

# Role of Plant U-BOX (PUB) Protein in Stress and Development

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## ABSTRACT

During the past few years, the significance of regulated protein degradation has become increasingly apparent in plants. There has been a remarkable exploration of information on the proteins of the ubiquitin/proteasome system and their role in protein degradation throughout the eukaryotes. The proteasome ubiquitination system selects a number of proteins for post-translational modification and subsequently, subjects them to degradation. E3 ubiquitin ligases play a central role in determining the target specificity in this system. Interestingly, most recently discovered, RING/U-box represents a type of E3 ligases and shows greater prevalence in plants (PUBs, Plant U-box E3 ubiquitin ligases) in comparison to animals. Hence, suggesting their involvement in a range of indispensable processes in plant system. U-box is a highly conserved domain whose physiological function remains unclear, but it has been implicated as a regulator of fundamental cellular processes ranging from cellular growth, damage responses and apoptosis. Besides, on the basis of assorted accessory domains or protein binding motifs, PUBs can be classified into several subclasses, which bestow functional divergence to them. Moreover, genome wide homology searches in monocots and dicots have revealed similar domain organizations in several of the U-box genes suggesting their evolution through a common ancestor. The participation of PUBs in plant development is extensive, affecting processes related to development and signal transduction cascades. Moreover, increasing evidences points towards the association of PUBs in defense against biotic as well as abiotic stresses. Here, we will be emphasizing the current knowledge about the aspects of cellular responses shared by PUB proteins in plants under stress and developmental conditions.

**Keywords:** abiotic stress, biotic stress, protein degradation, phytohormones, signal transduction, U-box

**Abbreviations:** DUB, deubiquitinating enzymes; PUB, plant U-box proteins; Ub, ubiquitin

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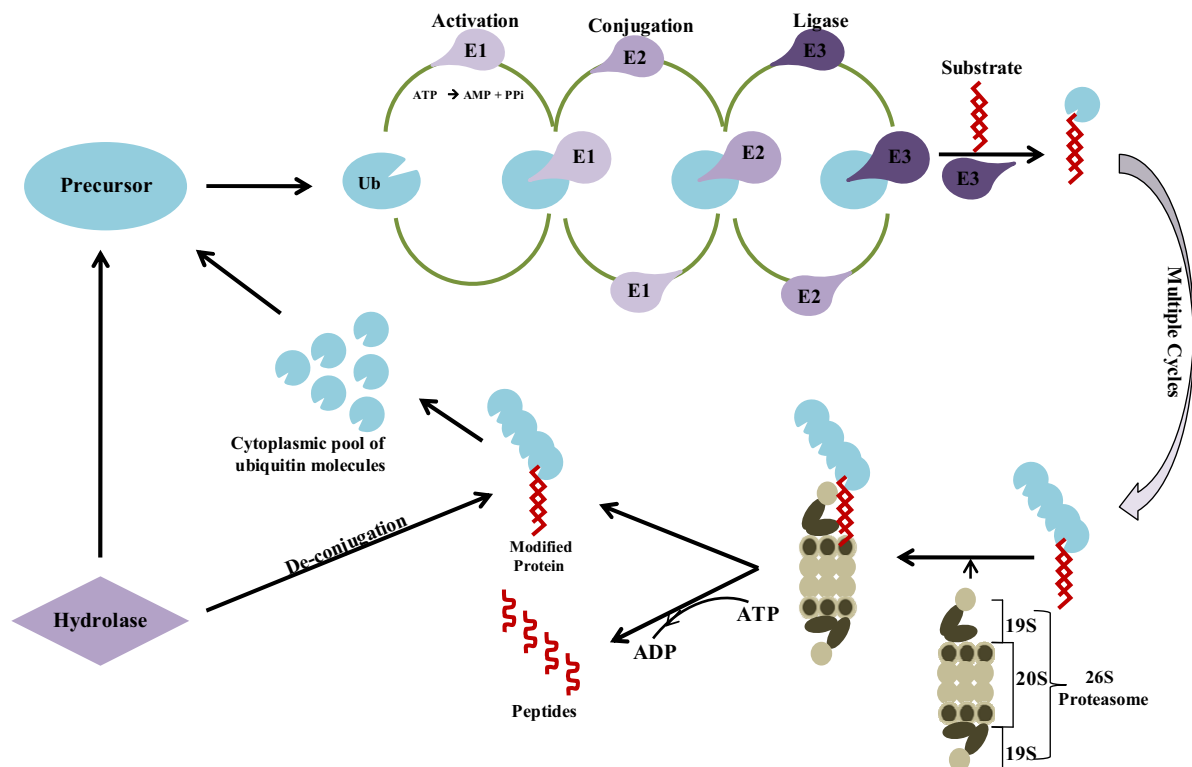
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## INTRODUCTION

In plants, growth, development and physiological processes are governed by the selective synthesis and removal of short-lived regulatory proteins. Protein degradation is a post-translational regulatory mechanism essential for cellular growth and regulation. One such important proteolysis mechanism is ubiquitin (Ub)/26S proteasome pathway. Aaron Ciechanover, Avram Herskko and Irwin Rose were awarded a Nobel Prize in chemistry (2004) for discovering the importance of ubiquitin degradation pathway to cellular regulation in eukaryotes. Recent understanding of the protein modification in plants has considerably increased, owing to the identification of plethora of genes involved in this pathway. Perhaps, ubiquitin/26S proteasome is a major catabolic pathway in plants that contributes significantly to development by affecting many fundamental cellular processes, including autophagic recycling, transcriptional regulation, RNA metabolism, embryogenesis, senescence, circadian clock, stress and hormone signaling (Kirschner 1999;

Dunlap 1999; Azevedo *et al.* 2001). The ubiquitin system is complex in terms of the number of substrate protein, it regulate and target the complex enzymatic machinery for the selective turnover of unwanted proteins by the 26S proteasome (Vierstra 2012).

