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REVIEW ARTICLE

Ubiquitination: a tool for plant adaptation to changing environments

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Abstract
Post-translational modifications namely ubiquitination, phosphorylation, methylation and acetylation play distinct roles in regulating the growth and development of plants. Among these, the ubiquitination regulates the abundance, activities, subcellular compartmentalization and trafficking of regulatory proteins involved in diverse developmental as well as stress-responsive processes. The ubiquitin–proteasome system (UPS) involves five essential components namely ubiquitin, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin ligase (E3) and the intact 26S proteasome. The E3 ubiquitin ligase is the major component of UPS that recognizes and tethers poly-ubiquitins on the target proteins. Owing to its specificity of substrate recognition, the E3 ubiquitin ligase contributes not only to the proteome plasticity of the cell but also regulates the plant's response to environmental cues. In this context, the review summarizes the components involved in UPS and elaborates the role of E3 ubiquitin ligase in biotic and abiotic stress responses.

Keywords Ubiquitination · E3 ligase · Plants · Biotic stress · Abiotic stress · Adaptation

Introduction

Being sessile, plants are continually challenged by several biotic and abiotic stresses, and the severity of these stresses are projected to increase with the change in global climatic conditions. To counteract, plants have also evolved several sophisticated physiological as well as molecular mechanisms that act in a coordinated fashion conferring tolerance characteristic to the plant [48, 50, 72]. Amidst these, the morphological and physiological barriers have been extensively studied [4, 7, 48, 50, 72]; however, the molecular aspects of defense response majorly remain elusive [8, 18, 19, 24, 38, 72]. Ubiquitination is one such process which was initially perceived as a process of selective protein degradation; however, recent studies have highlighted the role of protein ubiquitination in dormancy and germination, tissue and organelle development, self-incompatibility, and biotic and abiotic stress response [1, 54, 64, 66, 71].

Post-translational modifications namely ubiquitination, phosphorylation, methylation and acetylation play distinct roles in regulating the growth and development of all the eukaryotic species. These processes are also reported to interact with each other to form complex cross-talk networks [68]. Among these, the ubiquitination regulates the abundance, activities, subcellular compartmentalization and trafficking of regulatory proteins involved in diverse developmental as well as stress-responsive processes [1, 54, 64, 66, 71]. The central component of UPS is the ubiquitin molecule (Ub) which is transferred to target protein via ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). Further, the intact 26S proteasome is involved in the identification and degradation of polyubiquitinylated target protein [64]. Ubiquitin is the central component of UPS and is the highly conserved protein consisting of ~ 76 amino acids [1, 64]. The E1 activates the inactive ubiquitin, and the activated ubiquitin is transferred onto E2. The E3 mediates the further transfer of the activated ubiquitin onto the target protein. This transfer could either be direct [homology to the E6-associated protein C-terminus (HECT)-type E3s] or indirect [really interesting new gene

This article is dedicated to the memory of Profs AK Sharma and Archana Sharma.

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(RING), U-box and Cullin-based types E3s], which leads to the degradation of polyubiquitinated-target protein by the 26S proteasome [42, 64].

In plants, the gene families encoding the core components of UPS have been extensively studied. *Arabidopsis thaliana* contains two E1 genes, thirty-seven E2 genes and more than 1300 E3-related genes [22, 29, 65, 76]. The relatively higher number of E3 genes is justified by their roles in maintaining substrate specificity [64], and thus, several studies were conducted to delineate the roles of E3 ubiquitin ligases [14, 20, 23, 26, 35, 46, 55, 77, 79]. Among these, several reports highlight their involvement in biotic and abiotic stress responses of plants [2, 12, 23, 34, 37, 49, 55, 63, 80]. Given this, the present review summarizes the components involved in UPS and elaborates the role of E3 ubiquitin ligases in biotic and abiotic stress responses.

