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Protein Ubiquitination: An Emerging Theme in Plant Abiotic Stress Tolerance

Wendy J. Lyzenga • Sophia L. Stone*

Department of Biology, Dalhousie University, Halifax NS B3H 4J1, Canada

Corresponding author: * **Sophia.Stone@dal.ca**

ABSTRACT

The ubiquitin proteasome system (UPS) effectively and efficiently controls the abundance of regulatory proteins, removes abnormal proteins and regulates the activity of signalling proteins. This allows for control of regulatory networks and adaptation to external stimuli. Plants utilize the UPS to alter their proteome, to modulate cellular activity and thus cope with unfavourable growth conditions. Recent studies demonstrated that the UPS plays a critical role in abiotic stress tolerance. Using the model research plant *Arabidopsis thaliana*, E3 ubiquitin ligases, the substrate-recruiting component of the ubiquitination pathway, have been identified as regulators of salinity, cold, heat and drought stress tolerance. E3 ubiquitin ligases also play a central role in regulating the signalling pathway initiated by the stress phytohormone abscisic acid. These studies establish a direct link between ubiquitination and plant response to environmental stresses. This work has been extended to other model plants and provides a strategy for enhancing plant stress tolerance utilizing the regulatory enzymes of the UPS. This review focuses on the recent progress in understanding the role of the UPS in abiotic stress tolerance and discusses strategies for improving stress tolerance by targeting E3 ubiquitin ligases.

Keywords: abiotic stress, abscisic acid, E3 ubiquitin ligase, hormone signaling, ubiquitination
Abbreviations: ABA, abscisic acid; **ABI**, abscisic acid insensitive; **CRL**, Cullin RING ligase; **E1**, ubiquitin activating enzyme; **E2**, ubiquitin conjugating enzyme; **E3**, ubiquitin ligase; **HECT**, Homology to E6-Associated Carboxy-Terminus; **RING**, really interesting new gene; **UPS**, ubiquitin proteasome system

CONTENTS

INTRODUCTION

As sessile organisms plants must cope with unfavourable conditions such as water scarcity (drought), temperature fluctuations (heat or chilling), high salt conditions (salinity), radiation (high intensity ultra-violet light), heavy metal toxicity, oxidative stress and nutrient deprivation in soil. Understanding how plants adapt to the changing environment is of great interest as abiotic stresses cause significant crop losses each year and, thus, threaten the sustainability of the agricultural industry. Cold, salinity and drought are three key abiotic stresses which adversely affect plant growth and productivity and are among the principle causes of reduction in crop yield. To this end, deciphering the underlying genetic and molecular mechanisms for abiotic stress perception, transduction and tolerance remains an intensely studied area of research.

In response to environmental stimuli plants alter their cellular milieu 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 physiological and cellular responses (**Fig. 1**). Stress responses tend to be controlled by a large number of genes, which has made under-