Regulated proteolysis is a housekeeping mechanism, which maintains cellular homeostasis by removing abnormal proteins and supplying free amino acids during growth and developmental conditions (Vierstra 1996; Hellmann and Estelle 2002; Vierstra 2003). This pathway destines target proteins for degradation; many of these are short-lived and have a regulatory function associated with them. Further, in addition to the eradication of aberrant protein, ubiquitination also regulates the amount of active proteins in the cell by maintaining synthesis to degradation ratio. Recent work in plants has proven the role of ubiquitination in controlling the activity and level of key proteins, vital for many physiological and developmental response pathways.



**Fig. 1** Depiction of the basic steps of ubiquitin mediated protein degradation and modification pathway. Initially, the ubiquitin precursor molecules are processed and are activated by an E1 enzyme. Upon activation, the ubiquitin molecule is transferred to the E2 enzyme, which catalyzes its conjugation with the target protein. In the next step, another ubiquitination factor, E3 ligating enzyme recognizes and ligate the appropriate substrate with the ubiquitin molecule. Multiple cycles results into the formation of polyubiquitin chain on the protein substrate. At the end, ubiquitin tagged protein molecule is then either degraded into small peptides by the 26S proteasome or the modified protein molecule are de-ubiquitinated by a number of different enzymes. This cycle is reversible, as at each step, the participating molecules are recycled for the next turn.

## Mechanism of ubiquitin proteasome pathway

Ubiquitin is a key player of this protein degradation pathway. As the name suggests, Ub is ubiquitous among eukaryotes with a highly conserved (76 amino acids polypeptide) structure (Callis *et al.* 1995). Multiple ubiquitin molecules are tagged to the target proteins before degradation by the 26S proteasome. The ubiquitin conjugation cascade majorly engages three enzymes E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin-protein ligase enzyme). Tagging of the target protein with ubiquitin is carried out by these three ubiquitination factors, which function in concert at each successive step in an ATP (Adenosine triphosphate) dependent manner. In the initial step, E1 activates an ubiquitin molecule and transfer it to the active site of the ubiquitin-conjugating enzyme (E2). Next, ubiquitin-ligase (E3) binds E2 and catalyzes the formation of an isopeptide linkage between the activated ubiquitin and the lysine residue of the substrate protein. Consequently, destines ubiquitinated proteins to degradation by the 26S proteasome (Hatakeyama and Nakayama 2003). E3 ligases are responsible for recruiting precise target molecule for degradation and thus confer substrate specificity to the system (Fig. 1). As a result, E3 ligase family is the most diverse and numerous in plant system.

Both single and multi-protein E3 ligases are known to be present in higher plant system (Petroski and Deshaies 2005). Another ubiquitination factor E4 has also been characterized, which facilitates polyubiquitination of the target proteins (Pickart 2000). Several rounds of ubiquitin conjugation to target yield a protein with chains of polyubiquitin on it. Finally, the polyubiquitinated proteins are recognized by the 26S proteasome. The 26S proteasome is a remarkable complex comprising a proteolytic core and two regulatory particles associated with it. The target protein is escorted into the hollow proteolytic core and subjected to degradation into small peptides and ubiquitin moieties are

released and subsequently reused by the cell (reviewed in Smalle and Vierstra 2004; Yee and Goring 2009).

Ubiquitination process has been identified as a reversible process and the presence of an antagonist pathway can be expected in the plant system. Deubiquitination of the tagged substrates by the deubiquitinating enzymes (DUBs) is emerging as a key mechanism for the regulation and modification of tagged protein (Nijman *et al.* 2005). Several deubiquitinating enzymes are found in the cell that cleave ubiquitin molecules conjugated via either isopeptide or linear peptide bonds. DUBs play a functionally reciprocal role to the ubiquitination as it contributes to the maintenance of the cytoplasmic pool of free ubiquitin molecules after degradation of the substrate, proofreading ubiquitin-protein conjugates and are essential for the processing of ubiquitin precursor molecules (Wilkinson 1997). Subsequently, inactivation of deubiquitinating enzymes results in the inhibition of ubiquitin mediated proteolysis. Deubiquitination can also accelerate proteolysis by shortening polyubiquitin chains to a length that is easily recognized by the 26S proteasome (Wilkinson 1997).

In contrast to the ample information available for the ubiquitination, not much is known about deubiquitination process in plants. While few emerging studies have confirmed diverse role of DUBs under plant immunity (Ewan *et al.* 2011) and developmental processes, however, nothing significant have been reported on their role under stress signaling in plants (Yang *et al.* 2000; Doelling *et al.* 2001, 2007; Sridhar *et al.* 2007; Liu *et al.* 2008; Luo *et al.* 2008; Schmitz *et al.* 2009; Isono *et al.* 2010; Katsiarimpa *et al.* 2011).

Many plant genomic studies have identified numerous E3 ubiquitin ligases in plants, specifically, in *Arabidopsis* and rice. E3 ubiquitin ligases can be divided into various families based on the presence of specific HECT, RING, or U-box domains (reviewed in Smalle and Vierstra 2004; Stone and Callis 2007). The abundance of these proteins in

plants suggests a diverse role of each of them in conferring specificity to the process by ubiquitinating a particular target. The vast size of this gene family and their involvement in a range of biological processes including plant reproduction, hormonal response, cell death and DNA repair, biotic and abiotic stress tolerance indicates a major role of protein degradation in regulating the plant growth and development.