The components of ubiquitin–proteasome system

The ubiquitination process involves three distinct classes of enzymes namely E1, E2 and E3 ligase. The ubiquitin-activating enzyme (UBA;E1) activates the ubiquitin in the presence of ATP. The cysteine residue of E1 is utilized for the formation of thioester-linked intermediate E1-ubiquitin (E1-Ub). The ubiquitin-conjugating enzyme (UBC;E2) interacts with E1-Ub followed by the transfer of activated Ub to an active cysteine residue of E2, forming a thioester-linked E2-Ub intermediate. Subsequently, the Ub is transferred to the target protein by ubiquitin ligase (E3) [76]. The E3 interacts with both the target protein and E2-Ub to establish an isopeptide bond between the glycine residue at the C terminal of Ub and lysine residue of the target [76]. The process is repeated several times to generate polyubiquitinated target proteins, and it is reported that a minimum of four Ub molecules is required to develop the polyubiquitin chain that activates proteasomal degradation (Fig. 1) [17, 73]. The ubiquitin gene family of *A. thaliana* contains fourteen members belonging to three gene types namely, polyubiquitin genes, ubiquitin-like genes and ubiquitin extension genes [10]. The polyubiquitin and ubiquitin-like genes possess tandem repeats of the 228-bp ubiquitin coding region [10]. Post-translation, the Ub monomers are generated through proteolytic cleavage [75]. Each Ub contains seven lysine residues at different positions; Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63, and all these lysine residues are capable of forming structurally different polyubiquitin chains [26, 27]. The fate of modified protein depends on the topological attachment of polyubiquitin chain. The chain with Lys48 linkage is targeted for degradation by the 26S proteasome, whereas the ones having Lys63-linked ubiquitin chains are targeted for endocytosis and intracellular trafficking [53]. The monoubiquitinated target proteins are destined for vesicle sorting [47].

The ubiquitin-activating enzyme (E1) regulates the rate of ubiquitination, and given its importance, the genes encoding for E1 are extensively studied. In yeast, E1 is encoded by a single gene, whereas two E1 genes were reported to be present in *A. thaliana* and tobacco [22, 70]. However, three genes were identified in wheat, and in soybean, a maximum of four genes was reported [82]. The size of E1 proteins ranges from 110–125 kDa, and these proteins possess four conserved domains [22]. First is the adenylation domain that contains two ThiF-homology motifs, followed by the catalytic cysteine domain (which possesses the first and second catalytic cysteine half-domains denoted as FCCH and SCCH, respectively). The third is a four-helix bundle (4HB) followed by the C-terminal ubiquitin-fold domain (UFD) which determines the specificity of E1 towards E2 [82].

Similar to E1, the E2 enzymes also possess a highly conserved region of ~ 140–150 amino acids called the ubiquitin-conjugating (UBC) domain. The UBC domain contains the cysteinyl residue of the active site [29]. Besides 37 E2-encoding genes of *A. thaliana*, the UBC domain was also found to be present in two RUB-conjugating enzymes (AtRCE1 and AtRCE2) and a SUMO-conjugating enzyme (AtSCE1) [29]. E2 enzymes were initially inferred to be involved in Ub transit; however, recent studies have also identified their role in regulating the polyubiquitination process [82]. E2 activates chain elongation and also governs the topology of chain formation [78], thus determining the fate of the target proteins.

Unlike E1 and E2 proteins, the E3 ubiquitin ligases form the largest and highly diverse group of proteins. The presence of large number of E3 ligases in *A. thaliana* accounts for ~ 5% of the genome [64]. E3 ubiquitin ligases are classified into single-polypeptide proteins and multi-subunit complexes. The former group includes the HECT-, RING- and U-box-domain containing E3s [15]. The HECT-type E3 possesses a ~ 350 amino acid HECT domain at its C-terminal which mediates the covalent transfer of Ub from E2 to the target protein. In contrary to HECT proteins, the RING and U-box type E3 ligases interact noncovalently with the E2-Ub through a conserved RING- or U-box domain [15]. The RING and U-box type E3 ligase thus facilitate the transfer of Ub to the target protein through zinc-chelating domain and hydrogen bonds/salt bridges, respectively [15, 67]. Structurally, RING and U-box ligases are similar where the conserved Zn-coordinating residues are absent in U-box domain of the latter. Among the HECT-, RING- and U-box-domain containing E3s, the RING-type E3 ligases are capable of forming complex multi-subunits E3s, such as Skp1-Cullin-F-box (SCF), the anaphase-promoting complex/cyclosome (APC/C) and the Cullin-Elongin-BC-VHL (CBC VHL)-type E3 ligases [5, 15, 33, 67].

