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REVIEW PAPER

Abiotic stress tolerance mediated by protein ubiquitination

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Abstract

Plant growth and development is largely influenced by ubiquitin-mediated regulation of protein stability. Specificity of the ubiquitination pathway is controlled mainly by the substrate-recruiting E3 ubiquitin ligases, and consequently, E3 ligases control numerous cellular processes. Recent evidence that ubiquitination plays a critical role in regulating plant responses to abiotic stresses has launched intensive efforts to identify E3 ligases that mediate plant tolerance of adverse environmental conditions. Most stress-related E3 ligases identified to date facilitate responses to environmental stimuli by modulating the abundance of key downstream stress-responsive transcription factors. In this review, the regulatory roles of ubiquitin during the plant's response to abiotic stress are summarized and highlighted.

Key words: Abiotic stress, abscisic acid, E3 ligases, ubiquitination, 26S proteasome.

Introduction

Ubiquitination serves as a versatile post-translational modification that mediates growth and development of all eukaryotic species. Ubiquitin is a stable, highly conserved, and universally expressed protein. The covalent attachment of ubiquitin to a lysine residue of select proteins can regulate stability, activity, and trafficking. Genome sequencing has revealed the extent to which plants rely on protein ubiquitination to regulate organismal processes. For example, over 6% of *Arabidopsis thaliana* protein-coding genes are dedicated to the ubiquitin 26S proteasome system (UPS) (Vierstra, 2009). In plant species, the UPS regulates fundamental processes such as embryogenesis, photomorphogenesis, and organ development (Thomann *et al.*, 2005; Sonoda *et al.*, 2009; Pokhilko *et al.*, 2011). In addition to regulating these fundamental processes the UPS has recently emerged as a major player in plant responses to abiotic stresses.

Plants are consistently exposed to unfavourable growth conditions throughout their life cycle. Abiotic stresses such as drought, temperature fluctuations, high salinity, radiation, and nutrient deprivation adversely affect growth, development, and productivity. To ensure survival plants must effectively and efficiently sense, respond, and adapt to their ever-changing environment. Following the perception of environmental stimuli, plants adjust their physiology to mitigate any adverse effects that may result from exposure to abiotic stresses. This is accomplished via signal transduction events leading to changes in gene expression which facilitates various cellular responses. Understanding the molecular basis of abiotic stress perception and signal transduction is of great interest to plant researchers and is an intensely studied area of plant biology. Recent reports in this field have identified ubiquitin conjugation as a major regulator of stress-responsive transcription factors and other regulatory proteins. By modulating the amount and activity of regulatory proteins, ubiquitination plays a central role in regulating the transcriptional changes required for adaption to abiotic stresses. In this review, recent advances made in our understanding of the role the UPS plays during plant responses to various abiotic stresses are discussed.

The ubiquitination enzymes

Ubiquitin is attached to selected proteins through a conjugation cascade consisting of the following three enzymes: the ubiquitin-activating (UBA; E1) enzyme, ubiquitin-conjugating (UBC; E2) enzyme, and ubiquitin ligase (E3) (Fig. 1). Ubiquitin is first activated in an ATP-dependent reaction by the E1. A conserved E1 catalytic cysteine is used

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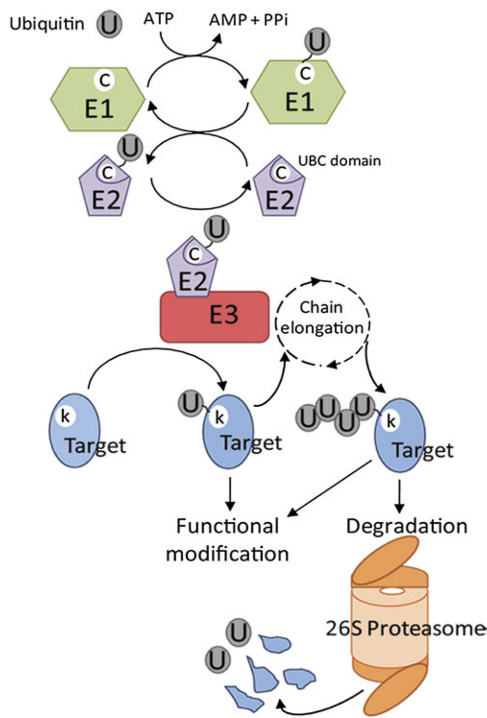

Fig. 1. The ubiquitination pathway. Ubiquitin is activated by the E1 and then transferred to a conserved cysteine residue on the E2 forming an E2-ubiquitin intermediate. Ubiquitin is transferred from the E2-ubiqiutin intermediate to an internal lysine of a target protein bound to the E3 (mono-ubiquitination). Additional ubiquitin molecules can be added to the mono-ubiquitinated target (polyubiquitination).

to form an E1-ubiquitin (E1-Ub) thioester linked intermediate. The E1-Ub interacts with the E2 and the activated ubiquitin is transferred to the active-site cysteine of the E2 forming a thioester linked E2-ubiquitin (E2-Ub) intermediate. Transfer of ubiquitin to the target protein is facilitated by the E3 which interacts with both the E2-Ub and the target protein. There are three major types of E3s: Really Interesting New Gene (RING)-type, Homology to E6-Associated Carboxyl-Terminus (HECT)-type or U-box-type (Fig. 2). The RING-type and U-box-type E3s mediate transfer of ubiquitin directly from the E2-Ub to the target protein. HECT-type E3s are unique in that they form an E3-Ub intermediate prior to the transfer of ubiquitin to the target protein (Scheffner *et al.*, 1995) (Fig. 2). Both mechanisms attach ubiquitin through an isopeotide bond using the carboxyl terminal glycine of ubiquitin and a lysine residue on the target protein.

The attachment of the initial ubiquitin molecule is followed by the assembly of different types of polyubiquitin chains. The first ubiquitin on the target protein acts as an 'acceptor' to which additional molecules are attached during repeated cycles (Fig. 1). Although various models have been proposed, the exact mechanism of chain assembly is not very well understood (Hochstrasser, 2006; Deshaies and Joazeiro, 2009). During chain elongation, ubiquitin molecules may be added sequentially to the growing chain on the target protein or the ubiquitin chain may be pre-assembled upon the E2 and then transferred as a whole to the substrate (Wang and Pickart, 2005; Li *et al.*, 2007; Kim and Huibregtse, 2009; Maspero *et al.*, 2011). Ubiquitin contains seven conserved lysine (Lys) residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) all of which can be used to produce structurally diverse polyubiquitin chains (Kirkpatrick *et al.*, 2006; Kim *et al.*, 2007). The topology of the attached polyubiquitin chain (polyubiquitination) determines the fate of the modified protein. A Lys48 linked ubiquitin chain serves as a signal for degradation by the 26S proteasome. By contrast, Lys63 linked ubiquitin chains have been implicated in endocytosis, protein activation, and intracellular trafficking (Pickart and Fushman, 2004). Another aspect of ubiquitination is the attachment of a single (mono-ubiquitination) or multiple mono-ubiquitins (multi-ubiquitination) to a target protein. These types of modifications serve as a signal for membrane protein internalization, vesicle sorting, DNA repair, and gene silencing (Mukhopadhyay and Riezman, 2007). In the case of RING-type and U-box-type E3s, the E2 enzymes mainly determine the specificity of chain assembly (Kim *et al.*, 2007; Rodrigo-Brenni *et al.*, 2010). However, there are some cases where the E2–E3 combination plays a role in determining the topology of the chain (Kim *et al.,* 2007; Deshaies and Joazeiro, 2009). By contrast, the HECT-type E3 alone determines lysine specificity during ubiquitin chain synthesis (Wang and Pickart, 2005; Maspero *et al.*, 2011).

Eukaryotes usually possess one or two E1s, tens of E2s, and hundreds of E3 ligases. Analysis of the *Arabidopsis* genome identified two E1s, 37 E2s, and over 1300 E3 encoding genes (Kraft *et al.*, 2005; Craig *et al.*, 2009). *Arabidopsis* E1 isoforms, *AtUBA1* and *AtUBA2*, are encoded by two distinct genes. They share 81% amino acid sequence identity, have similar expression patterns, and they have almost identical E2 interaction specificity (Hatfield *et al.*, 1997). E2 enzymes are defined by the presence of a 140 amino acid UBC domain that contains the conserved cysteinyl residue required for accepting the ubiquitin molecule from the E1-Ub (Wu *et al.*, 2003; Kraft *et al.*, 2005) (Fig. 1). The UBC domain also mediates interaction between the E3 and the E2-Ub intermediate (Kalchman *et al.*, 1996; Wu *et al.*, 2003; Kraft *et al.*, 2005).

