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REVIEW

Regulation of NLR stability in plant immunity

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Abstract Plant nucleotide binding domain and leucine-rich repeat (NLR) receptors recognize pathogen effectors directly or indirectly and mediate innate immune responses. NLR-mediated immunity also has direct impacts on plant growth and development, as well as yield and survival. The levels of NLR proteins are therefore intricately controlled in plants to balance defense responses and other processes. In recent years, the ubiquitination-26S proteasome system and the HSP90 chaperones have emerged as having key functions in the regulation of NLR stability. The N-end rule pathway of protein degradation is also directly linked to NLR stability. Recent progress in the regulation of NLR stability and turnover is summarized here, focusing on the key components and pathways.

Keywords E3 ubiquitin ligase, degradation, nucleotide-binding leucine-rich repeat receptor, plant immunity, proteasome, protein stability, ubiquitination

1 Introduction

Plants have evolved an innate immune system for pathogen recognition and subsequent defense activation[1]. This immune system mainly relies on two classes of immune receptors, i.e., the pattern recognition receptors (PRRs) and the intracellular nucleotide binding domain and leucine-rich repeat receptors (NLRs). The PRRs recognize pathogen-associated molecular patterns (PAMPs) and initiate PAMP-triggered immunity (PTI)[2], while the NLRs intercept, directly or indirectly, strain-specific pathogen effectors (also known as avirulent effectors) and mediate effector-triggered immunity (ETI)[3,4]. Both PTI and ETI can converge in downstream signaling pathways and culminate in similar cellular responses like reactive oxygen species burst, Ca^{2+} spikes, MAP kinase activation and cell death. However, the differences observed in the activation kinetics and robustness can lead to qualitatively different defense responses[5–7].

Plant and animal intracellular NLRs superfamily proteins share a similar modular domain architecture, including the core NBD and C-terminal LRR domain[1,4,8]. Plant NLRs can be classified into two major types, one with an N-terminal Toll/interleukin-1 receptor domain and the other with a coiled-coil domain. NLRs detect directly or indirectly isolate-specific pathogen effectors and trigger ETI that can amplify PTI basal transcriptional programs and defense responses, often resulting in localized cell death, namely hypersensitive response[9]. Over-accumulation and autoactivation of NLRs can also cause cell death that is detrimental to plant growth and development[10–12]. It is therefore critical for plants to tightly control the expression, stability and activity of NLRs in the absence of pathogens[13–15].

Ubiquitination is a key post-translational modification that covalently attaches ubiquitin (Ub) moiety to the lysine residues of a substrate protein in an ATP-dependent manner[16–18]. Ubiquitination is a three-step reaction cascade that involves the sequential action of three enzymes, the Ub-activating enzyme (E1), the Ub-conjugating enzyme (E2), and the Ub ligase (E3). The E3 ligases mainly determine substrate specificity and can be classified into four major subfamilies, HECT (homologous to E6-associated protein carboxyl terminus), RING (really interesting new gene), U-Box and CRL (cullin-RING ligase)[18]. Polyubiquitination, in some cases, relies on E4 ligase that facilitates polyubiquitin chain assembly in collaboration with E1, E2 and E3[19]. Two major types of polyubiquitin chains are well characterized, with Lys48- and Lys63-linked polyubiquitination in proteasomal degradation and vacuole endocytosis[20,21]. Polyubiquitination mediated substrate degradation has a central role in controlling protein stability and turnover in numerous biological processes, including plant hormone signaling and immunity[18].

The N-end rule pathway of protein degradation, as a set

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of ancient proteolytic mechanism present in both prokaryotes and eukaryotes[22–25], relates the half-life of a protein to the nature of its amino(N)-terminus (Nt)[26]. In eukaryotes, the N-end rule pathway engages the ubiquitin-proteasome system to target protein substrate for destruction by the 26S proteasome. Protein substrates for this pathway contains an N-degron (as a degradation signal), which consists of a destabilizing Nt-residue, a downstream Lys as a ubiquitination site, and a structural conformation that exposes the N-terminus of the protein[25]. The N-degron of a substrate is recognized by a N-recognin (a single-subunit E3 ligase) and results in polyubiquitination and proteasomal degradation of the substrate[24,25].