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Invited Review

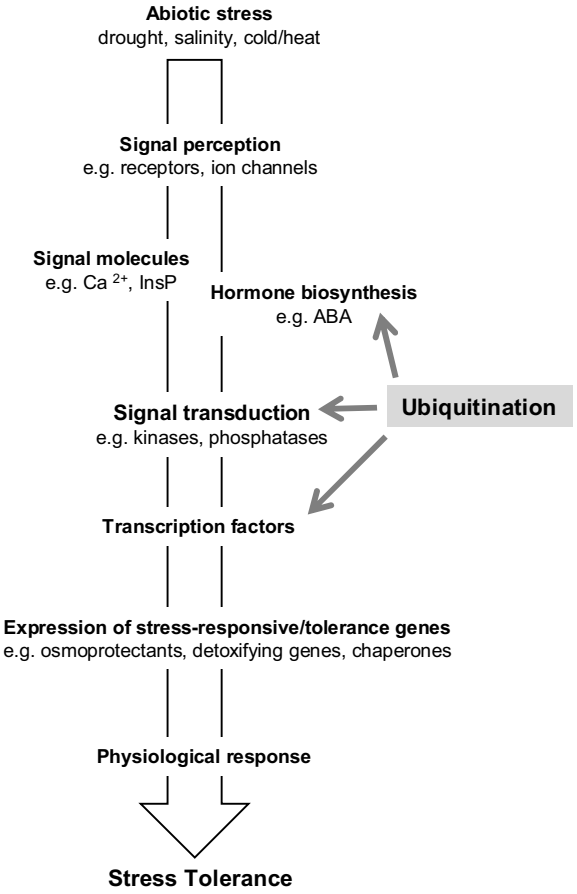

Fig. 1 A generic signal transduction pathway for response to abiotic stresses. Stresses such as cold, drought and salinity activate downstream signaling components via generation of second messengers such as calcium or accumulation of stress hormones. The signaling pathway target transcription factors that regulate the expression of stress-responsive genes. The expression of these genes leads to stress tolerance allowing the plant to survive unfavourable conditions. Protein ubiquitination may modulate the stress-responsive mechanism by regulating, hormone biosynthesis, the level or activity of components of the signaling pathway and the abundance of transcription factors.

standing the molecular basis of stress tolerance a difficult process. Generally, stress-responsive genes can be grouped into two categories (Wang *et al*. 2003; Bhatnagar-Mathur *et al*. 2008). The first category includes signalling proteins which relay the stress signal and transcription factors that regulate gene expression. The second category consists of gene products such as osmoprotectants and antioxidants that function to alleviate stress. For example, one of the main effects of cold stress is damage to the plant cell membrane (Steponkus 1984). In response to colder temperatures, plants modulate lipid composition so as to stabilize the cell membrane (reviewed in Mahajan and Tuteja 2005).

Engineering stress tolerant plants can target either of these two groups of stress-responsive genes. However, the multigenic nature of stress responses has made improving stress tolerance by traditional breeding methods a difficult process (Bohnert *et al*. 1995; McKersie *et al*. 1999; Vinocur and Altman 2005). Compared to altering the expression of a single stress-related gene, targeting transcription factors may be a more useful approach as it would allow for the control of multiple downstream stress-responsive genes (Chinnusamy *et al*. 2005; Bhatnagar-Mathur *et al*. 2008). A number of genes that respond to different stresses utilize the same transcription factor. Therefore, modulating the expression these transcription factors may enhance tolerance to multiple stresses. Other potential targets for engineering plants with enhanced stress tolerance are protein modifiers. Post-translational modification of stress-responsive signalling proteins and transcription factors by phosphorylation, farnesylation, sumoylation or ubiquitination is important for the regulating the expression of stress-responsive genes. Ubiquitination, for example, has been repeatedly shown to be involved in both biotic and abiotic stress response (for a recent review on ubiquitination and biotic stress see Dreher and Callis 2007). The ubiquitin-proteasome system (UPS) allows for rapid and efficient responses to abiotic stresses by regulating hormone biosynthesis and perception and the abundance of signalling proteins particularly transcription factors (**Fig. 1**) (Stone and Callis 2007). In this review we emphasize the role of the UPS in abiotic stress response and the potential of targeting the system as an approach to developing plants with enhanced stress tolerance.

THE UBIQUITIN PROTEASOME SYSTEM

Post-translationally modifying proteins via the attachment of one or more ubiquitin molecules is an extremely resourceful way to regulate protein abundance, cellular location and activity. Ubiquitin is a very stable, highly conserved, ubiquitously expressed molecule which can be linked to other proteins as well as itself, via one of seven lysine residues, producing structurally diverse polyubiquitin chains. The major function of ubiquitination is to selectively target proteins for proteasomal degradation, however recent studies have greatly expanded the cellular role of ubiquitination. The attachment of a single ubiquitin molecule to a target protein (monoubiquitination) has been shown to be sufficient to act as a signal for membrane protein internalization, vesicle sorting, DNA repair and gene silencing (Sun and Chen 2004; Mukhopadhyay and Riezman 2007). The attachment of a polyubiquitin chain to a target protein (polyubiquitination) has varying consequences depending upon which lysine residue of ubiquitin is used to produce the chain. The function of two types of polyubiquitination, lysine 48 (lys48) and lysine 63 (lys63) linked chains, have been extensively studied. Proteins modified by the attachment of a lys48 polyubiquitin chain are targeted for degradation by the 26S proteasome, a large ATP-dependent protease complex consisting of a 20S catalytic core capped on either end by a 19S regulatory particle. Lys63 polyubiquitination has been implicated in non-proteolytic functions such as endocytosis, protein kinase activation and DNA damage repair (Sun and Chen 2004; Mukhopadhyay and Riezman 2007). However, lys63 polyubiquitination can also serve as a signal to target proteins to the 26S proteasome for degradation (Saeki *et al*. 2009).