## EVOLUTION AND EXPANSION OF THE UBIQUITIN LIGASE IN PLANTS

Plant ubiquitin ligases function during diverse aspects of plant development and morphogenesis processes controlled by multiple intracellular signaling pathways. Previous studies have made significant contribution to our understanding of the ubiquitin proteasome pathway in plants. However, it was not until the whole genome sequencing of *Arabidopsis* and rice was accomplished that we understand significance of this pathway in plant cells. Intriguingly, genomic studies have predicted nearly 1,200 genes of the *Arabidopsis* proteome to directly participate in the UPS (ubiquitin proteasome system) together with thousands of additional target proteins (Vierstra 2003; Smalle and Vierstra 2004). The ubiquitinating system has been considered as the most complex system in terms of targets and enzymatic actions for the selective turnover of unnecessary proteins by the 26S proteasome (Vierstra 2012). On the basis of subunit composition and action mechanism, E3s can be classified into four groups: the Anaphase Promoting Complex (APC), Skp-Cullin-F-box (SCF) complex, the Homology to E6-associated protein Carboxyl Terminus (HECT) domain and the Really Interesting New Gene (RING)/U-box domain (Aravind and Koonin 2000). SCF and APC are defined as multimeric E3s and the other two groups comprise monomeric E3s and are recognized by their E3 interacting domain (HECT, RING or U-Box) (Smalle and Vierstra 2004). Plant constitutes multigenic E3 families when compared with other eukaryotic species. The F-box E3 protein family has predominantly experienced an expansion in plants followed by U-box E3 ligases (Gagne *et al.* 2002). However, compared to RING and HECT domain E3 ligases, plants have been predicted with larger number of U-box E3 ligases (Patterson 2002). U-box, originally identified in the yeast UFD2 protein (Koegl *et al.* 1999) is related to the RING-finger domain, even though it lacks the zinc-chelating residue, which is required to stabilize its secondary structure (Aravind and Koonin 2000).

Alternatively, U-box scaffold is stabilized by a system of salt bridges and hydrogen bond forming a structure similar to the RING finger domain (Azevedo *et al.* 2001). Moreover, compared to animals, which have only few, the huge expansion of U-box proteins in plants indicates their influence on every aspect of plant growth and development. Genomic studies in *Arabidopsis* and rice have helped to annotate 64 and 77 U-box genes respectively, in their genomes (Azevedo *et al.* 2001; Wiborg *et al.* 2008; Zeng *et al.* 2008). The plant U-box (PUB) family again, can be divided into five groups based on the presence of other distinctive domains, such as the UFD2, ARM repeats, UND, Ser/Thr kinase, WD40 repeats (Azevedo *et al.* 2001; Mudgil *et al.* 2004; Wiborg *et al.* 2008). The presence of large number of E3 ligases in plants with respect to other eukaryotes underlines the importance of E3s in regulating vital plant processes. Several studies aimed to identify regulatory roles associated with PUB proteins deciphered the involvement of directed-ubiquitination in many interesting processes in plants.

## REGULATORY ROLES OF PLANT U-BOX E3 LIGASE PROTEINS

### PUBs as signaling proteins for receptor kinases

Receptor kinases symbolize a large family consisting of

around 600 members in *Arabidopsis* (Shiu and Bleecker 2001a, 2001b). Many of these receptors have been implicated in a range of developmental and regulatory pathways in plants (Li and Chory 1997; Gomez-Gomez and Boller 2000; Clouse 2002; Morris and Walker 2003; Osakabe *et al.* 2005). Several PUB proteins have been identified as the interacting partners of receptor kinases (Bower *et al.* 1996; Gu *et al.* 1998; Stone *et al.* 1999, 2003). One of the first receptor kinase activated E3 ligase protein to be functionally characterized in plants was *Brassica napus* ARC1 (ARM Repeat-Containing 1) (Gu *et al.* 1998). ARC1 consists of a U-box domain along with several ARM repeats. It is required in the *Brassica* pistil for the rejection of self-pollen and to promote cross-pollination (Takayama and Isogai 2005). ARC1 functions downstream to the SRK (S Receptor Kinase), which is responsible for detecting self-pollen and initiating a signaling pathway for pollen rejection (Takasaki *et al.* 2000; Silva *et al.* 2001). ARC1 interacts with the kinase domain of SRK via its C-terminal ARM repeat domain in a phosphorylation-dependent manner (Gu *et al.* 1998). ARC1 is considered to be a positive regulator of the self-incompatibility system, as antisense-suppression of ARC1 in self-incompatible wild type *Brassica* plants resulted in a partial-breakdown of this mechanism and allows the successful germination of self-pollen on the stigma (Stone *et al.* 1999). However, the cellular mechanism by which ARC1 participates in the self-incompatibility system to bring about self-pollen rejection was not clear until Stone *et al.* (2003) experimentally proposed it to be an E3 ligase protein. Thus, as a part of self-incompatibility response, ARC1 tags compatibility factors in the pistil with ubiquitin and target it for proteasomal degradation, which in turn leads to pollen rejection (Stone *et al.* 2003). As a matter of the fact, *Arabidopsis* is a self-compatible plant; no functional orthologue of ARC1 was found in its genome. However, another kinase interacting gene *AtPUB8* has been implicated in the pseudo self-incompatibility by the regulation of mRNA levels of *Arabidopsis lyrata* SRK genes (Liu *et al.* 2007). Both *AtPUB8* interacting with SRK along with tobacco *NtPUB4* protein interacting with CHRK1 (chitinase-related receptor-like kinase) are functionally correlated to ARC1, implicated in the regulation of mRNA levels in the cell (Kim *et al.* 2003; Liu *et al.* 2007). Besides two CHRK1 related, SD1 receptor kinases ARK1 and ARK2 have been known to phosphorylate AtPUB9 and 13. Though the functional significance of this interaction is not well understood yet but it alters the subcellular localization of these proteins in the cell signifying a potential site-specific regulatory role. Given that *Arabidopsis* contains multiple SD1 receptor kinases and PUB genes, it is more likely that they interact in the same manner and control several other specific biological functions (Samuel *et al.* 2008). The above events suggest a conservation of signaling and interaction components across the plant species.