The RING-type E3s are the most abundant in the predicted *Arabidopsis* proteome followed by the U-box-type and HECT-type E3s. The canonical RING domain is defined by an octet of metal-binding cysteine and histidine residues that co-ordinate two zinc ions in a cross brace globular structure (Freemont, 1993). The spacing between the cysteine and histidine residues is also well conserved. The structure of the RING domain is essential for E2 binding and ubiquitin ligase activity (Lorick *et al.*, 1999). However, the domain does allow for some variability utilizing less conserved amino acids and changes in spacing between key metal binding residues, without loss of E3 ligase activity (Kosarev *et al.*, 2002; Stone *et al.*, 2005). Eleven percent of the predicted *Arabidopsis* RING domain-containing proteins (RING proteins) contain a modified RING domain (Stone *et al.*, 2005). Despite these differences, proteins containing a modified RING domain are capable of mediating ubiquitin conjugation (Stone *et al.*, 2005). Plant genomes contain significantly more RING

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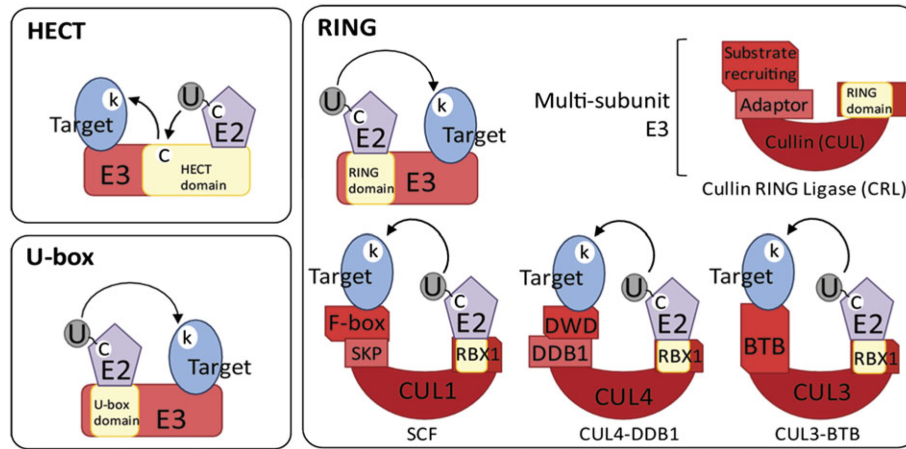

Fig. 2. E3 ubiquitin ligases. E3s are categorized based on the presence of a RING, HECT or U-box E2 binding domain. The RING-type E3s are subdivided into groups depending on whether the E2 and substrate-binding functions are found in the same protein (monomeric E3s) or on different proteins (multi-subunit E3s; CRLs). The multi-subunit CRL uses a CUL protein as a scaffold to interact with the E2 binding RING protein and a substrate-recruiting protein. The CUL3-based CRLs utilize the BTB substrate-recruiting proteins. CUL1- and CUL4-based CRLs use SKP and DDB1 adaptor proteins, respectively, to bind the F-box and DWD substrate-recruiting proteins.

protein encoding genes than that found in other eukaryotes. *Arabidopsis*, rice, and poplar contain 469, 378, and 399 RING-type E3 encoding genes, respectively, compared with 300 human and 47 *Saccharomyces cerevisiae* genes (Kraft *et al.*, 2005; Li *et al.*, 2008; Du *et al.*, 2009). Bioinformatic analysis of the *Arabidopsis* RING proteins identified a number of additional domains including protein–protein interaction, transmembrane, kinase, and DNA and RNA binding domains. Based on the presence and organization of these additional domains, the *Arabidopsis* RING family can be subdivided into 39 distinct groups (Stone *et al.*, 2005).

While the majority of RING proteins are predicted to function as monomeric E3s, RING proteins also participate in multiple subunit Cullin RING E3 ligases (CRLs) (Fig. 2). In the CRL family, functional E3s are composed of four or five different protein subunits. A Cullin (CUL) protein, CUL1, CUL3a/3b or CUL4, act as a scaffold to bring together the E2-Ub binding RING protein RING Box 1 (RBX1/ROC1/HRT1) and the substrate-recruiting protein (Schwechheimer and Villalobos, 2004; Hotton and Callis, 2008) (Fig. 2). The substrate-recruiting subunit can either bind directly to the CUL protein or via an adaptor protein (Fig. 2). CUL1 uses the adaptor protein S-Phase Kinase-associated Protein (SKP or ASK for *Arabidopsis*) to bind to substrate-recruiting F-box proteins. CUL4 uses DNA-damage Binding (DDB1) as an adaptor to bind substrate-recruiting DDB1 binding WD40 (DWD) proteins (Bai *et al.*, 1996; Lechner *et al.*, 2006; Lee *et al.*, 2008). The substrate-recruiting Broad complex Tramtrack Bric-a-Brac (BTB) proteins bind directly to CUL3a/b (Gingerich *et al.*, 2005). Because CRLs can be composed of one of three CULs and one of numerous substrate-recruiting proteins, they are the most diverse and numerous families of E3 ligases. For example, an *Arabidopsis* CUL1-based Skp-Cullin-F-box (SCF)-type E3 complex may be assembled using any of 700 substrate-recruiting F-box proteins (Fig. 2) (Lechner *et al.*, 2006). The CUL3 scaffold can probably associate with the 80 predicted BTB proteins and CUL4-DDB1 can potentially interact with 85 predicted DWD proteins (Gingerich *et al.*, 2005; Lee *et al.*, 2008).

The U-box domain forms a scaffold structure similar to the RING domain. However, the U-box structure is stabilized via salt bridges and hydrogen bonds instead of metal binding residues (Aravind and Koonin, 2000). Compared with other eukaryotic species there are significantly more U-box protein-encoding genes in plant genomes. The *Arabidopsis* and rice genome contains 64 and 77 U-box-type E3 encoding genes, respectively, compared with eight human and two *Saccharomyces cerevisiae* genes (Li *et al.*, 2008; Yee and Goring, 2009). The 64 members of the *Arabidopsis* plant U-box (PUB) E3 family can be placed into 13 groups based on the presence or organization of additional domains (Azevedo *et al.*, 2001; Mudgil *et al.*, 2004; Wiborg *et al.*, 2008; Yee and Goring, 2009). The vast majority of PUB proteins (64%) contain armadillo repeats as a potential substrate-binding domain. This is in contrast to the RING proteins that contain a variety of protein–protein interaction domains including, ankyrin repeats, WD40, BRCT, and VWA (Stone *et al.*, 2005). In addition, only a single RING domain-containing protein contains a kinase domain compared with 23% of PUB proteins (Stone *et al.*, 2005, 2006; Wiborg *et al.*, 2008; Yee and Goring, 2009).

HECT-type E3s proteins are distinguished by the presence of a conserved catalytic HECT domain that contains the invariant cysteinyl residue used to form the E3-Ub intermediate (Fig. 2) (Huibregtse *et al.*, 1995). Among E3s, the HECT-type family is usually the smallest across all plant species with only seven and eight members found in the *Arabidopsis* and rice genomes, respectively (Downes *et al.*, 2003).

The UPS is essential for plant response to abiotic stresses

The UPS functions within the cytoplasm and nucleus to modulate the levels of regulatory proteins and to remove

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misfolded or damaged proteins that may accumulate as a result of exposure to abiotic stress. One of the first indications that the UPS was involved in regulating plant stress tolerance was the observation that expression of polyubiquitin genes is stress-regulated (Christensen *et al.*, 1992; Genschik *et al.*, 1992; Sun and Callis, 1997). Ubiquitin is encoded by multiple polyubiquitin genes (*UBQ3*, *UBQ4*, *UBQIO*, *UBQ11*, and *UBQ14*) that contain 3–6 ubiquitin-coding regions in tandem (Callis *et al.*, 1995). Following translation, nascent polyubiquitin proteins are proteolytically processed into ubiquitin monomers (Vierstra, 1996). The pool of free ubiquitin molecules is regulated through differential expression of the polyubiquitin genes (Christensen *et al.*, 1992; Genschik *et al.*, 1992; Sun and Callis, 1997). Specifically, transcript abundance of *Arabidopsis UBQ14* is increased during heat stress (Sun and Callis, 1997). Similarly, high temperatures also induce the expression of multiple polyubiquitin genes in tobacco, potato, and maize (Christensen *et al.*, 1992; Garbarino *et al.*, 1992; Genschik *et al.*, 1992). In fact, over-expression of a single mono-ubiquitin gene enhances tolerance to multiple stresses without adversely affecting growth and development under favourable conditions (Guo *et al.*, 2008). Transgenic tobacco over-expressing a wheat polyubiquitin gene, containing a single ubiquitin repeat, were more tolerant of cold, high salinity, and drought conditions compared with control plants. The stress-induced expression of polyubiquitin genes is consistent with the role of the UPS in turning over damaged proteins to mitigate the negative effects of environmental stress.