The covalent attachment of ubiquitin to a target protein involves an enzymatic cascade mediated by three enzymes, E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme) and E3 (ubiquitin ligase) (**Fig. 2**). The conjugation cascade is initiated by E1 which activates the ubiquitin molecules by forming an E1-ubiquitin thioester intermediate. The activated ubiquitin is then transferred to the E2 forming an E2-ubiquitin intermediate via a thioester linkage. The E3 enzyme mediates the transfer of ubiquitin from the E2-ubiquitin intermediate to the target protein. Ubiquitin attachment is facilitated by the formation of an isopeptide bond between the carboxyl terminus of ubiquitin and an internal lysine residue on the target protein.

Plant genomes examined so far contain two or more E1 enzymes, tens of E2s and a large number of E3s (**Table 1**). A single E1 is able to produce enough activated ubiquitin for the entire system (Pickart 2001a). The Arabidopsis genome contains two E1 encoding genes that share a similar expression pattern and E2 interaction specificity (Hatfield *et al*. 1997). E2s are characterized by the presence of a conserved core domain (UBC domain) which contains the cysteinyl residue required for accepting the ubiquitin molecule from the E1 (Pickart 2001a; Wu *et al*. 2003; Kraft *et al*. 2005). The UBC domain also facilitates interaction with the ubiquitin ligase. In addition to the UBC domain, a few E2s contain an amino and/or carboxyl-terminal extension which may mediate E3 ligase interaction specificity (Jentsch 1992;

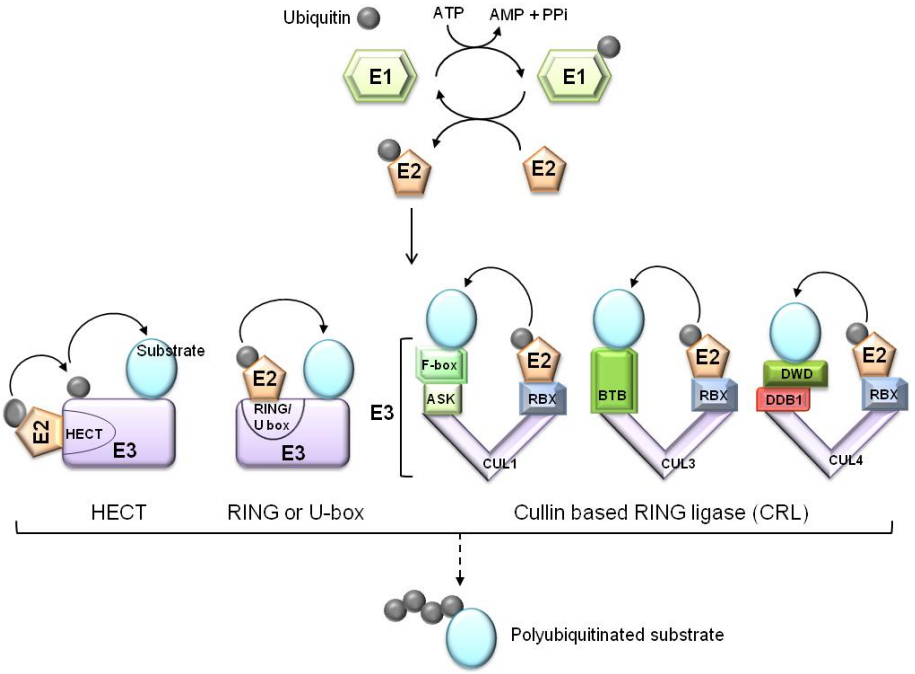

Fig. 2 The ubiquitination pathway. ATP-dependent activation of ubiquitin by the E1 (ubiquitin activating enzyme), is followed by transfer to the E2 (ubiquitin conjugating enzyme). The E2-ubiquitin intermediate then interacts with the E3 (ubiquitin ligase) and jointly transfers ubiquitin to the substrate. HECT-type E3 ligases form a intermediate with ubiquitin prior to transfer of ubiquitin to the substrate, while U-box and RING E3 ligases, including CRLs, facilitate direct transfer of ubiquitin to the substrate. CRLs are grouped into three categories according to substrate recognition protein; F-box, BTB, and DWD. The cycle is repeated to generate a polyubiquitin chain.

Table 1 Comparison of ubiquitination enzymes gene families in *Arabidopsis*, rice and poplar.