### U-box proteins in regulation of hormonal signaling in plants

Plant hormones are comprised of a group of structurally unrelated small molecules and are indispensable for plant survival. Plants depend on hormones to induce changes in growth and development and mediate responses to both biotic and abiotic stress in response to a wide range of external stimuli. Several hormones are known to regulate protein synthesis; especially transcription, prompting that whether hormone are also involved in proteolysis. Hormone perception, synthesis, signaling and response can be exquisitely regulated through protein modulation via the ubiquitin proteasome pathway. Emerging advances in plant biology have established a novel mechanism of hormone perception in which the hormone signal is sensed by components of the ubiquitin proteasome system that is commonly found in all eukaryotic cells. To date, many phytohormones have been identified including auxin, abscisic acid (ABA), cytokinin (CK), gibberellin (GA), ethylene,

brassinosteroids (BR), jasmonate (JA), salicylic acid (SA), nitric oxide, strigolactones and karrikins (Davies 1995; Van Staden *et al.* 2000; Browse 2005; Vert *et al.* 2005; Grun *et al.* 2006; Loake and Grant 2007; Gomez-Roldan *et al.* 2008; Umehara *et al.* 2008; Santner and Estelle 2010).

Gibberellin is known to be involved in various physiological processes in plants such as growth, germination, stem elongation and fruit development (Olszewski *et al.* 2002). The PHOTOPERIOD RESPONSIVE 1 (PHOR1) was one of the first PUB/ARM protein found to be involved in hormone signaling. PHOR1 is known as the key regulator of photoperiod response in potato. PHOR1 similar to ARC1 was identified as a positive regulator of GA signaling in *Solanum tuberosum* ssp. *Andigena* (Amador *et al.* 2001) prior to being confirmed as a U-box protein (Monte *et al.* 2003). A PHOR1 transcript shows up-regulation in a photoperiod-dependent manner.

The PHOR1 loss of function plants shows a phenotype of semi-dwarf plants with impaired responsiveness to exogenous GA. The tuberization process was also accelerated in the absence of PHOR1 as compared to control plants suggesting that PHOR1 inhibits tuberization under short day conditions. All results were indicative of GA responsiveness of PHOR1 (Amador *et al.* 2001). Conversely, over expressing plants showed an altogether different phenotype with longer internodes and insensitivity towards the inhibitor of GA biosynthesis (Amador *et al.* 2001). The PHOR1 protein has a U-box motif followed by seven ARM repeats in its structure. Intracellular localization of PHOR1 is also found to be regulated under the control of GA. PHOR1 is largely a cytosolic protein, however, in response to exogenous GA, transiently translocate into the nucleus (Amador *et al.* 2001). *Arabidopsis* has also been reported with few uncharacterized PHOR1 related members (Monte *et al.* 2003). Hence, we can say that PHOR1 acts as a potential player in GA response, proteasomal degradation integrating all along with the photoperiodism i.e. environmental stimuli (Amador *et al.* 2001). It has been speculated that similar to PHOR1 involvement in GA responses in potato, there might be several other plants U-box proteins involved in phytohormone responses. Likewise, *NtPUB4* interacting CHRK1 has been shown to be involved in a signaling pathway regulating endogenous cytokinin level in tobacco. Functional disruption of the CHRK1 activity affects plant development and cytokinin homeostasis (Lee *et al.* 2003). Recent studies to identify any new hormone related regulation by PUB proteins have yielded interesting results in terms of ABA signaling component. ABA is an important phytohormone that directs seed maturation and control seed dormancy to ensure that seeds germinate only under favorable growth conditions. The accumulation of ABA in plants protects them from abiotic (drought, salinity) and biotic (pathogenic) stress (Lopez-Molina *et al.* 2001; Finkelstein *et al.* 2002). Even though some aspects of ABA signaling have been elucidated, the involvement of the E3 ubiquitin ligases especially, U-box, help us gain further insight into the entire network of ABA signal transduction.

For instance, animal CHIP (Carboxyl-terminus of Hsp70 Interacting Protein) related tetratricopeptide repeats (TPR) / U-box protein characterized in *Arabidopsis*, *AtCHIP* was found to be highly expressed under temperature stress. *AtCHIP* interacts with protein phosphatase 2A (PP2A), in such a way that increased activity of this protein lead to an altered response to ABA and auxin in the *AtCHIP* over-expressing plants (Luo *et al.* 2006). A possible role of *AtPUB9* interacting with ARK1 receptor in ABA response was subsequently established. Exogenous treatment with ABA resulted in the re-localization of the *AtPUB9* from the nucleus to the plasma membrane (Samuel *et al.* 2008). The biological role for both ARK1 and *AtPUB9* in ABA signaling was established by the ABA germination assays, where the mutant seeds displayed hypersensitivity towards ABA (Samuel *et al.* 2008). Further investigation identified a relative role of both the genes with the well-characterized ABA responsive ABI3 (Nambara *et al.* 1995), where, a

cross was generated between *abi3-6* and *pub9* mutant. This epistatic analysis confirmed that *AtPUB9* functions upstream or at the same level of the transcription factor ABI3 in ABA signaling (Samuel *et al.* 2008).