Defects in 26S proteasome function also alter plant tolerance to various environmental stresses. The 26S proteasome is an ATP-dependent protease complex consisting of a proteolytic 20S complex capped on one or both ends by a 19S regulatory particle (RP). Access to the active sites of the 20S complex is regulated by the RP that mediates substrate recruiting, unfolding, translocation into the proteolytic chamber of the 20S, and recycling of ubiquitin molecules (Strickland *et al.*, 2000; Navon and Goldberg, 2001). The RP is composed of two subcomplexes referred to as the Base and the Lid. The Base sits directly on the 20S and contains six AAA-ATPases (RPTs) and three non-ATPase (RPNs) subunits. The Lid subcomplex contains an additional eight RPNs (Fu *et al.*, 2001). Mutations of RP subunits that affect 26S proteasome function can decrease complex accumulation, reduce the rate of ubiquitin-dependent proteolysis, and also alter plant response to abiotic stresses (Smalle *et al.*, 2003; Smalle and Vierstra, 2004; Ueda *et al.*, 2004; Kurepa *et al.*, 2008). *Arabidopsis rpn10-1*, *rpn1a-4*, and *rpn1a-5* plants are less tolerant of salt stress (Smalle *et al.*, 2003; Wang *et al.*, 2009). *rpn10-1* plants are also hypersensitive to UV radiation and DNA damaging agents (Smalle *et al.*, 2003). *rpn1a-4*, *rpn1a-5, rpn10-1*, *rpn12a-1*, and *rpt2a-2* all exhibit decreased heat shock tolerance (Kurepa *et al.*, 2008; Wang *et al.*, 2009). The sensitivity of RP mutants to various abiotic stresses suggest that the 26S proteasome plays a crucial and general role during plant responses to adverse growth conditions.

With the identification of a growing number of E3 ligases that regulate abiotic stress responses, the mechanisms of E3 mode of action during stress signalling is becoming more defined (Fig. 3; Table 1). E3 ligases may function by suppressing the stress signalling pathway during favourable growth conditions, by eliminating negative regulators of the stress signalling pathway in response to a stimulus, or by attenuating the signalling pathway to allow for further growth once conditions have improved (Fig. 3). E3 ligases may also function within a positive feedback loop to enhance stress signalling (Fig. 3). Not much is known about how abiotic stress signalling regulates the activity of these E3 ligases. In some cases, the expression and cellular localization of E3 ligases is stress-regulated (Ko *et al.*, 2006; Zhang *et al.*, 2007; Molinier *et al.*, 2008).

Known targets of the E3 ligases include many transcriptional regulators (Table 1). A typical example are the *Arabidopsis* DELLA proteins that repress gibberellin (GA)

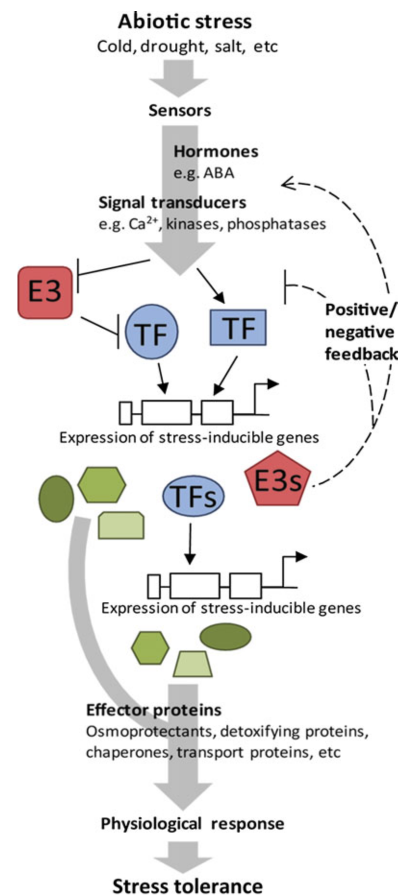

Fig. 3. Regulation of abiotic stress signalling by E3 ligases. Plant perceive stress signal via sensors (unknown) and the signal is transduced via plant hormones, secondary messengers, and transcriptional regulators. The expression of stress-inducible genes is facilitated by transcription factors (TF) many of which are stress-regulated. E3 ligases tend to regulate components of the signalling pathway, mainly stress-responsive TFs. In the absence of a stress signal, E3 ligases may suppress the signalling pathway by, for example, promoting the degradation of a TF. E3 ligases may function within a feedback mechanism to enhance or attenuate the stress signal.

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Table 1. Ubiquitination enzymes with known or predicted roles in ABA-dependent or independent stress tolerance

Enzyme	Name	Species[a]	Biological function	Targets	References
E3					
RING	AIP2	*At*	ABA signalling	ABI3	Zhang *et al.*, 2005
	AIRP1	*At*	ABA-dependent drought stress tolerance		Ryu *et al.*, 2010
	BIRF1	*Os*	Drought and oxidative stress tolerance		Liu *et al.*, 2008
	BTS	*At*	Iron deficiency response	ILR-3?	Long *et al.*, 2010
	ATL31	*At*	Carbon and nitrogen stress		Sato *et al.*, 2009
	COP1	*At*	Possible regulation of ABA signalling via HY5	HY5, HFR1, BIT1, LAF1	Chen *et al.*, 2008, Duek *et al.*, 2004, Hong *et al,* 2008, Seo *et al.*, 2003
	DRIP1/2	*At*	Drought stress tolerance	DREB2A	Qin *et al.*, 2008
	DSG1	*Os*	ABA signalling	ABI3	Park *et al.*, 2010
	HOS1	*At*	Cold stress tolerance	ICE1	Dong *et al.*, 2006
	KEG	*At*	ABA signalling	ABI5	Stone *et al.*, 2006
	NLA	*At*	Nitrogen deficiency stress		Peng *et al.*, 2007
	RFP1	*Ca*	Osmotic stress tolerance		Hong *et al.*, 2007
	RFP1	*Gm*	Cold, salinity and drought tolerance		Du *et al.*, 2009
	RHA2a	*At*	ABA signalling		Bu *et al.*, 2009
	RING-1	*Os*	Drought and heat stress tolerance		Meng *et al.*, 2006
	Rma1	*At*	Drought stress tolerance	PIP2;1	Lee *et al.*, 2009
	Rma1H1	*Ca*	Drought stress tolerance	PIP2;1	Lee *et al.*, 2009
	SAP5	*At*	Drought and salinity stress tolerance		Kang *et al.*, 2011
	SDIR1	*At*	Drought and salinity stress tolerance, ABA signalling		Zhang *et al.*, 2007
	XERICO	*At*	Drought stress tolerance, ABA biosynthesis		Ko *et al.*, 2006
	ZF1	*Zm*	Drought and salinity stress tolerance		Huai *et al.*, 2009
	ZFP1	*Ad*	Drought stress tolerance		Yang *et al.*, 2008
CRL	DDB1	*At*	UV radiation tolerance	DDB2	Molinier *et al.*, 2008; Castells *et al.*, 2011
	FBP7	*At*	Cold temperature tolerance		Calderón-Villalobos *et al.*, 2007
	DWA1/2	*At*	ABA signalling	ABI5	Lee *et al.*, 2010
	DOR	*At*	Drought stress tolerance		Zhang *et al.*, 2008
U-box	CHIP	*At*	Temperature fluctuation tolerance	PP2A,ClpP4, FtsH	Luo *et al.*, 2006 Shen *et al.*, 2007*a* Shen *et al.*, 2007*b*
	PUB1	*Ca*	Drought and salinity stress tolerance	RPN6	Cho *et al.*, 2006
	PUB9	*At*	ABA signalling		Samuel *et al.*, 2008
	PUB15	*Os*	Oxidative stress tolerance		Park *et al.*, 2011
	PUB22/23	*At*	Drought and salinity stress tolerance	RPN12a	Cho *et al.*, 2008
E2	UBC2	*Ah*	Drought stress tolerance		Wan *et al.*, 2010
	UBC2	*Gm*	Drought and salinity stress tolerance		Zhou *et al.*, 2010
	UBC13	*At*	Iron deficiency response		Li and Schmidt, 2010

[a] Species: *Ad, Artemisia desertorum; Ah, Arachis hypogaea* (peanut); *At, Arabidopsis thaliana; Ca, Capsicum annuum* (hot pepper); *Gm, Glycine max* (soybean); *Os, Oryza sativa* (rice); *Zm, Zea mays* (maize).

responses in the absence of the growth hormone. In the presence of bioactive GA, DELLA proteins are targeted for proteasomal degradation by the $SCF^{SLY/GID2}$ E3 ligase complex (Dill *et al.*, 2004). The level of bioactive GA is regulated by environmental conditions, suggesting that plants may utilize GA signalling to modulate DELLA protein stability and growth in response to abiotic stresses (Yamauchi *et al.*, 2004; Achard *et al.*, 2006). Other potential targets of E3 ligases may include stress hormone biosynthesis enzymes and effector proteins that mediate tolerance of abiotic stresses. Proteome analysis of the UPS system from *Arabidopsis* identified a number of different types of stress related proteins (Manzano *et al.*, 2008; Igawa *et al.*, 2009). In fact, the majority of ubiquitinated proteins that were isolated in each study were functionally categorized as stress response or abiotic stress proteins. The fact that plants place such an emphasis on the UPS to facilitate abiotic stress response is not surprising. The UPS allows for rapid and efficient responses to abiotic stresses by regulating stress hormone biosynthesis and the abundance of regulatory proteins.