In this review, we summarize the recent findings about the regulation of NLR stability and homeostasis in plants, focusing on the involvement of Ub-26S proteasome system, the N-end rule pathway and the HSP90 chaperone machinery.

2 NLR stability regulated by ubiquitination

2.1 Distinct E3 ligases regulate NLR stability

In *Arabidopsis*, the stability of two NLRs, SNC1 and RPS2, was found to be controlled by the F-box protein CPR1 (constitutive expressor of pathogenesis-related genes 1). CPR1 is a subunit of the SCF(SKP1-CULLIN1-F-box) E3 ligase complex[11,27]. In *cpr1* mutants, the accumulation of SNC1 and RPS2 is increased to the levels that can induce autoimmune responses, while overexpression of *CPR1* results in decreased levels of SNC1 and thus suppressed autoimmunity triggered by the autoactive *snc1* mutant[11,28]. CPR1 interacts with the SCF complex subunits (ASK1 and ASK2), as well as SNC1 and RPS2, implying that both SNC1 and RPS2 are substrates of SCF^{CPR1} complex-mediated degradation (Fig. 1(b)).

Many plant NLRs form heteropairs with another atypical NLR for pathogen recognition and defense activation[29]. However, SNC1 forms heteropair with three typical NLRs, SIKIC1, SIKIC2 and SIKIC3 that are redundantly required for *snc1*-mediated autoimmunity[30]. Further studies demonstrated that the protein levels of these SIKCs are controlled by two paralog RING-type E3 ligases, MUSE1 (mutant, *snc1*-enhancing 1) and MUSE2 that have overlapping functions in negative regulation of defense responses. These findings indicate that distinct E3 ligases target different NLR components of the same immunocomplex, which may provide tight and dynamic regulation of the homeostasis of the NLR complexes.

In rice, NLR protein Piz-t recognizes rice blast fungal effector AvrPiz-t and triggers ETI to *Magnaporthe oryzae*[31,32]. Two rice RING-type E3 ligases, APIP6 and APIP10, were shown to interact with AvrPiz-t and to be involved in regulating PTI against *M. oryzae*[33,34]. Further, Park and colleagues also demonstrated that APIP10 promoted the degradation of Piz-t when these two proteins were coexpressed[33]. Conversely, silencing of *APIP10* in the *Piz-t* background led to increased accumulation of Piz-t, enhanced cell death and disease resistance to *M. oryzae*. This study indicates that the RING-type E3 ligase APIP10 may serve to control the abundance of Piz-t to avoid autoimmunity (Fig. 1(c)), while targeting of APIP10 by AvrPiz-t may induce the accumulation of Piz-t and the activation of ETI to *M. oryzae*.

In barley, the *Mla* locus encodes about 30 allelic NLRs, each recognizing distinct barley powdery mildew isolate and mediating ETI to *Blumeria graminis* f.sp. *hordei*[35]. Wang and co-authors reported that a RING-type E3 ligase, MIR1 (MLA-interacting RING-type ligase 1), directly interacts with several functional MLAs and negatively affects the stability of these MLAs[36]. MIR1 can directly promote the ubiquitination and proteasomal degradation of MLAs (Fig. 1(c)). Silencing of *MIR1* led to higher MLA1 protein levels in barley. This study suggests that RING-type E3 ligase MIR1 is involved in the degradation of functional MLAs, thus the attenuation of MLA-mediated defense signaling in barley[36].

2.2 E4 ligase and ATPase regulate NLR stability

Genetic screening for enhancers of *snc1* autoimmunity isolated a series of *muse* (mutant, *snc1*-enhancing) mutants[37]. The identification of *muse3* indicates that the MUSE3 E4 ubiquitin ligase is involved in the polyubiquitination and degradation of NLR targets[38]. Knockout of *MUSE3* resulted in increased levels of SNC1 and RPS2, while overexpression of *MUSE3* with *CPR1* enhanced the polyubiquitination and degradation of the NLRs. It is believed that the MUSE3 E4 ubiquitin ligase may act downstream of the E3 ligase SCF^{CPR1}[38].