Two more PUB proteins, which are regulated at the transcript level by phytohormone ABA, include *AtPUB18* and *19*. Both *AtPUB18* and *19* are homologous to each other and coordinately modulate ABA signaling during germination (Bergler and Hoth 2010). A recent study generated some insight into the involvement of one more U-box E3 ligases (*AtPUB19*) to be involved in phytohormone responses. Expression analysis shows that the transcript level of *AtPUB19* is highly upregulated under abiotic stress conditions. It was shown to be rapidly induced by drought and ABA. *AtPUB19* overexpressing plants shows hypersensitivity towards ABA during germination while mutant showed reverse phenotype when treated with ABA (Liu *et al.* 2011). Recently characterized *AtPUB43* and *44* were proposed to be involved in plant growth and development pathways. Based on plant mutant analysis, it was shown that both function during seed germination and early seedling growth. This has been speculated that perhaps, both *PUB43* and *44* functions as part of inhibitory effect of ABA on seed germination as increased level of ABA was detected in the mutant plants (Raab *et al.* 2009; Salt *et al.* 2011). In view of the large number and diversity of E3 ubiquitin ligases in plant genomes, participation of more number of E3 ligases in the regulation of hormone signaling can be anticipated. However, the exact mechanism of their regulation is still need to be resolved.

### Biological significance of PUB proteins under abiotic stress

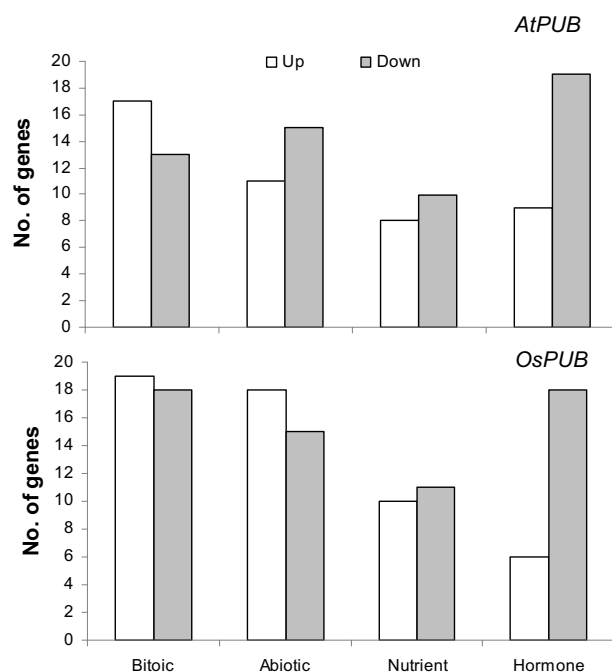
The crosstalk between stress and hormone signaling during the plant development requires tight regulation of signaling components, potentially by protein turnover mechanism. Various consistent observations in number of *AtPUB* genes expressing under hormonal action served as a preliminary clue in determining other stress related regulation by PUB proteins in plants. As a first step toward answering this question, study in mangrove (*Bruguiera gymnorhiza*) gave an indication of a PUB gene to be highly expressed under abiotic stress conditions (Banzai and Karube 2002). Mangroves are able to survive under high salt growth environments; this prompted scientists to identify salinity tolerance genes in its genome.

A PUB gene, *BG55*, was identified to show transient increase in expression in response to salt treatment (Banzai and Karube 2002). Salinity tolerance of mangroves is known to be tightly linked to the regulation of gene expression (reviewed by Parida 2010). Using this information many PUB genes have been identified in plants that were up regulated in response to salinity conditions and closely related to *BG55*. Many of the U-box genes in *Arabidopsis* such as *AtPUB23* and *24* were known to show strong up-regulation in the roots under salt and drought conditions (Cho *et al.* 2008). Microarray analysis has shown that many of these were responsive toward a range of abiotic stresses such as pathogenicity, wounding, heat, cold, drought and salinity. Interestingly, presence of U-box in these stress responsive genes suggests that they induce adaptability in plants against various environmental stresses by direct degradation of proteins to modulate its proteome. The framework of their biological functions can also be established by another U-box protein found in *Capsicum annum* L. cv. 'Pukang', *CaPUB1*. This has been shown to be involved in increased expression during dehydration, cold and salt and mechanical wounding stresses (Cho *et al.* 2006). The constitutive expression of *CaPUB1* gene in *Arabidopsis* under 35S promoter caused a distinct phenotype of transgenic plants in comparison to wild type in terms of enhanced growth of hypocotyls and roots, early bolting, and hypersensitivity to water and salt stresses. However, rapid growth phenotype of constitutively expressing transgene of

*CaPUB1* indicated it to be antagonizing the adaptive stress response (Cho *et al.* 2006). In *Arabidopsis*, *AtPUB22* and *AtPUB23* were identified to co-ordinate cellular responses to tolerate stress conditions such as cold, drought and salt, related to *CaPUB1* function. *AtPUB23* was found to be much more induced than *AtPUB22* under drought and cold stress conditions. Phenotypic analysis of the overexpressing *AtPUB22* and *23* plants confirmed their hypersensitivity towards salt and water deficient conditions (Cho *et al.* 2008). Similarly, coordinated regulation has recently been recognized in two homologous ABA induced genes, *AtPUB18* and *19* in *Arabidopsis*. These two PUB/ARM genes are regulators of ABA and, apparently salt mediated inhibition of seed germination (Bergler and Hoth 2010).