	Arabidopsis	Rice*	Poplar**	References
E1	2	6	6	Hatfield *et al*. 1997; Du *et al*. 2009b
E2	37	49	70	Smalle and Vierstra 2004; Du *et al*. 2009
E3				
HECT	7	8	7	Kraft *et al*. 2005; Du *et al*. 2009b
U-box	61	77	93	Kraft *et al*. 2005; Zeng *et al*. 2008; Du *et al*. 2009b
RING	476	378	399	Kraft *et al*. 2005; Du *et al*. 2009
Cullin RING Ligase (CRL)				
BTB	80	149	81	Gingerich *et al*. 2005; Gingerich *et al*. 2007; Du *et al*. 2009b
F-box	600-700	687	320	Kuroda *et al*. 2002; Gagne *et al*. 2002; Jain *et al*. 2007; Yang *et al*. 2008a
DWD	85	78	nr	Lee *et al*. 2008

* Numbers for rice E1, E2, RING and HECT E3 were taken from http://bioinformatics.cau.edu.cn/plantsUPS (Du *et al*. 2009b).
** With the exception of the F-box proteins (Yang et al. 2008a), numbers for the poplar E1, E2 and E3 family of ubiquitin ligases retrieved from http://bioinformatics.cau.edu.cn/plantsUPS (Du *et al*. 2009b).
nr, number of DWD proteins in poplar was not reported.

Kraft *et al*. 2005). There are usually dozens of E2s; the Arabidopsis genome for example contains 37 E2 encoding genes (**Table 1**) (Kraft *et al*. 2005). The specificity of the ubiquitination pathway is governed mainly by the substrate-recruiting E3 ubiquitin ligases. The Arabidopsis genome is predicted to encode for over 1300 E3s which can be sub-divided into distinct groups depending on their mode of action and subunit composition (**Table 1**). The abundance and diversity of E3 ligases allows the ubiquitination pathway to regulate the activity of a large number of proteins.

E3 UBIQUITIN LIGASES

Ubiquitin ligases can be classified into three groups based on the presence of either a Homology to E6-Associated Carboxy-Terminus (HECT), U-box or Really Interesting New Gene (RING) E2 binding domain (**Table 1**). The HECT-type E3s are the smallest group with only seven members in Arabidopsis (Downes *et al*. 2003). Unlike the other groups of E3s, the HECT-type E3s form a E3-ubiquitin intermediate prior to the transfer of ubiquitin to the target protein (Scheffner *et al*. 1995) (**Fig. 2**). The U-box-type and RING-type E3 ligases facilitate transfer of the ubiquitin molecule directly from the E2-ubiquitin intermediate to the target protein (**Fig. 2**). The Arabidopsis genome contains 61 U-box-type and 476 RING-type E3s (Azevedo *et al*. 2001; Stone *et al*. 2005; Yee and Goring 2009). The RING and U-box proteins can be divided into thirty and five different groups, respectively, based on domain composition and organization (Azevedo *et al*. 2001; Stone *et al*. 2005). The diversity of the RING E3s is further reflected in variations within the RING domain itself (Stone *et al*. 2005). The RING domain uses an octet of cysteine and histidine amino acids as metal ligand residues to co-ordinate two zinc ions in a cross brace structure essential for E3 ligase activity (Freemont 1993). Five modified RING domains have been identified which have variability in the positioning of key metal ligand residues within the RING domain (Stone *et al*. 2005). The variability does not seem to affect function as E3 ligase activity has been demonstrated for a number of the modified domains.

Though the majority of E2-binding RING domains are found in monomeric E3 proteins, RING domain-containing proteins are also components of multi-subunit E3 ligases such as the Cullin based RING E3 ligases (CRLs) (Smalle and Vierstra 2004) (**Fig. 2**). Three types of CRLs have been described in plants, each utilizing a different Cullin subunit (CUL1, CUL3a/3b or CUL4), which functions as a scaffold

that interacts with the E2 binding RING protein and a substrate-recruiting protein (Schwechheimer and Villalobos 2004; Hotton and Callis 2008). Families of substrate-recruiting proteins utilized by the CRLs include the F-box, Broad complex Tramtrack Bric-a-Brac (BTB) and DDB1 binding WD40 (DWD) motif containing proteins (**Table 1**). The Skp1-Cullin-F-box (SCF)-type CRL, for example, contains CUL1, RBX1a/b RING protein, ASK1/Skp1 adaptor protein that facilitates interaction with the substrate-recruiting subunit, a F-box protein of which there are over 700 in the predicted Arabidopsis proteome (Gagne *et al*. 2002; Lechner *et al*. 2006) (**Fig. 2**). The diversity of substrate-recruiting subunits and the ability to utilize one of three Cullin proteins makes the CRL group the largest class of ubiquitin ligases.