Regulation of abscisic acid-dependent stress signalling requires multiple E3s

The phytohormone abscisic acid (ABA) functions during adaptive response to environmental stresses. ABA regulates

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seed maturation and prolongs dormancy to ensure that seeds germinate under conditions favourable to growth and development. Immediately following germination, ABA suspends the growth of young seedlings exposed to abiotic stresses such as salinity or drought. Seedling development is slowed until better environmental conditions arise. In adult plants, ABA mediates various protective responses that help to alleviate stress-induced damage (Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003). A well-studied ABA-mediated event is the regulation of stomatal closure in response to drought stress. During times of water scarcity ABA prevents transpirational water loss by promoting stomatal closure (Hetherington, 2001).

Perception of environmental stimuli leads to increased biosynthesis and accumulation of ABA (Cutler and Krochko, 1999; Taylor *et al.*, 2000). ABA triggers intracellular signalling which culminates in the expression of ABA-responsive genes. Transcriptional analyses of ABA-responsive genes identified hundreds of genes that are either up- or down-regulated in response to ABA (Hoth *et al.*, 2002; Seki *et al.*, 2002). Changes in gene expression generated by drought and high salinity are mediated by ABA-responsive transcription factors such as the basic leucine zipper (bZIP) transcriptional activators. These transcriptional activators interact with the ABA-regulatory elements (ABRE) found in the promoter of stress-responsive genes (Hattori *et al.*, 2002; Narusaka *et al.*, 2003). The UPS regulates ABA-responsive transcription by modulating the abundance of these transcription factors.

The observation that ABA promotes the accumulation of the short-lived bZIP transcription factor Abscisic Acid Insensitive 5 (ABI5) provided evidence for UPS involvement in regulating ABA signalling (Uno *et al.*, 2000; Lopez-Molina *et al.*, 2003; Smalle *et al.*, 2003). Ubiquitinated ABI5 accumulates in seedlings treated with proteasome inhibitors and ABI5 is stabilized in *rpn10-1* (Lopez-Molina *et al.*, 2003; Smalle *et al.*, 2003). The ABA-dependent stabilization of ABI5 is proposed to serve as an early developmental checkpoint to delay growth during adverse environmental conditions (Lopez-Molina *et al.*, 2001). This proposal is based on the fact that ABA is able to induce ABI5 protein accumulation and seedling growth arrest only within a short period of time following germination (Lopez-Molina *et al.*, 2001). In addition, ABI5 protein accumulation is also induced by salt and drought stress. These observations also support the notion that under favourable growth conditions the UPS is required to maintain low levels of ABI5 and thus permits growth.

Significant strides have been made in understanding the role of ubiquitination in regulating ABI5 function. E3 ligases Keep on Going (KEG), DWD hypersensitive to ABA 1 (DWA1) and DWA2 have been implicated in modulating ABI5 protein abundance (Fig. 4) (Stone *et al.*,

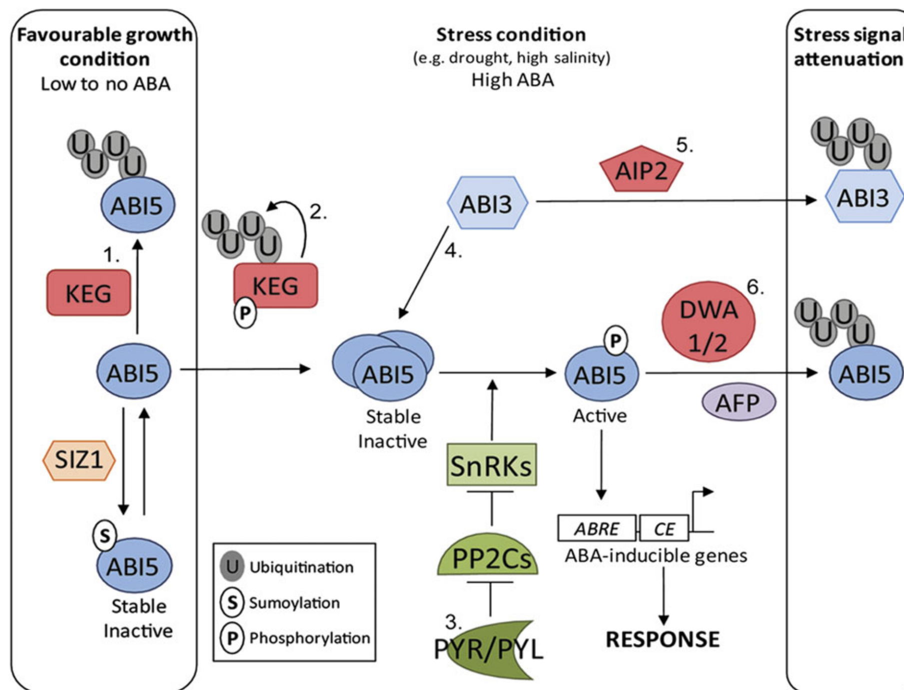

Fig. 4. Ubiquitin-mediated regulation of the ABA signalling. Under normal growth conditions (in the absence of stress) KEG is involved in preventing ABI5 accumulation while SIZ1 sumoylation maintains a small pool of inactive ABI5 (1). Under stressed conditions ABA levels increase. ABA promotes the self-ubiquitination and degradation of KEG. Reduction in KEG protein levels assist in the accumulation of ABI5 protein levels (2). ABA binds to its receptor (PYR/PYL/RCAR) which inactivates PP2C resulting in SnRK activation and phosphorylation of ABA-responsive transcription factors such as ABI5 (3). Activated ABI5 promotes the expression of ABA-inducible genes which mediate various responses including post-germinative growth arrest. ABI3 function upstream of ABI5 (4). ABA induces expression of AIP2 which promote the degradation of ABI3 (5). ABI5 is turned over via ubiquitination by DWA1/2 (6). AFP may also be required for the degradation of ABI5. The ubiquitin-mediated degradation of ABI3 and ABI5 attenuate the ABA signal.

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2006; Liu and Stone, 2010; Lee *et al.*, 2010). KEG is a large multi-domain protein that contains functional RING and kinase domains followed by a series of ankyrin and HERC2-like repeats that facilitate protein–protein interactions (Stone *et al.*, 2006; Gu and Innes, 2011). KEG is a negative regulator of ABA signalling and is required for maintaining low levels of ABI5 in the absence of ABA (Stone *et al.*, 2006; Liu and Stone, 2010). *KEG* mutants (*keg-1/2/3*) are hypersensitive to ABA, accumulate extremely high levels of ABI5 and undergo growth arrest shortly after germination. KEG mediates ABI5 ubiquitination *in vitro* and the reduction of ABI5 protein levels in *keg* mutants is dependent on the presence of a functional KEG RING domain (Liu and Stone, 2010). The fact that ABI5 accumulates in *keg* seedlings without ABA treatment suggests that KEG targets ABI5 for degradation to suppress ABI5-dependent post-germinative growth arrest in the absence of the hormone.

The mechanism of ABA-dependent stabilization of ABI5 is not very well understood. However, a recent study by Liu and Stone (2010) has proposed a possible mechanism. As observed with many E3 ligases, KEG is capable of autoubiquitination (Stone *et al.*, 2006). The autocatalytic process can serve as a negative regulatory mechanism leading to the down-regulation of the E3 via degradation by the 26S proteasome (Fang *et al.*, 2000). ABA may manipulate this intrinsic ability of KEG to promote autoubiquitination and subsequent degradation. In the presence of ABA the turnover of KEG protein increases significantly (Liu and Stone, 2010). Mutations in KEG's RING domain and inhibition of 26S proteasome activity prohibit ABA-induced degradation of KEG (Liu and Stone, 2010). These results suggest that ABA promotes the accumulation of ABI5 by reducing KEG protein levels via self-ubiquitination and degradation by the 26S proteasome (Fig. 4). The mechanism wherein ABA directs KEG towards self-ubiquitination over substrate ubiquitination remains to be determined. Mutations within KEG's kinase domain or treatments with kinase inhibitors also inhibit ABA-induced ubiquitination and degradation of KEG suggesting that phosphorylation may be involved in this process. Phosphorylation has been shown to regulate E3 ligase activity via modification of the substrate or the E3 ligase itself. In some cases, phosphorylation of E3 ligases, such as Parkin, promotes enzyme activation (Sha *et al.*, 2010). In contrast, phosphorylation of other E3s, such as Mdm2, leads to down-regulation of the E3 and substrate accumulation (Cheng *et al.*, 2009).