A recent report contributed more to elucidate the functional role of *AtPUB19* under abiotic stress. *AtPUB19* was found to be induced under all abiotic stress conditions where suppression of *AtPUB19* results in enhanced drought tolerance and overexpression resulted in the hypersensitive phenotype (Liu *et al.* 2011). The U-box domain regulates ubiquitin activity in various ways; one of these is by interacting with the equally diverse E2 proteins (Pringa *et al.* 2001). Different reports in soybean (*GmUBC2*) and peanut (*AhUBC2*) ascertain the role of E2 Ub-conjugating enzyme (UBC) in abiotic stresses in addition to U-box E3 ubiquitin ligase, which further establish a link between ubiquitination and stress tolerance. Overexpression of *GmUBC2* induces the expression of several abiotic stress responsive genes, which specifically, affect *AtNHX1* ( $\text{Na}^+/\text{H}^+$  antiporter) expression to sequester  $\text{Na}^+$  in plants resulting in enhanced salt tolerance. *Arabidopsis* was used as a model for the overexpression study of both *AhUBC2* and *GmUBC2*, which confirmed them to be as the positive regulator of salt and drought tolerance (Wan *et al.* 2010; Zhou *et al.* 2010). Interestingly, under drought conditions, the mRNA accumulation for both the genes was found predominantly in leaves and stems of soybean and peanut plant suggesting ubiquitination might occur in leaves but not in roots under water-stressed condition, hence triggering the degradation of target proteins to control drought response in plants (Wan *et al.* 2010; Zhou *et al.* 2010).

Many reports suggested that oxygen radicals stimulate ubiquitin-dependent degradation of newly synthesized proteins (Medicherla and Goldberg 2008). TPR repeat containing *Arabidopsis* U-box protein *AtCHIP* is eponymous to animal CHIP proteins and in a similar way interacts with cytosolic Hsp70 chaperons to carry out ubiquitination of foreign proteins (Ballinger *et al.* 1999; Shen *et al.* 2007b). Moreover, in response to temperature stress, there is a rapid increase in the transcript level of *AtCHIP* gene (Yan *et al.* 2003; Christmann *et al.* 2004; Luo *et al.* 2006). Owing to this, *AtCHIP* is thought to regulate cellular responses to tolerate stress conditions (Dai *et al.* 2003; Yan *et al.* 2003; Qian *et al.* 2006). Surprisingly, increase in the transcript level under stress conditions showed no correlation with the increase in the stress tolerance and in fact, rendered plants to become more sensitive to both low and high temperatures. This observation was peculiar in the sense that unlike plant CHIP protein, animal CHIP overexpression confers stress protection and recovery in animal cells (Dai *et al.* 2003). Apart from *AtCHIP* an additional gene was found to suppress protein oxidation from oxidative stress. Mutation in *OsPUB15*, yield an increase in ROS level in the cell indicating it to be responsible for reducing cellular oxidative stress during seed germination. More than this, transcript level of *OsPUB15* was also found to be increased upon salt and drought stresses (Park *et al.* 2011). The ubiquitin-26S proteasome system regulates diverse aspects in plant development including nutrient deprivation. A new study reported the role of a U-box protein *OsUPS*, under phosphate deprivation condition. In response to phosphorous and iron deficiency, transcript level of *OsUPS* gene was found to be strongly induced (Hur *et al.* 2011). The expression analysis of rice (77 genes) and *Arabidopsis* (41 genes) U-box proteins was performed *in silico* using microarray data



**Fig. 2 Gene expression analyses of U-box E3 ligase genes of *Arabidopsis* (35 out of 41) and *Oryza sativa* (54 out of 77) under different conditions.** Numbers of up- and down-regulated genes are indicated for each condition. The conditions described are biotic stress, abiotic stress, and nutrient deprivation, and hormone treatments. Expression data was obtained from Genevestigator (<https://www.genevestigator.ethz.ch>).

available at the GENEVESTIGATOR web-site (<https://www.genevestigator.ethz.ch>) (Fig. 2). A gene was considered up- or down-regulated when transcript amount increased or decreased at least two times in treated samples with respect to control samples. The physiological conditions tested included biotic, abiotic, hormone response, and nutrient starvation. Probeset were not found for 6 and 23 genes of *Arabidopsis* and rice, respectively in the GENEVESTIGATOR dataset. Based on this *in silico* analysis, several of the rice and *Arabidopsis* genes were found to be differentially expressing (either up- or down-regulation) under different stress conditions such as abiotic, biotic and nutrient deficient conditions (Fig. 2).

### Significance of PUB proteins in plant defense

Pathogen infection poses a life threatening challenge to plants. Consecutively, plants have developed sophisticated mechanisms to perceive such attacks, and to translate that perception into an adaptive response. Plant PUB proteins have been implicated in defense against such pathogens by regulated degradation. In order to combat such attacks plants generate a hypersensitive response (HR) with the associated PUB proteins, which control the level of key proteins by ubiquitination. Several E3 ligases are known to be involved in nodule formation and to coordinate bacterial infection (reviewed in Hervé *et al.* 2011). One of the first example appeared was *ACRE276*, (*Avr9/Cf-9* Rapidly Elicited) gene in tobacco (*Nicotiana tabacum*) where it showed high expression in response to treatment with the fungal pathogen (*Cladosporium fulvum*). In *Nicotiana*, one more PUB gene *NtACRE74* was discovered to be involved in response to pathogen along with *NtACRE276* (Durrant *et al.* 2000). Subsequent investigation conducted in *Arabidopsis* to identify FLARE (FLAgellin Rapidly Elicited) genes, which showed rapid change in gene expression when exposed to the flg22 peptide, a conserved component of bacterial flagellin involved in activating the innate immunity response (Navarro *et al.* 2004). Among the genes, which were induced upon flg22 treatment, several genes potentially involved in protein ubiquitination were iden-