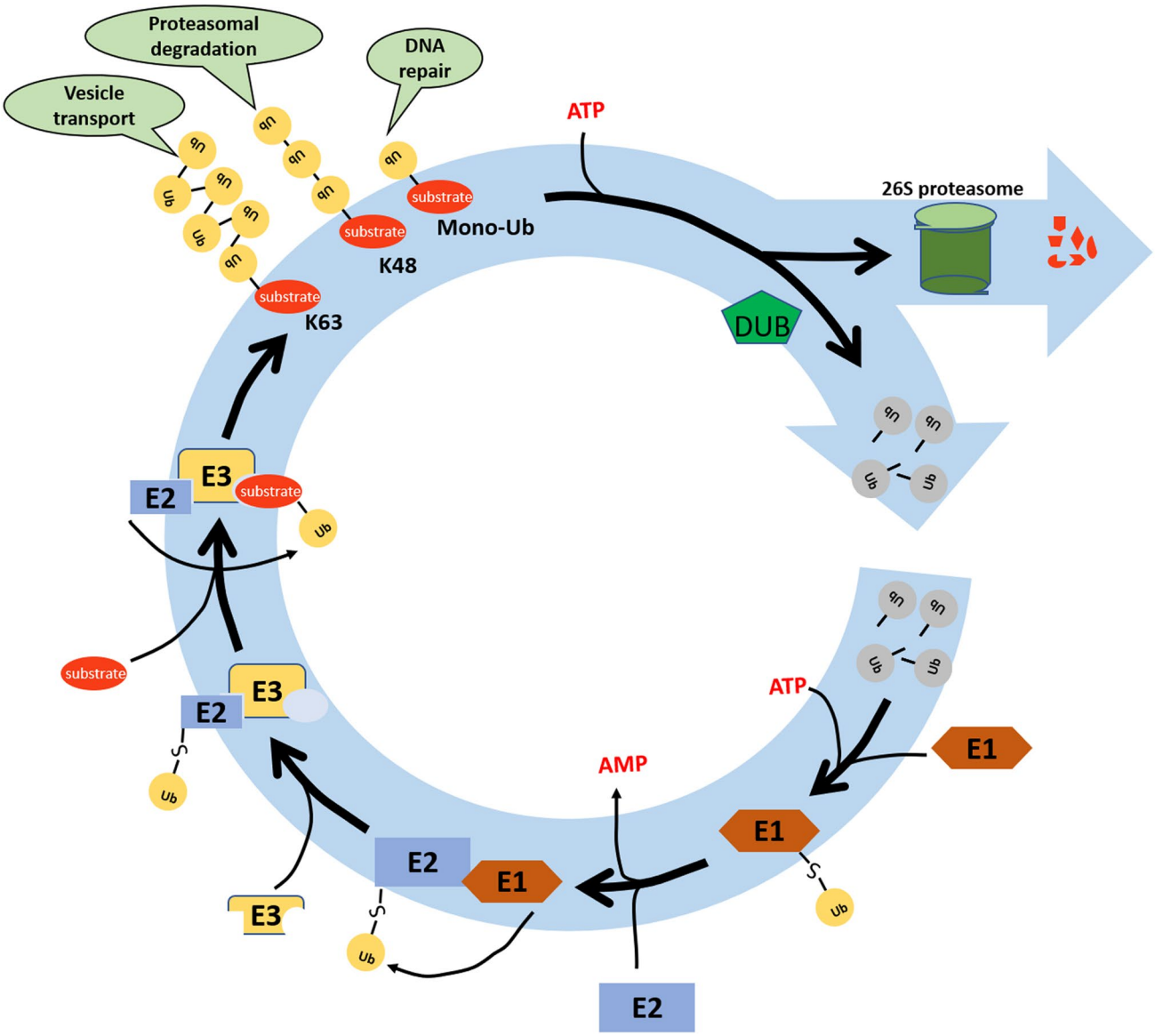

Fig. 1 The schematic representation of ubiquitin modification cycle. Ubiquitination of a substrate protein happens through sequential cascade of enzymes. Initially ubiquitin-activating enzyme (E1) forms a thioester linkage with ubiquitin with the expense of an ATP molecule. Through a transesterification reaction ubiquitin is transferred to ubiquitin conjugating enzyme (E2). The E3 ligase associates with E2-Ub complex. The ubiquitin protein is transferred to lysine residue of the substrate from E2-Ub complex. Ub can be attached to one another via one of its seven lysine residues. The site of attachment of Ub determines its fate, polyubiquitin chains originating from Lys48, Lys63 and monoubiquitin are destined for proteosomal degradation, vesicular transport and DNA repair, respectively. The deubiquitination enzymes transforms the polyubiquitin chain to monomeric inactive ubiquitin to be used in next round of ubiquitination process. Finally, the substrate is degraded by 26S proteasome complex

Among the gene families encoding for different E3 ligases, HECT-type E3 ligase family is the smallest having seven and nineteen genes in *A. thaliana* and soybean, respectively [6, 16, 64, 82]. In the case of RING-, U-box and F-box gene families, the numbers ranged from 469, 64 and 694 in *A. thaliana* and 760, 124 and 472 in soybean, respectively [64, 82]. In case of rice, 77 and 687 genes encoded for U-box and F-box gene families [18, 82]. Integrated computational pipelines and advanced bioinformatics tools play a systematic role in the identification of gene families, and several genome-wide studies are being undertaken in sequenced crops that reveal the characteristic features of corresponding gene family members. In this