligases such as PRT6 may require different enzymatic modifications of the N-terminal residue, such as, for example, cysteine oxidation by PCO enzymes, or conjugation of arginine by so-called arginine transferases. Different components of the N-end rule pathway have been shown to play important roles in plant adaptation to different abiotic and biotic stresses and also in the regulation of the JA and ABA hormone signaling pathways. Note that this figure does not provide a complete overview of the N-end rule pathway, but focuses on components whose functions have been dissected in more detail in plants. Ubiquitin is depicted in yellow. O_2, oxygen; NO, nitric oxide; C*, oxidized cysteine.

The first roles of the N-end rule pathway in the regulation of plant responses to environmental cues were shown to be linked to both oxygen (O_2) and nitric oxide (NO) sensing and signaling (Gibbs *et al.*, 2011, 2014, 2015; Licausi *et al.*, 2011; Abbas *et al.*, 2015). N-end rule function in this process depends on the degradation of a set of transcription factors that belong to the group VII of ERFs (noted ERF-VII), including RELATED TO APETALA2.2 (RAP2.2), RAP2.3, RAP2.12, HYPOXIA RESPONSIVE ERF1 (HRE1), and HRE2. These ERF-VII transcription factors start with the sequence Met-Cys, but the initial methionine is cleaved by methionine aminopeptidases, thus exposing the second cysteine residue at the N-terminus. Under normal oxygen conditions, this N-terminal cysteine is oxidized into cysteine sulfinic acid by the action of PLANT CYSTEINE OXIDASES (PCOs) (Weits *et al.*, 2014; White *et al.*, 2017), which is sufficient to target these transcription factors for degradation through the N-end rule pathway (Fig. 5). Although not all steps have been biochemically dissected in detail, genetic analysis, as well as the monitoring of protein stability in different mutant backgrounds for the N-end rule pathway indicate that the N-terminal oxidized cysteine results in the conjugation of arginine by arginine transferases to the N-terminus of these ERF-VII transcription factors. This is followed by their recognition by the RING-domain E3 ligase PROTEOLYSIS6 (PRT6), which targets them for proteasomal degradation (Gibbs *et al.*, 2011; Licausi *et al.*, 2011). In contrast, under low oxygen conditions, which may arise during germination in the soil (e.g. Abbas *et al.*, 2015), as well as upon waterlogging or flooding (reviewed in Voesenek and Bailey-Serres, 2015; Loreti *et al.*, 2016), the N-terminal cysteine residue of the ERF-VII transcription factors remains unoxidized, leading to stabilization of these transcription factors and the regulation of their target genes (Gibbs *et al.*, 2011; Licausi *et al.*, 2011; Gasch *et al.*, 2016). In addition, the recent analysis of *prt6* mutant Arabidopsis and barley plants in response to a range of abiotic stresses, including high salinity, drought, and heat, shows that the N-end rule pathway plays a broader role in co-ordinating plant responses to a wide range of environmental cues and stresses (Vicente *et al.*, 2017). However, the molecular mechanisms have not yet been elucidated in detail.

Recent studies have also highlighted the role of the N-end rule pathway in the regulation of plant responses to biotic stresses. Gravot *et al.* (2016) observed a link between the activity of the above-mentioned ERF-VII transcription factors, their N-end rule-dependent degradation, and infection by the protist *Plasmodiophora brassicae*. Indeed, hypoxia-responsive genes, many of which are thought to be under the control of the ERF-VII transcription factors (Gasch *et al.*, 2016), are induced at early stages of infection by *P. brassicae*. Interestingly, Arabidopsis plants mutant for arginine transferases or *PRT6* are known to accumulate the ERF-VIIs and are more susceptible to this pathogen. Altogether, these observations point to a role of the ERF-VIIs and their N-end rule degradation in the regulation of root responses to *P. brassicae*.

Furthermore, a pathogen susceptibility screen on different mutants of the N-end rule pathway revealed its role as a positive regulator of plant defenses against pathogens (de Marchi *et al.*, 2016). Indeed, several enzymatic components of this pathway, including the two RING-domain E3 ligases PRT6 and PRT1, and the above-mentioned arginine transferases were shown to be important for the regulation of plant defenses against fungal and bacterial pathogens with different lifestyles (de Marchi *et al.*, 2016). Furthermore, it was observed that the regulation (i.e. activation and repression) of genes involved in plant defense against a strain of *P. syringae* pathovar tomato DC3000 that expresses the effector protein AvrRpm1 is dampened in plants mutant for the arginine transferases or for *PRT6*. These results suggest that the increased susceptibility to pathogens could be due to a reduced amplitude of the defense response in these mutants (de Marchi *et al.*, 2016). Interestingly, de Marchi *et al.* (2016) also established that phytohormone signaling

pathways such as that of JA could be regulated by the N-end rule pathway, thus adding to the known roles of this pathway in the regulation of ABA signaling (Holman *et al.*, 2009; Vicente *et al.*, 2017; Zhang *et al.*, 2018).

Conclusion

Our understanding of the roles played by the ubiquitin system as a signaling hub has greatly progressed in the last two decades. Much of the knowledge that has been gained has led to a detailed understanding of how the ubiquitin system, and in particular E3 ligases, regulate signaling pathways that are involved in the response to a specific stress or signal. This knowledge now serves as a foundation, as the community shifts from studying plant responses to individual stresses, to understanding how plants sense and integrate combined environmental signals and stresses. Both proteolytic and non-proteolytic roles of the ubiquitin system are already emerging as essential in this context. Areas that are likely to develop quickly in the coming years include the study of ubiquitin chains involving lysine residues other than Lys48, as well as the identification of protein substrates that may be targeted for degradation by E3 ligases. The latter has so far proven difficult because (i) of the weak and dynamic nature of the interactions between an E3 ligase and its cognate substrates; and (ii) these substrates are targeted for degradation and hence might not accumulate to detectable levels *in vivo*. In addition, exposure of a degradation signal, which is essential for substrate recognition by an E3 ligase, also typically involves one or several post-translational modification(s) of the substrate (e.g. phosphorylation, protease cleavage, etc.). These modifications and the recognition of the substrate by an E3 ligase might only occur upon a particular signal, within a short time frame after signal detection, or in a specific cell type. Hence, identifying E3 ligase substrates might require a detailed knowledge of where and when E3 ligase activity is required. The potential need to focus on specific cell types and hence the limited amount of tissue or cells that can be used for substrate identification using proteomics approaches might also hamper the detection of E3 ligase substrates. Nevertheless, recent advances in proteomics and in the strategies used to tag and affinity purify ubiquitylated proteins with different ubiquitin chains will continue to facilitate this task greatly in the future (Harper and Tan, 2012; O'Connor *et al.*, 2015; Tan *et al.*, 2013; Zhuang *et al.*, 2013; Iconomou and Saunders, 2016). Finally, the study of the ubiquitin system and its role as a signaling hub needs to be viewed in the context of crop improvement and the need to maintain crop yields in a sustainable manner, and has a high potential for the development of applications of agricultural interest.

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