More recently, MUSE8/*At*CDC48A, a conserved AAA-ATPase among eukaryotes, was found to be involved in NLR turnover in *Arabidopsis*[39]. In *Atcdc48A-4* mutant the protein level of SNC1 is increased, and *At*CDC48A can interact with the MUSE3 E4 ligase. AtCDC48A may process the polyubiquitinated NLR substrate for degradation by the 26S proteasome (Fig. 1(b)). Notably, studies in other organisms showed that CDC48 can form a functional proteasome complex with the 20S core particle and function in post-ubiquitination and protein degradation[40,41].

2.3 TRAF proteins as adaptors regulate NLR stability

In mammals, tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) are cytosolic adaptor proteins that act as signaling transducers for the TNFR superfamily and the Toll-like receptor superfamily[42]. Some mammal TRAFs have been shown to be important in adaptive and

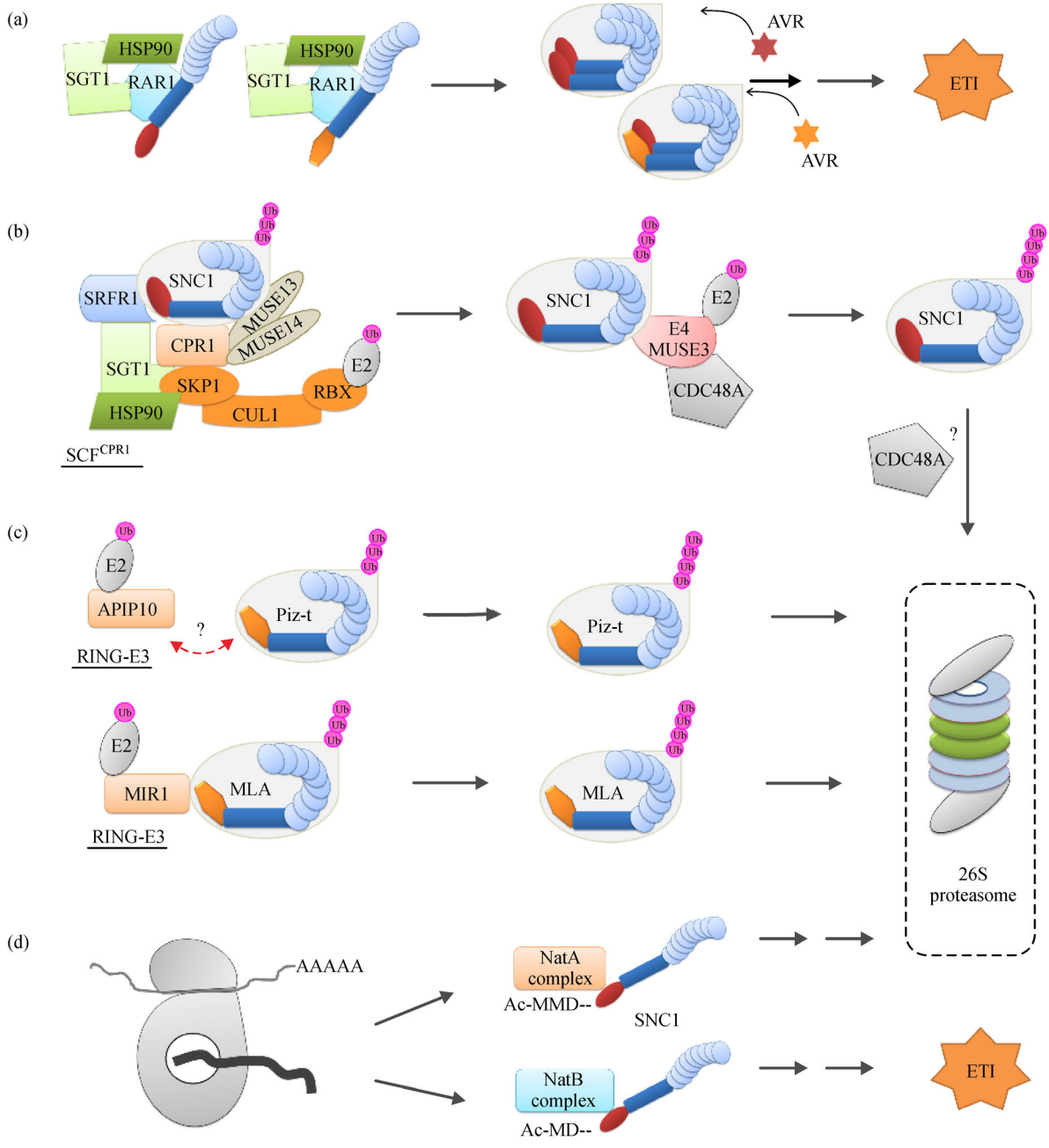

Fig. 1 Major components/pathways in the stability regulation of nucleotide binding domain and leucine-rich repeat receptors (NLRs) in plants. (a) HSP90-SGT1-RAR1 chaperones regulate the folding, maturation and/or activation of NLRs. The NLRs can form homocomplex or heterocomplex to detect strain-specific AVR effectors and to trigger ETI; (b) a hypothetical pathway depicting the regulation of *Arabidopsis* SNC1 by the E3 Ub-ligase complex SCF^{CPR1}. RPS2 and RPS4 can similarly be regulated (not shown). SGT1, HSP90, and SRPR1 are associated with the SCF complex. MUSE13/14 are two TRAF-domain-containing proteins. The MUSE3 E4 ligase and CDC48A may act downstream of the SCF complex; (c) the RING-type E3 ligases, APIP10 and MIR1, control the stability of rice Piz-t and barley MLA, respectively; (d) Nt-acetylation antagonistically regulates the homeostasis of SNC1. At least two