context, similar studies should also be performed to identify the genes encoding core components of the ubiquitin system in crop plants which will further enable their functional characterization towards delineating the potential role for ubiquitination in stress endurance.

Role of E3 ligase in plant defense against biotic stresses

The involvement of UPS in stress perception and activating downstream responses is well reported [21]. The UPS suppress the stress signalling pathway during the absence of stress, thus eliminating the negative regulators of signalling responses. Also, UPS functions as a positive regulator depending on the nature of the biotic stress (Table 1) [9, 13, 19, 30, 63, 74]. Several studies have underlined the differential expression of RING-type genes during treatment with different elicitors [reviewed by 40]. Tobacco plants treated with fungal Avr9 effector showed enhanced expression of an ATL gene, *AVR9/CF-9-RAPIDLY ELICITED132* (*NtACRE132*) [57]. Three different E3 ligases namely *NtACRE132*, *NtACRE276* and *NtACRE189* were identified through cDNA-AFLP analysis of Avr9 treated Cf9 tobacco cell cultures. Reduced *Avr4/Cf4*- or *Avr9/Cf9*-induced hypersensitive response were observed in *NtACRE189* and *NtACRE276* silenced background, which highlights the role of E3 ligases as a positive regulator of *Cf4*- and *Cf9*-mediated cell death and resistance against fungus infection [57]. Similarly, induced expression of *AtATL2*, *AtATL6*, *AtATL9*, *SlATL6* and *OsEL5* were observed during elicitor treatment in *A. thaliana, Solanum lycopersicum* and *Oryza sativa*, respectively [45, 60, 62, 69, 74]. A loss-of-function mutant of *AtATL9* (*atl9*), an active E3 ubiquitin ligase resulted in susceptibility to *Golovinomyces cichoracearum* [40].