**Table 1** Predicted role of some of the PUB genes in plants.

Species	PUB name	Function in plants	References
<i>Arabidopsis thaliana</i>	<i>OsPUB13</i>	Flowering, biotic stress	Li <i>et al.</i> 2012; Liu <i>et al.</i> 2012
<i>Oryza sativa</i>	<i>OsPUB15</i>	Oxidative stress tolerance	Park <i>et al.</i> 2011
<i>A. thaliana</i>	<i>AtPUB19</i>	Negatively regulates ABA and drought stress	Liu <i>et al.</i> 2011
<i>Medicago truncatula</i>	<i>PUB1</i>	Negatively regulates infection and nodulation	Mbengue <i>et al.</i> 2010
<i>A. thaliana</i>	<i>SAUL1</i>	Suppresses premature senescence	Raab <i>et al.</i> 2009
<i>M. truncatula</i>	<i>LIN</i>	Prevents early infection in legumes	Kiss <i>et al.</i> 2009
<i>Brassica napus</i>	<i>BnARC1</i>	Self-incompatibility	Gu <i>et al.</i> 1998; Stone <i>et al.</i> 1999, 2003
<i>A. thaliana</i>	<i>AtPUB8</i>	Pseudo self-compatibility	Liu <i>et al.</i> 2007
<i>Solanum tuberosum</i>	<i>StPHOR1</i>	Responsive to gibberellin	Monte <i>et al.</i> 2003
<i>A. thaliana</i>	<i>AtPUB9</i>	Responsive to abscisic acid	Samuel <i>et al.</i> 2008
<i>A. thaliana</i>	<i>AtCHIP</i>	Temperature stress tolerance and ABA response	Yan <i>et al.</i> 2003; Luo <i>et al.</i> 2006
<i>Capsicum annuum</i>	<i>CaPUB1</i>	Negatively regulates drought and salt stress	Cho <i>et al.</i> 2006
<i>A. thaliana</i>	<i>AtPUB22,23</i>	Abiotic stress regulation	Cho <i>et al.</i> 2008
<i>A. thaliana</i>	<i>AtPUB22,23,24</i>	Negatively regulates ROS level, biotic stress	Trujillo <i>et al.</i> 2008
<i>Nicotiana tabacum</i>	<i>NtACRE276</i>	Positively regulates biotic stress	Yang <i>et al.</i> 2006
<i>Solanum lycopersicum</i>	<i>SlACRE276</i>	Positively regulates biotic stress	González-Lamothe <i>et al.</i> 2006
<i>A. thaliana</i>	<i>AtPUB17</i>	Positively regulates biotic stress	Yang <i>et al.</i> 2006
<i>N. tabacum</i>	<i>NtACRE74/CMPG1</i>	Positively regulates biotic stress	Durrant <i>et al.</i> 2000
<i>S. lycopersicum</i>	<i>CMPG1/PUB20/PUB21</i>	Positively regulates biotic stress	González-Lamothe <i>et al.</i> 2006
<i>A. thaliana</i>			
<i>O. sativa</i>	<i>OsSPL11</i>	Negatively regulates biotic stress	Zeng <i>et al.</i> 2004
<i>P. syringae</i>	<i>AvrPtoB</i>	Plant defense	Abramovitch <i>et al.</i> 2006; Janjusevic <i>et al.</i> 2006
<i>A. thaliana</i>	<i>PUB5, PUB12</i>	Plant defense	Navarro <i>et al.</i> 2004
<i>A. thaliana</i>	<i>PUB27, PUB28, PUB29</i>	Responsive to gibberellin	Monte <i>et al.</i> 2003
<i>N. tabacum</i>	<i>NtPUB4</i>	Cytokinin homeostasis?	Kim <i>et al.</i> 2003
<i>A. thaliana</i>	<i>AtPUB12,13,14</i>	Negative regulation of PCD by pub12 pub13 pub14 mutants	Zeng 2005

tified. These include *AtPUB20/AtPUB21* (*CMPG1*, *NtACRE74*) and *AtPUB17* (*NtACRE276*) along with *AtPUB12* and *AtPUB5* (Navarro *et al.* 2004). Consequently, a large number of *AtPUB* genes are known to be involved in plant defense responses. Parsley *CMPG1*, tobacco *ACRE276* and *Arabidopsis PUB17* were subsequently confirmed to be U-box E3 ligases, which act as positive regulators of the hypersensitive responses to pathogenic infections (González-Lamothe *et al.* 2006; Yang *et al.* 2006).

Related to tobacco *ACRE276* and tomato *ACRE74* gene, which acts to control cf-9-dependent responses, *Arabidopsis AtPUB20*, *AtPUB21* and *CMPG1* from parsley have been known as early defense responsive genes activated immediately after bacterial infection (Kirsch *et al.* 2001; Heise *et al.* 2002; González-Lamothe *et al.* 2006; Yang *et al.* 2006). All of these identified ACRE genes, which are essential for cf-mediated hypersensitivity response encodes a U-box domain as a components of ubiquitylation machinery (Rowland *et al.* 2005). Mutation in the U-box domain abolishes the HR response indicating it to be crucial for the physiological role of the ACRE protein (González-Lamothe *et al.* 2006). Another ACRE ortholog, a novel UND/PUB/ARM protein *StPUB17* isolated from leaves of potato infected with *Phytophthora infestans*. This gene plays a substantial role in late blight resistance and salt tolerance in potato crops. *StPUB17* encodes an E3 ubiquitin ligase activity protein, which is implicated in protein degradation in response to biotic and abiotic stress (Ni *et al.* 2010). *StPUB17* shares more than 90% similarity in UND/U-box/ARM domain with tobacco *ACRE276*, *BnARC1* and *OsPUB4*. The expression of *StPUB17* was analyzed after treating with defense related signal molecules such as SA, MeJA and on infection by *P. infestans* using RT PCR. Transcript levels of *StPUB17* were found to be rapidly induced under these conditions supporting its role in conferring late blight resistance (Ni *et al.* 2009). The biochemical mechanism underlying this response is still not clear and requires further investigation to identify upstream and downstream partners. Consistent with these findings a similar protein in *Arabidopsis AtPUB17* was found to be positively regulating cell death and defense responses (Yang *et al.* 2006). Predicted functions of few of the plant U-box genes are listed in **Table 1**.