DWA1 and DWA2 both function as the substrate recruiting component of a CUL4 based CRL (Lee *et al.*, 2008). DWA1 and DWA2 are also responsible for targeting ABI5 for proteasome-dependent degradation and may do so to attenuate the stress signalling pathway (Fig. 4). ABI5 is more stable in ABA treated *dwa1 dwa2* seedlings compared with the wild type and *DWA1 DWA2* mutants display hypersensitivity to ABA. This is consistent with the model of DWA1 and DWA2 acting as negative regulators of ABA signalling (Lee *et al.*, 2010). Interestingly, ABI5 does not accumulate in *dwa1 dwa2* in the absence of ABA. This is in contrast to *keg* mutants which accumulates extremely high levels of ABI5 without the application of ABA. KEG may function to maintain low levels of ABI5 in the absence of ABA and abiotic stress, while DWA1 and DWA2 may function to attenuate ABA signalling so that plants can readily re-establish growth once environmental conditions improve (Fig. 4).

Adding to the complexity of the ubiquitin-mediated regulation of ABI5 is the ABI5 binding protein (AFP). Upon ABA treatment, an increase in AFP protein levels closely follows that of ABI5 (Lopez-Molina *et al.*, 2003). Co-expression of AFP with ABI5 promotes the localization of both proteins to nuclear bodies. Even though AFP is not an E3 ligase it has been proposed to promote the proteasomal degradation of ABI5 (Lopez-Molina *et al.*, 2003). DWA1 and DWA2 interact with each other in the nucleus, although not in nuclear bodies (Lee *et al.*, 2010). It is possible that AFP may facilitate DWA1/2-mediated degradation of ABI5 (Fig. 4). Recently, the relationship between ABI5 and AFP has been proposed to be at the level of transcription. The AFP family of proteins (AFP1–4) are similar in domain organization to the adaptor protein Novel Interactor of JAZ (NINJA) that represses expression of jasmonoyl-isoleucine responsive genes by facilitating interactions between jasmonate ZIM-domain (JAZ) repressor proteins and the co-repressor TOPLESS (TPL) (Pauwels *et al.*, 2010). Similarly, AFP interacts with TPL and may function to recruit TPL to ABI5 and generate a transcriptional complex that represses the expression of ABA-responsive genes (Pauwels *et al.*, 2010).

Other ABA-responsive transcription factors are also regulated by the UPS. ABI3, a B3-type transcription factor, functions upstream of ABI5 to mediate ABA-dependent processes (Finkelstein and Lynch, 2000; Lopez-Molina *et al.*, 2002). ABI3 protein is unstable in most stages of plant development but does accumulate during specific developmental windows (Lopez-Molina *et al.*, 2001, 2002; Zhang *et al.*, 2005). The RING-type E3 ABI3-Interacting Protein 2 (AIP2) is required for the ubiquitin-mediated degradation of ABI3 (Fig. 4). Zhang *et al.*, (2005) demonstrated that ABA promotes the expression of AIP2 which results in a reduction in ABI3 protein levels. Similar to DWA1/2, AIP2-mediated degradation of ABI3 may function to attenuate ABA signalling.

Although there is no direct evidence of ubiquitination, studies suggests that ABA-responsive transcription factors ABI4, ABRE Binding Factor 2 (ABF2) and ABF3 are also regulated by the UPS. ABI4 has long been known to regulate ABA signalling (Finkelstein, 1994). However, evidence that ABI4 may be regulated by the UPS has only recently emerged. The abundance of ABI4 was observed by examining the activity of the *β-glucuronidase* (*GUS*) reporter in plants over-expressing a *ABI4–GUS* transgene. The activity of GUS (and therefore ABI4 protein level) increased after treatment with proteasome inhibitors (Finkelstein *et al.*, 2011).

Arm Protein Repeat Interacting with ABF2 (ARIA), a BTB protein which may function as a component of a CRL, interacts with ABF2 and both share a similar gene expression pattern (Kim *et al.*, 2004). Consistent with the hypothesis that ARIA regulates ABF2 and, therefore, ABA responses, *ARIA* over-expressing plants displayed

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hypersensitivity to ABA and *ARIA* mutants are insensitive to ABA (Kim *et al.*, 2004).

The ABF3 protein levels are stabilized by the application of exogenous ABA or the inhibition of proteasome activity. Phosphorylation of ABF3 by Open Stomata 1 (OST1) is involved in the ABA-mediated stabilization of ABF3 (Sirichandra *et al.*, 2010). OST1 is a member of the Suc non-fermenting1-related protein kinase subfamily 2 (SnRK2) (Yoshida *et al.*, 2002). SnRK2s along with the ABA receptor family, Pyrabactin resistance 1(PYR1)/PYR1-like (PYL)/ Regulatory component of ABA receptor (RCAR), and clade A Protein Phosphatase type 2Cs (PP2Cs) represent the core regulatory network of the ABA signalling pathway (Weiner *et al.*, 2010) (Fig. 4). Under non-stress conditions SnRK2s are inhibited by PPC2 driven dephosphorylation. An increase in ABA levels result in ABA-bound PYR/PYL/RCAR receptors binding to and inhibiting the activity of PP2Cs leading to the activation of SnRK2s (Weiner *et al.*, 2010) (Fig. 4). The ABA-activated SnRK2s phosphorylate transcription factors and possibly other regulatory proteins that regulate the expression of ABA-responsive genes. In response to ABA, OST1 phosphorylates ABF3 within a 14-3-3 protein binding motif found in most ABF proteins (Sirichandra *et al.*, 2010). Mutant ABF3 protein lacking the 14-3-3 phosphorylation site is only detected after plants are treated with proteasome inhibitors (Sirichandra *et al.*, 2010). This study suggests that phosphorylation by the ABA-activated SnRK2 is required for stabilization of ABF3 and this may be accomplished via binding of a 14-3-3 protein. More importantly, this study demonstrates that the role of ABA-activated kinases is not limited to the activation of transcription factors, but they may also be required for stability.

In addition to the above mentioned ubiquitin ligases there is a growing list of E3s that function in response to ABA but targets remain to be identified (Table 1). These E3 ligases were isolated via efforts to identify stress-responsive genes. Some E3 ligases have received attention because their mRNA transcript abundance is regulated by stress and/or ABA. Other E3s have surfaced in screens for mutants with aberrant ABA-related phenotypes. Examples of E3 ligases in these categories are Salt and Drought Induced RING Finger 1 (SDIR1), *Arabidopsis thaliana* ABA-insensitive RING protein 1(AtAIRP), RING-H2 E3 ligase (RHA) 2a, RHA2b, Drought tolerance repressor (DOR), and XERICO.

RING-type E3 ligases SDIR1, AtAIRP1, RHA2a, and RHA2b are positive regulators of ABA signalling. Plants lacking these RING-type E3 encoding genes are insensitive to ABA while transgenic over-expressing plants are hypersensitive to the effects of ABA (Zhang *et al.*, 2007; Bu *et al.*, 2009; Ryu *et al.*, 2010; Li *et al.*, 2011). Over-expression of *SDIR1*, *AtAIRP1*, or *RHA2b* enhances drought tolerance via an increase in ABA-induced stomatal closure (Zhang *et al.*, 2007; Ryu *et al.*, 2010; Li *et al.*, 2011). SDIR1 is a salt and drought stress-regulated membrane bound protein that functions upstream of ABA-responsive transcription factors (Zhang *et al.*, 2007). Expression of *AtAIRP* is induced by ABA, cold, salt, and drought stresses (Ryu *et al.*, 2010). Interestingly, ABA-induced expression of *AtAIRP* does not occur in the SnRK2 triple mutant, *srk2d/snrk2.2 srk2e/snrk2.6/ ost1 srk2i/snrk2.3*, suggesting that expression of *AtAIRP* in response to stress is regulated via the ABA-activated protein kinases (Fujita *et al.*, 2009; Ryu *et al.*, 2010). AtAIRP is also suggested to act upstream of ABA-responsive transcription factors (Ryu *et al.*, 2010). RHA2a and RHA2b function redundantly and parallel to ABA-responsive transcription factors such as ABI3 and ABI5 (Bu *et al.*, 2009; Li *et al.*, 2011).