In parsley, rapid induction in the expression of two RING-type E3 ligase genes namely *CMPG1* and *CMPG2* was observed during treatment with Pep25 oligopeptide elicitor. The CMPG1 and CMPG2 consist of N-terminal RING domain (31–96 amino acids) and C-terminal leucine-rich repeats [28], and in *A. thaliana,* these proteins were reported to have roles in conferring tolerance to pathogen infection [25]. In tobacco, upregulation of ubiquitin-activating enzymes, *NtE1A* and *NtE1B* during tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV) infection suggested the involvement of ubiquitination in plant-virus interaction [70]. Higher expression of UPS component genes during *Tomato leaf curl New Delhi virus* infection in tomato suggested the possible involvement of UPS in defense

Table 1 Members of ubiquitin ligases involved in plant defense during biotic and abiotic challenges

Gene (protein type)	Substrate	Host	Stressor	Reference
Biotic challenges				
ACRE132 (RING)		Tobacco/tomato	*C. fulvum*	[57]
ACRE189 (F-box)		Tobacco/tomato	*C. fulvum*	[57]
ACRE276 (U-box)		Tobacco/tomato	*C. fulvum*	[57]
CMPG1/		*Arabidopsis*	Oligopeptide elicitor or *P. syringae*	[25]
CMPG2 (U-box) ATL2/ mATL6 (RING)		*Arabidopsis*	Elicitor chitin	[60, 62]
BRH1 (RING)		*Arabidopsis*	Elicitor chitin	[69]
NtRING1 (RING)		Tobacco	Fungal pathogen	[20]
EL5 (RING)		*Oryza sativa*	N-acetylchitooligo-saccharide elicitor	[45]
CMPG1 (U-box)		Parsley (*P.crispum*)	Oligopeptide elicitor or *P. sojae*	[28]
BOI1 (RING)	BOS1	*Arabidopsis*	Fungal treatment	[37, 43]
RFP1 (RING)	βc 1 of TYLCCNV	Tobacco	*TYLCCNV*	[63]
MIEL1 (RING)	MYB30	*Arabidopsis*	Fungal pathogen	[39]
EIRP1 (RING)	VpWRKY11	*Vitis pseudoreticulata*	Fungal elicitor	[80]
SPL11 (U-box)	Pre-mRNA processing protein	*Oryza Sativa*	*M. grisea* and *X. oryzae* pv *oryzae*	[81]
Abiotic challenges				
DRIP1/2 (RING)	DREB2A	*Arabidopsis*	Drought stress	[55]
Rma1H1 (RING)	PIP2;1	*Capsicum annuum*	Drought stress	[31]
PUB23/22 (U-box)	RPN12a	*Arabidopsis*	Drought and salinity stress	[12]
HOS1(RING)	ICE1	*Arabidopsis*	Cold stress	[14]
PUB1 (U-box)	RPN6	*Capsicum annuum*	Drought and salinity stress	[12]
OsHCI1 (RING)		*Oryza sativa*	Heat and cold stress	[34]
OsDIS1 (RING)	OsNEK6	*Oryza sativa*	Drought stress	[49]
NLA (RING)		*Arabidopsis*	Nitrogen deficiency stress	[52]
CNI1 (RING)		*Arabidopsis*	C/N stress	[61]

response [58]. Functional characterization of *SlRPT4*, a component of 19S regulatory particle showed its involvement in activating hypersensitive response and programmed cell death, thus restricting the viral spread [32, 58].

In tobacco, a RING-type E3 ligase, NtRFP1 has been shown to positively regulate *Tomato yellow leaf curl China virus* resistance. NtRFP1 interacts with viral βC1 protein and initiates degradation of the viral protein. The NtRFP1 overexpressing plants showed reduced symptoms after virus infection. However, NtRFP1 silenced plants showed significant symptom development [63]. Similarly, another RING-type E3 ligase, NtRING1 has been shown to restrict pathogen spread by enhancing HR response against elicitin, *Ralstonia solanacearum* and TMV in tobacco [20].