N-terminal isoforms of SNC1 exist in *Arabidopsis*. The Met-Met-Asp-SNC1 isoform is acetylated (Ac-MMD) by the NatA acetylase complex, which serves as a degron for the destabilization of the protein. The Met-Asp-SNC1 isoform is acetylated (Ac-MD) by the NatB complex, which stabilizes the proteins that may subsequently trigger ETI. Polyubiquitinated NLRs are degraded through the 26S proteasome.

innate immune signaling[43]. In *Arabidopsis*, there may be more than 70 genes encoding for TRAF-domain-containing proteins, but the function of the majority of them is still unknown[44]. Huang et al.[45] identified two *muse* mutants,

muse13 and *muse14*, with the enhanced *snc1* autoimmune phenotype. *MUSE13* and *MUSE14* encode two TRAF-domain-containing proteins that are functionally redundant. Loss of both *MUSE13/14* resulted in increased accumulation of SNC1 and RPS2, while overexpression of *MUSE13* decreased levels and activities of the NLRs. MUSE13 was also shown to associate with the E3 ligase SCF^{CPR1} and the NLRs. These pieces of evidence indicate that MUSE13 and MUSE14 may function as adaptors to facilitate the targeting of the SCF^{CPR1} complex to SNC1 and RPS2 for ubiquitination and degradation[45] (Fig. 1(b)).

Notably, a recent study indicates that the degradation of MUSE13 and MUSE14 is also controlled by the Ub-26S proteasome system. Huang et al.[46] identified an F-box protein SNIPER4 via a reverse genetic approach, and showed that SNIPER4 regulates the turnover of MUSE13 and MUSE14. Overexpression of the wildtype SNIPER4 could decrease the levels of MUSE13 and MUSE14, whereas the dominant-negative SNIPER4 had an opposite effect. These data indicate that SNIPER4 functions to fine-tune the stability of NLR such as SNC1, leading to an optimal immune output[46].