The F-box protein DOR functions as a negative regulator of ABA-mediated stomata closure (Zhang *et al.*, 2008). *DOR* mutant plants display enhanced drought tolerance and accumulate higher levels of ABA than the wild type in response to drought. DOR can associate with, two CRL subunits, ASK14 and CUL1. Transcriptome analysis of *dor* plants under drought stress revealed that a variety of ABA biosynthesis genes and ABA-responsive genes were up-regulated compared with wild type. In particular, a key enzyme in ABA biosynthesis, *9-cis-epoxycarotenoid dioxygenase 3* (NCED3), is significantly up-regulated in the *DOR* mutants. RING-type E3 XERICO is another ubiquitin ligase with links to ABA biosynthesis. Over-expression of *XERICO* resulted in drought-tolerant plants that were hypersensitive to ABA and accumulated more cellular ABA than wild type (Ko *et al.*, 2006). Interestingly, the accumulation of ABA in *XERICO* over-expressing plants occurs without a concomitant increase in the expression of ABA biosynthetic genes such as *NCED3* (Ko *et al.*, 2006). In addition, compared with wild type, a stronger, more sustained, expression of *NCED3* was observed in *XERICO* over-expressing plants following ABA treatment. This suggests that XERICO acts post-translationally to regulate ABA biosynthesis.

E3 ligase function during drought and salt stress

The abundance of the drought-responsive transcription factor Dehydration-responsive Element Binding Protein 2A (DREB2A) is regulated by two RING-type E3 ligases, DREB2A Interacting Protein 1 (DRIP1) and DRIP2 (Qin *et al.*, 2008) (Fig. 5). DREB2A is usually unstable but accumulates during dehydration stress suggesting regulation by the UPS (Sakuma *et al.*, 2006*a*, *b*). DRIP1 mediates DREB2A ubiquitination *in vitro* and DREB2A protein levels are more stable in *drip1-1* plants compared with wild-type (Qin *et al.*, 2008). In addition, DREB2A accumulates upon inhibition of the 26S proteasome activity. *DRIP1 DRIP2* double mutants displayed enhanced drought tolerance which coincided with a significant increase in the expression of a number of drought-inducible genes specifically genes regulated by DREB2A (Qin *et al.*, 2008). In the absence of stress stimuli, DRIP1 and DRIP2 function redundantly to suppress drought signalling via the ubiquitin-mediated proteolysis of DREB2A.

DREB2A instability is due to a serine and threonine-rich 30–amino acid negative regulatory domain (Sakuma *et al.*, 2006*a*, *b*). Deletion of the negative regulatory domain stabilized DRBE2A indicating the presence of a degron. A degron is an amino acid sequence that serves as a signal for

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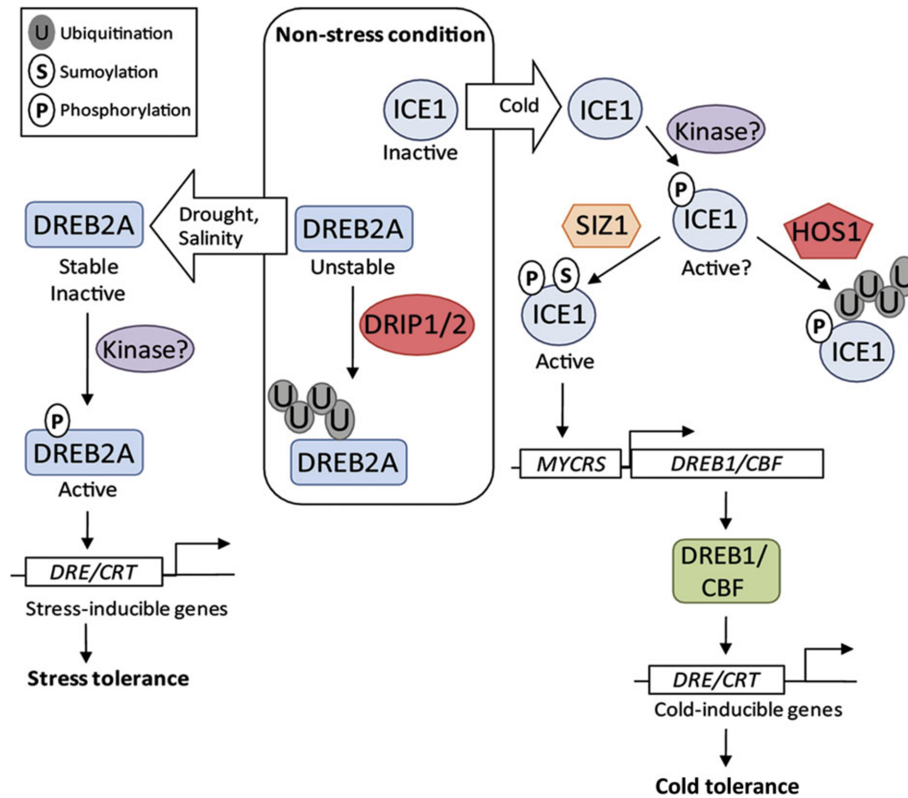

Fig. 5. Ubiquitin-mediated regulation of ABA-independent responses to drought, high salinity, and cold stresses. Under normal growth conditions, the DREB2A transcription factor is unstable due to DRIP1/2-mediated ubiquitination. During drought and salinity stress, DREB2A is stabilized, activated via phosphorylation, and initiates transcription of stress-inducible genes. Expression of cold-responsive genes is mediated by the ICE transcription factor. Activation of ICE under cold conditions requires phosphorylation and SIZ1-mediated sumolyation. Under cold conditions ICE1 is targeted for degradation via HOS1-mediated ubiquitination. Ubiquitin-mediated degradation of ICE1 may serve to attenuate the signal to ensure the transient expression of cold-responsive genes.

ubiquitin-mediated degradation (Varshavsky, 1991). The DREB2A degron may facilitate degradation of DREB2A under favourable growth conditions while it would be made unavailable to the degradation machinery under stress conditions. DREB2A protein would then accumulate and regulate the expression of stress-responsive genes. Another interesting untested possibility is that since stress conditions do not affect DRIP1/2 transcript levels, DREB2A accumulation may occur as a result of drought-induced relocalization of DRIP1/2.

The RING membrane-anchor 1 homologue 1 (Rma1H1) was originally identified as a dehydration-regulated gene in *Capsicum annuum* (hot pepper; Park *et al.*, 2003). Correspondingly, over-expression of *Rma1H1* in *Arabidopsis* enhanced drought tolerance (Lee *et al.*, 2009). A potential target for Rma1H1 is the plasma membrane aquaporin PIP2;1. Aquaporins have been suggested to enhance symplastic water transport which has a negative impact on plants during water stress (Jang *et al.*, 2004; Alexandersson *et al.*, 2005). In protoplasts co-transformed with *PIP2;1* and *Rma1H1* the protein level of PIP2;1 was lower than when PIP2;1 was transformed alone. The reduction of PIP2;1 protein levels could be blocked by treatment with proteasome inhibitors suggesting Rma1H1-mediated degradation of PIP2;1 via the 26S proteasome (Lee *et al.*, 2009). *Rma1H1* has three *Arabidopsis* homologues, *Rma1*, *Rma2*, and *Rma3* (Lee *et al.*, 2009). One striking difference between hot pepper Rma1H1 and its *Arabidopsis* counterparts is that while Rma1H1 was relatively stable in transgenic plants Rma1 is only detectable after inhibition of the 26S proteasome, suggesting that Rma1 is itself regulated by the UPS (Lee *et al.*, 2009). Similar to Rma1H1, *Rma1* over-expression reduced PIP2;1 levels in co-transfected protoplasts. Lee *et al.* (2009) propose a model in which Rma1H1 and Rma1 promote dehydration tolerance by mediating the degradation of aquaporins that may promote symplastic water transport.