Subsequent studies in *Arabidopsis* provide evidence that PUB proteins might be functioning in both abiotic stress and plant defense against pathogens. The *Arabidopsis pub22 pub23 pub24* triple knockouts showed increased resistance towards bacterial and oomycete pathogens by rapid acceleration in the release of reactive oxygen species also known as respiratory burst (Trujillo *et al.* 2008). Interestingly, the homozygous double mutant *pub22pub23* have already been implicated in increased drought tolerance (Cho *et al.* 2008). One notable effect here is that *Arabidopsis AtPUB22* and *23* proteins act as negative regulators of both plant defense response and abiotic stress, indicating that plant U-box proteins are involved in a diverse array of responses and developmental processes. In sync with the earlier identified PUB proteins in *Arabidopsis* and tobacco as positive regulators of the hypersensitive response, rice (*Oryza sativa*) *SPL11* (Spotted Leaf 11) gene was uncovered with similar regulatory role. *SPL11* is a U-box/ARM protein found to be a negative regulator of pathogenic defense (Zeng *et al.* 2004). The *spl11* mutation in contrast to ACRE proteins causes spontaneous development of necrotic lesions mimicking pathogen attack in the absence of pathogen stimuli (Singh *et al.* 1995). *SPL11* protein is a PUB protein and function as E3 ubiquitin ligase (Zeng *et al.* 2004). Further characterization revealed many *OsSPL11*-related negative regulators of plant defense responses in *Arabidopsis*, such as *AtPUB12*, *13*, and *14* (Zeng *et al.* 2005), and *AtPUB22*, *23*, and *24*, which were recently discovered by Trujillo *et al.* (2008). Besides both *AtPUB12* and *13* have been shown to play significant role in the attenuation of plant immune signaling. Upon treatment with flagellin, *pub12pub13* double mutant display an elevated immune response in *Arabidopsis* (Lu *et al.* 2011). Although, how these proteins negatively regulate cell death is largely unknown but the involvement of U-box suggests some insight into the complex cell death and disease resistance pathway. Recently, the dual role of *PUB13* was shown in plant innate immunity and in flowering. *PUB13* provided the first direct evidence that ubiquitination controls flowering time in plants. The *PUB13* mutation is characterized by spontaneous cell death, hydrogen peroxide and SA accumulation, increased resistance towards biotrophic pathogens, and early flowering under certain conditions (Li *et al.* 2012;



Liu *et al.* 2012).

In another instance, a positive regulator of plant defense response, *AtPUB44* was recently discovered by Salt *et al.* (2011). This gene showed a seedling lethal phenotype following pathogen challenge. This effect leads to widespread cell death lesions throughout the cotyledons and roots. Homozygous mutants of *AtPUB44* displayed a strong lethal phenotype compared to the heterozygous mutant (Salt *et al.* 2011). Amazingly, U-box mediated protein degradation pathway might also be functioning to inhibit cell death and immunity in plants to ensure bacterial virulence. The *Pseudomonas syringae* protein AvrPtoB is an example of a pathogen evolving molecular mechanism to imitate host ubiquitin system to suppress plant cell death (PCD) for their benefit. AvrPtoB targets a conserved component of PCD in fungi and eukaryotes and thus acts as a general cell death repressor (Janjusevic *et al.* 2006; Abramovitch *et al.* 2006). Thus, it is evident that PUB proteins can influence multiple plant responses under a range of external stimuli. More studies related to identification of ubiquitin ligase target molecule will definitely provide further insight into the functions of PUB proteins in plants.

## CONCLUSION AND FUTURE PERSPECTIVES

Plants in their immediate environment are subjected to multiple abiotic stress conditions. The only way to deal with these stresses is through physiological and morphological changes. Numerous ways of adaptation to abiotic stress are interlinked. Plants utilize a number of mechanisms to modulate protein level. Regulation of proteolysis by PUB proteins is a key process to plant development and environmental responses. Thus, it is essential to understand this protein degradation system and its involvement in the plant innate immunity against stress conditions. A growing number of E3s have been implicated in the abiotic stress responses, indicating a strong link between the UPS and plant stress tolerance. To date, only a small percentage of plant U-box family proteins have been functionally characterized. Those studied so far are functionally assorted, having signaling, developmental and structural roles in plants as well as in animals. The fact that the expression of many PUB genes is induced by stress and several of these have been identified indicated that there are more yet to be identified PUBs, essential for plant adaptation to abiotic stress. Unfortunately, despite of the huge knowledge obtained about the E3 ligase family at genomic level, our perception for their regulatory role during plant responses to abiotic stress is hindered by the lack of information about downstream target proteins. The positive or negative regulation of stress response depends on the nature of their downstream target proteins. Identification of downstream target proteins for PUB proteins is necessary to establish biological function of these proteins. At present, not much is known about how the activities of ubiquitin ligases are controlled in response to stress conditions.

The emerging picture is that these proteins provide modification and cell type-specificity to developmental signaling pathways. The quest for future research is to investigate upstream and downstream targets to understand the mechanisms by which this fascinating family of proteins performs its roles in plant growth, development and stress management.

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