3 NLR stability regulated by the N-end rule pathways

One branch of the N-end rule pathway is the Ac/N-end rule pathway that targets proteins with N-terminal acetylated residues for proteolysis[47–49]. Nt-acetyltransferases (Nat) catalyze the acetylation of Nt-residue of a substrate by transferring the acetyl groups from acetyl-CoA to the Nt α-amino group of the substrate[50,51]. In plants, loss or reduction of the Nt-acetylase activities in mutant of *NatA*, *NatB*, or *NatC* causes a range of pleiotropic defects[25], indicating a key role of the Nt-acetylation in plant growth and behavior.

The Nt-acetylation has recently been shown to be directly linked to NLR stability and plant immunity[52]. In *Arabidopsis*, SNC1 was found to be Nt-acetylated and likely regulated by the Ac/N-end rule pathway. In *natA* mutants, SNC1 accumulats to abnormal higher levels, which indicates that NatA complex may acetylate SNC1 at the N terminus and destabilize the protein. Importantly, the researchers found that SNC1 can undertake alternative translation initiation and create two distinct Nt-variants, namely Met-Met-Asp-SNC1 and Met-Asp-SNC1. Remarkably, the Met-Met-Asp-SNC1 form was shown to be specifically acetylated by NatA, which triggers the degradation of the protein, while the Met-Asp-SNC1 form was acetylated by NatB which unexpectedly stabilizes the protein[52] (Fig. 1(d)). Therefore, SNC1 acetylation by NatA creates an Ac/N-degron that will be recognized by an unidentified E3 ligase(s) only when the first Met is present. The Nt-acetylation of the NLR protein with antagonistic effects may provide an intricate regulation of the immune receptor to maintain NLR homeostasis in plants.

4 NLR stability regulated by HSP90 and co-chaperones

Heat shock protein 90 (HSP90) is a highly conserved protein that serves as molecular chaperone for diverse client proteins[53]. HSP90 interacts with specific proteins, so called co-chaperones, to form complexes that regulate the folding, maturation, stabilization and activation of client proteins, playing positive roles in NLR stability and/or activity[54]. So far, HSP90 and co-chaperones have been shown in many cases to contribute to NLR protein mediated immunity in plants (Fig. 1(a)). For example, HSP90 and its co-chaperone RAR1 are required for the folding and stabilization of a number of NLR proteins[15], including MLA1/6, Rx, RPM1, RPS5, Mi-1 and I-2[55–60]. In many cases, SGT1, as a co-chaperone interacting with RAR1 and HSP90, is required for the maintenance of steady-state levels of NLRs, including Rx and N[15,59,61,62].

Significantly, HSP90 and SGT1 also regulate the turnover of NLRs and have negative roles in NLR stability. It has been shown that, in *sgt1b* mutant plants, NLRs such as RPS5 and SNC1 accumulated to abnormally high levels, indicating that SGT1 contributes to the turnover of certain NLRs in plants[58,63]. SGT1 functions together with SRFR1 (a suppressor of RPS4-RLD1) to negatively regulate the accumulation of some NLRs[63]. SGT1 was also found to directly interact with SKP1 (S-phase kinase-associated protein 1), a highly conserved protein that is a core component of the SCF E3 ubiquitin ligase[64,65]. SGT1 was suggested to act as an adaptor linking HSP90 and SCF[66]. Moreover, in *Arabidopsis*, specific mutations in *HSP90.2* and *HSP90.3* led to heightened accumulation of NLRs such as SNC1, RPS2 and RPS4, indicating HSP90 is also involved in the turnover of the NLRs[67]. These lines of evidence indicate that the HSP90 chaperone machinery is also tightly associated with ubiquitin-dependent degradation pathway (Fig. 1(b)).

Other types of co-chaperones are also involved in the modulation of NLR stability. For example, the protein phosphatase 5 was reported to act as a co-chaperone that interacts with HSP90 and is required for the folding and functioning of the tomato NLR protein I-2[68]. Similarly, *Arabidopsis* phosphatase IBR5 can form complexes with HSP90-SGT1b and an atypical NLR protein CHS3 to stabilize the NLR[69–71].