A lesion mimic rice mutant *spotted leaf11* (*spl11*) identified from an ethyl methanesulfonate–mutagenized *indica* cultivar IR68 showed enhanced, non-race-specific resistance to blast (*Magnaporthe grisea*) and blight (*Xanthomonas oryzae* pv *oryzae*) diseases [81]. Further analysis showed that *Spl11* gene encodes a novel protein with both a U-box domain and six armadillo (ARM) repeats, and the point-mutation in *spl11* results in premature termination of translation. In vitro ubiquitination assay showed that the SPL11 protein has E3 ligase activity which requires the U-box domain [81]; and altogether, the study suggested the role of UPS in regulating cell death and defense. In *A. thaliana,* MIEL1, a RING-type E3 ligase is a negative regulator of defense response [39]. MIEL1 ubiquitinates a defense-activating transcription factor, Myb30. The proteasomal degradation of Myb30 results in the attenuation of defense and HR responses [39]. In normal conditions, Myb30 activates the biosynthesis of very long chain fatty acids (VLCFAs) that confers resistance to the pathogen [56].

BOI1 (Botrytis Susceptible1 Interactor) is a RING-type E3 ligase that interacts with an R2R3MYB transcription factor, BOS1 leading to its ubiquitination in *A. thaliana* [37, 43]. *BOI1* RNAi lines showed enhanced susceptibility to *Botrytis cinerea*; however, *BOI1* RNAi plants overexpressing BOS1 showed higher resistance to the pathogen [37, 43]. Another RING-type E3 ligase, EIRP1 of wild grapevine (*Vitis pseudoreticulata*) interacts with the transcription factor, VpWRKY11. This transcription factor activates the expression of JA-responsive genes that negatively regulate resistance to fungal infection [80]. Therefore, degradation of VpWRKY11 by EIRP1 results in lower JA-responsive signaling, followed by resistance to fungus [80]. These observations highlight the involvement of ubiquitin ligases in modulating the immune responses. Further, the involvement of UPS components in all the defense processes from pathogen perception to defense signalling during both pathogen- and effector-triggered immune responses demonstrate the versatile roles of UPS in biotic stress response. However, the targets of numerous defense-responsive E3 ligases are unknown, and therefore, the identification and functional characterization of these targets are imperative.

Role of E3 ligase in abiotic stress responses

In contrary to biotic stresses, the role of E3 ligases has been largely shown in abiotic stresses. Dehydration-responsive Element Binding Protein 2A (DREB2A) is a class of transcription factors that are involved in dehydration stress response. In *A. thaliana,* two RING-type E3 ligases namely DRIP1 and DRIP2 were shown to affect the accumulation of DREB2A protein, thereby negatively regulating the plant's response to drought [55]. Arabidopsis *drip1drip2* double mutants showed enhanced tolerance to drought stress as compared to wild-type plants underlines the DRIP1/DRIP2-mediated DREB2A ubiquitination and degradation in normal conditions. However, the degradation of DREB2A is inhibited during stress which results in the transcriptional activation of drought-responsive genes [59].

Another E3 ubiquitin ligase, Rma1H1 was initially characterized as a dehydration-responsive gene in *Capsicum annuum* [31, 51]. Overexpression of Rma1H1 resulted in enhanced drought tolerance in *A. thaliana* [31]. This protein targets a plasma membrane-localized aquaporin, PIP2 that helps in symplastic water transport leading to a negative impact on plants during water stress [3]. The protoplast co-transformed with PIP2;1 and Rma1H1 showed a significant reduction of PIP2;1 [3]. The reduction of PIP2;1 protein level via ubiquitinylation was validated with the addition of proteasomal inhibitor in the initial co-transformation experiment, which highlighted the role of Rma1H1 in proteasomal degradation of PIP2;1 [31]. In rice, OsDIS1 is a RING-type E3 ligase that negatively regulates a drought-responsive gene, OsNEK6 which has positive roles in drought tolerance [49].

Similar to RING-type E3 ligases, the U-box domain containing proteins (PUB) were also shown to be involved in abiotic stress responses. In *A. thaliana,* overexpression of two PUB genes, *PUB22* and *PUB23* conferred susceptibility to salinity and drought stresses [12]. Further downstream experiments revealed that PUB22 and PUB23 interact with RPN6 and RPN12a, respectively. RPN6 and RPN12a are the components of proteasome complex, and their ubiquitination disrupts the 26S proteasome cascade, thus making the plant vulnerable to environmental cues [12]. Similarly, the PUB genes, PUB46 and PUB48 have shown to play roles in drought stress response [11].