Another hot pepper drought stress-inducible E3 encoding gene is *CaPUB1*. Unlike Rma1H1, over-expression of *CaPUB1* renders transgenic *Arabidopsis* plants more sensitive to salt and drought stress (Cho *et al.*, 2006). Comparison of the protein profiles of wild type to *CaPUB1* over-expressing plants identified RPN6 as a potential substrate for the U-box-type E3. Subsequent experiments revealed that CaPUB1 was able to interact with and ubiquitinate RPN6. The significance of RPN6 ubiquitination by CaPUB1 is still unclear. One proposal is that ubiquitin-dependent regulation of RPN6 may regulate the activity of the 26S proteasome during drought stress response (Kurepa *et al.*, 2009). *Arabidopsis* PUB22 and PUB23 are homologues of CaPUB1 (Cho *et al.*, 2008). Similar to CaPUB1, over-expression of *PUB22* or *PUB23* rendered transgenic plants more sensitive

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to drought and salt stress, while *pub22 pub23* were very tolerant of drought and salt stress. PUB22 and PUB23 interact with and ubiquitinate RPN12a (Cho *et al.*, 2008). The importance of this interaction is unknown but it is possible that, similar to the ubiquitination of RPN6 by CaPUB1, ubiquitination of RPN12a may influence the properties of the 26S proteasome (Kurepa *et al.*, 2009). In *PUB22* or *PUB23* over-expressing transgenic plants RPN12a associates with a wide range of protein complexes (200 kDa to 900 kDa) (Cho *et al.*, 2008). In wild-type plants, RPN12a is only found within a specific protein complex (800 kDa to 900 kDa) that is consistent with the size of the RP. Interestingly, in drought-stressed plants, RPN12a associate with complexes that are similar in size to those of the *PUB22* or *PUB23* over-expressing plants (Cho *et al.*, 2008). During drought stress or increased expression of PUB22 or PUB23, the subunit composition of the PR seems to change and this may somehow influence the activity of the 26S proteasome.

Response to temperature fluctuations is mediated by ubiquitination

Inducer of CBF Expression 1 (ICE1), a MYC transcription factor, controls the expression of cold-responsive transcription factor CBF3/DREB1A, that regulates the transcription of numerous cold-responsive genes (Fig. 5). The expression of *ICE1*, which is normally constitutive, is up-regulated in response to cold temperatures. Over-expression of *ICE* leads to increased expression of its target genes but only under cold conditions (Chinnusamy *et al.*, 2003). This implies that cold signalling not only increases *ICE* expression but also activates the protein (Fig. 5). Another consequence of cold exposure is the reduction in ICE protein levels. The cold-mediated decrease in ICE abundance can be blocked by proteasome inhibitors which implicates the UPS (Dong *et al.*, 2006). Direct regulation of cold signalling by the UPS was confirmed by the identification of RING-type E3 High Expression of Osmotically Responsive Gene 1 (HOS1) as a mediator of ICE1 ubiquitination and subsequent degradation (Fig. 5). Consistent with a role in regulating ICE protein abundance *HOS1* over-expression results in reduced tolerance of freezing conditions as well as a decrease in the expression levels of ICE1 target genes. (Xiong *et al.*, 2002; Dong *et al.*, 2006). Although HOS1 contains a variant RING domain, it is capable of catalysing ICE1 ubiquitination *in vitro* and *in vivo* (Lee *et al.*, 2001; Stone *et al.*, 2005; Dong *et al.*, 2006). Degradation of nuclear localized ICE1 is facilitated by cold-induced relocalization of HOS1 from the cytoplasm to the nucleus (Lee *et al.*, 2001; Dong *et al.*, 2006). The HOS1-mediated degradation of ICE1 in response to cold may seem contradictory at first but cold-responsive genes are only transiently induced by cold treatment (Chinnusamy *et al.*, 2003).

Phosphorylation provides another level of regulation of ICE1 activity. Mutation of a potential phosphorylation site, Serine 403, increased transactivational activity, prohibited cold-induced degradation of ICE1 and enhanced freezing tolerance (Miura *et al.*, 2011). Stabilization of the ICE1 mutant against cold-induced degradation is due to inhibition of polyubiquitination (Miura *et al.*, 2011). Surprisingly, the mutation does not hinder HOS1-mediated ubiquitination of ICE1 *in vitro*. Phosphorylation seems to indirectly affect ICE1 stability possibly through another post-translational mechanism that modulates UPS regulation of ICE1. In any event, phosphorylation of ICE1 is involved in regulating protein activation and stabilization (Fig. 5).

Arabidopsis thaliana Carboxyl Terminus of Hsc70-Interacting Protein (AtCHIP) is a U-box-type E3 ligase named for its sequence similarity to mammalian co-chaperone CHIP which targets non-native or damaged proteins for degradation by the 26S proteasome (Meacham *et al.*, 2001; Murata *et al.*, 2001). Since cold and heat stress induce expression of *AtCHIP* one would predict that the E3 facilitates stress tolerance by targeting denatured and damaged proteins for degradation. On the contrary, over-expression of *AtCHIP* actually renders plants more sensitive to temperature stress (Yan *et al.*, 2003). An explanation put forward by Yan *et al.* (2003) is that high levels of AtCHIP protein facilitate the rapid turnover of misfolded proteins that could otherwise be refolded into functional proteins by the chaperone system. Identified AtCHIP substrates include A3 and RCN1, which are subunits of Protein Phosphatase 2A (PP2A) (Luo *et al.*, 2006). A3 and RCN1 protein levels are not altered in *AtCHIP* over-expressing plants, which is consistent with AtCHIP mono-ubiquitination of both proteins *in vitro*. Under cold temperatures, higher phosphatase activity was observed in *AtCHIP* over-expressing plants, which further suggest that AtCHIP-mediated ubiquitination of PP2A may serve a non-proteolytic function.

UV stress tolerance requires a Cullin RING ligase

Plants benefit from and require sunlight for photosynthesis but, at the same time, they must also protect themselves from damage caused by ultraviolet (UV) radiation. Two basic mechanisms are used by plants to repair DNA damage, photoreactivation and nucleotide excision repair (NER) (Tuteja *et al.*, 2009). Repair of UV-induced damaged DNA through the NER pathway involves a CUL4-DDB1 CRL (Groisman *et al.*, 2003; Wittschieben *et al.*, 2005). DDB2, which is turned over after UV exposure, is a target of CUL4-DDB1 E3 ligase activity (Molinier *et al.*, 2008). DDB2 is localized to the nucleus where it binds to bulky DNA lesions caused by UV radiation and presumably recruits NER machinery to the lesions (Luijsterburg *et al.*, 2007; Molinier *et al.*, 2008). Under non-stress conditions DDB1 is localized to the cytoplasm (Molinier *et al.*, 2008). Following UV radiation DDB1 is recruited into the nucleus where it promotes the degradation of DDB2. The reduction in DDB2 protein levels does not occur if components of the CRL, CUL4 or DDB1, are non-functional. CUL4-DDB1 mediated removal of DDB2 from the DNA lesion may be required to permit access of the NER machinery to the lesion. UV-induced degradation of DDB2 by CUL4-DDB1 is facilitated by the Ataxia Telangiectasia-mutated and Rad3-related (ATR) protein kinase that transmits DNA damage signal and by De-etiolated 1

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(DET1). Following UV exposure, ATR promotes the nuclear localization of DDB1, which is a prerequisite for DDB2 degradation (Molinier *et al.*, 2008). DDB2 is not degraded in UV-treated *det1* plants (Castells *et al.*, 2011). Adding to the complexity, UV-induced CUL4-DDB1-dependent degradation of DET1 occurs along with DDB2. The purpose for DET1 degradation and DET1 involvement in DDB2 degradation is not clear.

Ubiquitination and plant response to nutrient deprivation

Nitrogen is an essential macronutrient that contributes to plant biomass and influences various aspects of plant development. Plants adapt to low nitrogen availability by redistributing nitrogen from mature to younger actively growing organs and increasing accumulation of anthocyanin (Miller *et al.*, 2007). The RING-type E3 Nitrogen Limitation Adaptation (NLA) is a positive regulator of adaptive response to low nitrogen (Peng *et al.*, 2007). *NLA* mutants are hypersensitive to the effects of low nitrogen conditions and senesce much earlier than the wild type under the same conditions. Metabolite profiling suggests that *nla* plants are able to acquire nitrogen but fail to adapt to low nitrogen conditions by redirecting nitrogen from old to new tissue or by accumulating anthocyanin (Peng *et al.*, 2007).

After germination, nutrient availability determines if the seedling transits through the post-germinative developmental checkpoint described by Lopez-Molina *et al.* (2001). A high level of glucose stalls development while an increase in nitrogen and glucose permits growth. This demonstrates the importance of the ratio between carbon and nitrogen during this stage and the characterized carbon/nitrogen (C/N) response (Coruzzi and Zhou, 2001). *Arabidopsis* plants grown under high concentrations of glucose and low concentrations of nitrogen (severe C/N stress) arrest growth post-germination and do not survive (Martin *et al.*, 2002). Over-expression of RING-type E3 *ATL31*/Carbon-Nitrogen Insensitive 1-dominant (*CNI1*) rendered plants insensitive to C/N stress and these transgenic plants were able to pass through the early checkpoint despite the stress conditions (Sato *et al.*, 2009). Conversely, *ATL31* mutants grown under C/N stress are unable to progress through the post-germinative checkpoint. Similarly, mutations in the closely related *ATL31* genes, *ATL2* and *ATL6*, also produce hypersensitivity to C/N stress (Sato *et al.*, 2009). ATL31 is a functional E3 ligase and may function to reduce the level of proteins that stall growth during this checkpoint.