5 NLR stability regulated by sumoylation, scaffolding and other mechanism

SIZ1 is a small ubiquitin-like modifier (SUMO) E3 ligase that regulates SA-dependent immune responses and many

other processes in *Arabidopsis*[72]. The *siz1* loss-of-function mutant has an autoimmune phenotype that is dependent on SNC1, whereas the overexpression of SIZ1 attenuates the protein accumulation of SNC1 in *Arabidopsis*[73]. There are four predicted sumoylation sites and five putative SUMO-interaction motifs in SNC1, and consistently, SNC1 is sumoylated *in planta*[73]. These lines of evidence imply that SIZ1 regulates SNC1 turnover and plant immunity through sumoylation.

SRFR1 (a suppressor of RPS4-RLD1) is a tetratricopeptide repeat protein that also negatively regulates the stability of SNC1, RPS2 and RPS4 in *Arabidopsis*[63,74]. In *srfr1* mutants, the levels of these NLR proteins is increased, accompanied by constitutively activated immune responses. Importantly, SRFR1 can interact with SGT1, SNC1 and RPS4. It is believed that SRFR1 and SGT1 may work together to act as a scaffolding protein or chaperone in the SCF^{CPR1} complex to regulate the turnover of some NLRs[13] (Fig. 1(b)).

More recently, MUSE7, a putative kinase substrate that is evolutionarily conserved, has also been found to negatively impact NLR accumulation[75]. MUSE7 decreased the accumulation of the tested *Arabidopsis* NLRs, SNC1, RPS2 and RPM1. However, no interaction was detected between MUSE7 and CPR1 or HSP90.3 and SNC1, indicating that MUSE7 may not affect NLR stability through ubiquitination and degradation pathway.

As mentioned above, *M. oryzae* effector AvrPiz-t can interact with several rice proteins potentially as its virulence targets. AvrPiz-t interacts with the bZIP-type transcription factor APIP5, to suppress the transcriptional activity and the accumulation of APIP5 and to induce necrosis at the necrotrophic stage[76]. Notably, Piz-t can also interact with APIP5 to stabilize it and to prevent necrosis. Conversely, APIP5 can positively affect the accumulation of the rice NLR Piz-t through an as yet unknown mechanism.

6 Conclusions and perspectives

Here, we have summarized recent advances on plant components and cellular pathways controlling the stability and turnover of NLR receptors (Fig. 1). The Ub-26S proteasome system remains to be the key mechanism to degrade NLRs, with which other regulatory components are associated or related, such as adaptor, scaffolding protein and chaperone, as well as SUMO E3 ligase. In addition, the HSP90 chaperone complex and the Ac/N-end rule pathway can have either positive and negative effects on NLR stability. The maintenance of intracellular NLR homeostasis is so critical for plant survival and growth that it requires diverse components and different pathways to collaborate. Other components and molecular mechanisms remain to be identified in future studies on NLR stability regulation.

E3 Ub-ligases are the most diverse components in the Ub-26S proteasome system, for example, there are more than 1500 different E3s in *Arabidopsis* and rice[13,18,77]. The presence of these vast numbers of E3 ligases raise the questions that if there are other E3 ligases regulating the stability of other NLRs, what are these E3s and what are their targets. Moreover, as there is only one E4 ligase identified so far in *Arabidopsis*[19,38], the identification of new factors (e.g., E3s) that associate with this E4 ligase and NLRs may expand our understanding of NLR stability regulation in plants. With respect to the association between *At*CDC48 and MUSE3 E4 ligase, it would be interesting to know whether *At*CDC48-mediated degradation of SNC1 is indeed dependent on the 26S proteasome pathway and requires MUSE3 (Fig. 1(b)). Since many regulatory components and a series of processes are involved in the regulation of NLR stability, it is important to understand how plants coordinate the functions of these regulatory components to ensure enhanced yet balanced immunity upon detection of pathogens and activation of NLRs.

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