Inducer of CBF expression 1 (ICE1) regulates the transcription of a cold-responsive transcription factor, CBF3/DREB1A that subsequently controls the expression of numerous cold-responsive genes [2]. In *A. thaliana, high expression of osmotically responsive gene 1* (*HOS1*) is a

RING-type E3 ligase that interacts with ICE1 during cold stress and subsequent ubiquitination of ICE1 results in the attenuation of cold stress response [2, 14]. Another RING-type E3 ligase of rice, *Oryza sativa heat and cold-induced 1* (*OsHCI1*) has been shown to positively regulate heat and cold stresses by ubiquitinating six target proteins including 20S proteasome subunit α7 (OsPSA7), periplasmic beta-glucosidase (OsBGLU1), ethylene-responsive protein (OsbHLH065), glycine-rich cell-wall structural protein (OsGRP1), peroxidase (OsPOX1), and 14-3-3 protein (Os14-3-3) [34]. Overexpression of OsHCI1 has shown to confer enhanced tolerance to heat and cold stresses in transgenic *A. thaliana* plants [34].

In *A. thaliana*, *NLA* (*Nitrogen Limitation Adaptation*) gene has been reported to encode for a RING-type E3 ligase that regulates the adaptability of plants to limited nitrogen conditions [44]. This protein interacts with ubiquitin conjugase 8 (AtUBC8), a negative regulator of the nitrogen limitation sensing and signalling pathway. NLA mutants showed premature senescence phenotype, and during nitrogen-limiting conditions, the mutant lines failed to develop any response that is required for the survival of the plants [52]. The ratio of nitrogen to glucose is vital for development post germination. Plant growth halts at a higher level of glucose than nitrogen, but a slight increase in nitrogen level recovers the plant growth [36, 41]. In *A. thaliana*, it has been shown that a RING-type E3 ligase, Carbon–Nitrogen Insensitive 1-dominant (CNI1) is required for the C/N response during the early post-germinative growth of seedlings [61].

In addition, several other RING-type E3 ligases namely Salt- and Drought-Induced Ring Finger 1 (SDIR1), RING Zinc Finger 1 (RZF1), ABA-insensitive RING protein 1 (AIRP), Keep On Going (KEG), Ring finger protein with Microtubule-Targeting domain 1 (RMT1), Salt-Induced RING Finger Protein 1 (SIRP1) and Stress-related Ring Finger Protein 1 (SRFP1) were reported as either positive or negative regulators in response to several abiotic stresses. However, no much information is available on the precise regulatory roles of these proteins at molecular and biochemical levels. The major drawback is the non-availability of information about the target proteins. Also, the lack of efficient screening systems for the target substrates is a shortcoming. Thus, high throughput screening approaches are to be developed and introduced in this study for efficient screening of target proteins.

Conclusions

Plants inadvertently encounter biotic and abiotic stresses throughout their lifecycle, and this has enabled the plants to develop several sophisticated yet complex defense mechanisms. One such defense response is equipped via the UPS. Increasing evidence indicates that UPS could be a versatile tool which confers adaptability to diverse stressors. To date, very few E3 ligases have been identified to have roles in biotic as well as abiotic stresses. Although the expression profiles of these proteins during stress and non-stress conditions are available, but not much information about the target proteins of these ligases. Compared to the reports available in the animal system, relatively fewer studies have been performed on this aspect in the plant system, and much of the mechanistic part remains elusive. The knowledge on the timing and the site of ubiquitination requires a detailed study for gaining further insights. Not all the substrate proteins are destined for proteasomal degradation since some lead to vesicular transport and DNA repair functions; however, no much information is available on the mechanistic as well as biological perspective. Altogether, UPS remains an interesting yet challenging field to explore and gain a better understanding of their molecular as well as biological functions in stress responses.

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