Iron is an essential nutrient facilitating photosynthesis, chlorophyll biosynthesis and a variety of redox reactions. *Arabidopsis* responds to iron-limiting conditions by up-regulating the expression of bHLH transcription factors such as Fer-like Iron Deficiency-Induced Transcription Factor (FIT), Popeye (PYE), and PYE homologue IAA-Leu Resistant-3 (ILR-3). These transcription factors induce expression of genes required for increasing iron availability and maintaining iron homeostasis (Colangelo and Guerinot, 2004; Rampey *et al.*, 2006; Yuan *et al.*, 2008; Long *et al.*, 2010; Lingam *et al.*, 2011) A recent study demonstrating a link between ethylene signalling and the iron-deficient stress response provided evidence for the involvement of the UPS in regulating FIT protein levels (Lingam *et al.*, 2011). FIT protein accumulates in response to iron-deficiency stress. Inhibition of ethylene biosynthesis via aminoethoxyvinylglycine (AVG) treatment during iron-deficiency stress prohibits FIT accumulation suggesting that ethylene signalling is required for the stabilization of FIT protein (Lingam *et al.*, 2011).

Although ubiquitin ligase activity remains to be experimentally demonstrated, the RING type E3 ligase Brutus (BTS) is proposed to regulate the abundance of ILR-3 (Long *et al.*, 2010). *BTS* along with *PYE* were identified in cell type specific transcription profiling as genes induced during iron deficiency stress. Unlike *pye-1*, *BTS* partial loss of function mutation rendered plants more tolerant to iron deficiency, suggesting that BTS is a positive regulator of the iron-deficient stress response. It is also worth noting that complete loss of function of *BTS* is lethal under normal growth conditions suggesting that BTS function is not limited to iron homeostasis (McElver *et al.*, 2001). The opposing effects of *pye* and *bts-1* during iron-deficient stress suggest that BTS may regulate the abundance of PYE (Long *et al.*, 2010). Surprisingly, BTS does not interact with PYE but it does interact with ILR3, a potential dimerizing partner of PYE. BTS may influence the stability of ILR-3 during iron-deficient stress and therefore indirectly affect the activity of PYE (Long *et al.*, 2010).

E2 ubiquitin conjugating enzymes and abiotic stress tolerance

Research into plant ubiquitination has focused mainly on E3 ligases and therefore considerably less is known about the biological relevance of the E1 and E2 enzymes during abiotic stress tolerance. Recent analysis of E2 function demonstrated a requirement for these enzymes during abiotic stress response. Over-expression of E2 enzymes from *Glycine max* (soybean; *GmUBC2*) and *Arachis hypogaea* (peanut; *AhUBC2*) in *Arabidopsis* enhanced tolerance of drought stress (Wan *et al.*, 2010; Zhou *et al.*, 2010). In addition, *GmUBC2* is up-regulated in response to drought and salt stress and *AhUBC2* is up-regulated during drought conditions. *Cucumis sativus* UBC13 (cucumber; *CsUBC13*) accumulates under iron-deficient conditions (Li and Schmidt, 2010). Plants respond to iron scarcity by increasing root surface area. *Arabidopsis* plants respond by forming branched root hairs and over-expression of *CsUBC13* in *Arabidopsis* plants enhanced this response (Li and Schmidt, 2010). Conversely, plants carrying mutations in the *Arabidopsis* orthologues, *UBC13A* and *UBC13B* [also referred to as UBC35 and UBC36, respectively (Kraft *et al.*, 2005)], did not produce branched root hairs in response to iron-deficient conditions.

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Ubiquitin-like proteins in abiotic stress tolerance

Eukaryotic cells employ a variety of small polypeptides as post-translational regulators of protein function. In addition to ubiquitin, plants utilize a number of ubiquitin-like proteins such as Related to ubiquitin 1 (RUB1), Small ubiquitin-like modifier (SUMO) and Ubiquitin fold modifier (UFM), and Homology to ubiquitin (HUB) (Miura and Hasegawa, 2010). In contrast to ubiquitin, where the major function is to facilitate protein degradation, the ubiquitin-like proteins function as modifiers regulating protein activity, subcellular localization, and protein–protein interactions.

The conjugation of SUMO to target proteins increases dramatically in response to various stresses including cold, drought, heat, metal toxicity (copper) and nutrient deprivation (Miura *et al.*, 2005; Catala *et al.*, 2007; Saracco *et al.*, 2007; Chen *et al.*, 2011). Mutations which impair SUMO conjugation decrease tolerance of these stresses (Miura *et al.*, 2005; Chen *et al.*, 2011). Sumolyation is similar to ubiquitination in that it also utilizes the sequential action of three enzymes, E1, E2, and E3, to attach SUMO to an internal lysine of target proteins. In *Arabidopsis*, the pathway is initiated by an E1 heterodimer, SUMO-activating enzyme 1(SAE1), and SAE2 which together is equivalent to the ubiquitin E1, UBA1 (Miura and Hasegawa, 2010). Unlike ubiquitination, only a single E2 SUMO-conjugating enzyme (SCE1) is used by the pathway (Saracco *et al.*, 2007). Few SUMO E3s have been identified to date including SIZ1 [for SAP (scaffold attachment factor, acinus, protein inhibitor of activated signal transducer and activator of transcription) and Miz1 (Ms×2-interacting zinc finger) domain] and High Ploidy2 (HPY2)/Methyl Methane Sulphonate Sensitivity21 (MMS21) (Miura *et al.*, 2005; Ishida *et al.*, 2009; Huang *et al.*, 2009). Substrates identified for *Arabidopsis* SIZ1 include transcription factors phosphate starvation response 1 (PHR1) (phosphate starvation), ABI5 (ABA signalling), ICE1 (cold signalling), and flowering locus D (FLD) (flowering time) (Miura *et al.*, 2005, 2009, 2011; Jin *et al.*, 2008). Conjugation of SUMO to ABI5 by SIZ1 prohibits ABI5 turnover (Miura *et al.*, 2009). Substitution of ABI5 Lys319 for arginine blocks sumolyation and further destabilizes ABI5 indicating that sumoylated ABI5 is not a suitable substrate for ubiquitination. Miura *et al.* (2009) suggests that sumoylation results in the accumulation of an inactive form of ABI5. Desumolyation of ABI5 provides a readily available pool of ABI5 that can be activated by phosphorylation upon initiation of ABA signalling (Fig. 4). Conjugation of SUMO to ICE1 seems to be required for transcriptional activity repressing ubiquitination and enhancing stability (Fig. 5) (Miura *et al.*, 2011). Blocking ICE1 sumolyation via a Lys393 mutation decreased freezing tolerance and reduced cold-induced expression of ICE1 target genes. Instances of sumolyation machinery competing with ubiquitination for the same lysine residue to direct substrate stabilization or degradation has been described (Ulrich, 2005). It is not clear if a similar mechanism is used to regulate the abundance of ICE as well as ABI5.

Future perspectives

Plant tolerance of adverse growth conditions such as cold, drought, and high salinity involves developmental, physiological, and biochemical changes, which limit damage, re-establish homeostasis, and facilitate repair of damaged systems. Adaptability to the changing environment influences development, growth and yield. Thus it is important to understand the regulatory mechanisms involved in stress tolerance. The identification of E3 ubiquitin ligases which play a regulatory role in abiotic stress responses, establishes a direct link between the UPS and plant stress tolerance. Only a very small number of the over 1300 *Arabidopsis* E3 ligases have defined roles in abiotic stress tolerance. The fact that the expression of many E3-encoding genes is stress-regulated and numerous stress-related proteins have been identified in searches for ubiquitinated proteins ensures that other E3 ligases that are essential for plant adaptation to abiotic stress will be encountered.

Our understanding of the regulatory role of the E3s during plant responses to abiotic stress is hindered by the lack of substrate identity. The function of E3 ligase depends on the nature of their target protein. That is, whether or not the target proteins are positive or negative regulators of the stress response. Although substrate identification is essential for determining biological function, it is also very important for understanding the biochemical function of the E3 enzymes. Once a substrate is identified, the mechanism of regulation by ubiquitination can be determined. Currently, very little is known about how plant E3 ligases are regulated specifically in response to external stimuli. Understanding the mechanism of stress signal-mediated up or down-regulation of E3 ligase activity will broaden our knowledge of cellular changes required for adaptation to adverse environmental conditions.

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