THE UBIQUITIN-PROTEASOME SYSTEM AND ABSCISIC ACID SIGNALLING

The plant hormone abscisic acid (ABA) functions in adaptive response to environmental stresses. Salinity, drought and cold stress causes the accumulation and increased biosynthesis of ABA (Cutler and Krochko 1999; Taylor *et al*. 2000). ABA regulates seed maturation and prolongs seed dormancy to ensure that seeds germinate under favourable conditions. Immediately following germination, ABA suspends the growth of young seedlings exposed to stresses such as cold, salinity or drought. Seedling development is slowed until better environmental conditions arise. As plants mature further, stress-induced accumulation of ABA directs various protective responses that help ameliorate 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. Under drought conditions, ABA prevents transpirational water loss by promoting the efflux of potassium ions from guard cells which causes loss of turgor pressure leading to stomatal aperture closure (MacRobbie 1998; Hetherington 2001; Himmelbach *et al*. 2003).

ABA-mediated responses, such as growth arrest of early seedlings exposed to stress conditions, require changes in expression of a large subset of genes. Transcriptional analyses of ABA-responsive genes identified over 1350 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 cold, drought and high salinity are mediated by ABA-responsive transcription factors such as the basic leucine zipper (bZIP) transcriptional activators, which interact with the ABA-regulatory elements (ABRE) found in the promoter of stress-responsive genes (Hattori *et al*. 2002; Narusaka *et al*. 2003). The ABA-responsive transcription factors activate a subset of genes that function together to enhance stress tolerance. 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 Abscisic Acid Insensitive 5 (ABI5), a bZIP transcription factor that functions as a positive regulator of ABA responses, provided evidence for the involvement of the UPS in regulating ABA signalling (Uno *et al*. 2000; Lopez-Molina *et al*. 2003; Smalle and Vierstra 2004). Ubiquitinated ABI5 accumulates in seedlings treated with proteasome inhibitors and ABI5 is stabilized in *rpn10-1*, which has a defect in RPN10, a non-ATPase subunit of the 19S regulatory particle (Lopez-Molina *et al*. 2003; Smalle *et al*. 2003). ABA signalling results in ABI5 phosphorylation, a dramatic increase in ABI5 protein levels and seedling growth arrest. Interestingly, 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). These observations, along with the fact that ABI5 protein accumulation is also induced by salt and drought stress, suggests that ABA-dependent stabilization of ABI5 serves as an early developmental checkpoint to delay growth during adverse environmental conditions. Under favourable growth conditions the UPS is required to maintain low levels of ABI5, thus permitting growth.

Other ABA-responsive transcription factors are also regulated by the UPS. The B3 transcription factor ABI3 plays a central role in mediating ABA-dependent responses (Finkelstein and Lynch 2000). ABI3 function is required for desiccation tolerance, maintaining seed dormancy, plastid development and vegetative to reproductive phase transition (Rohde *et al*. 2000; Finkelstein *et al*. 2002). ABI3 protein is unstable in most stages of plant development and degradation of ABI3 can be blocked by proteasome inhibitors (Lopez-Molina *et al*. 2001, 2002; Zhang *et al*. 2005). Although there is no direct evidence for UPS-mediated degradation, preliminary evidence suggests that ABI4 and ABA-responsive ABRE Binding Factor 2 (ABF2) transcription factors may also be regulated by the UPS. Similar to ABI5, ABI4 is very unstable but unlike ABI5 treatment with ABA does not result in the accumulation of ABI4 protein (Finkelstein *et al.* 2011). However, treatment with proteasome inhibitors stabilizes the protein in transgenic plant constitutively expressing ABI4. Evidence for UPS regulation of ABF2 is based on ABF2 interaction with Arm Protein Repeat Interacting with ABF2 (ARIA), a BTB protein which may function as a component of a CRL E3 ligase complex (Kim *et al*. 2004).

E3 LIGASES AND ABSCISIC ACID SIGNALLING

Efforts to identify stress-responsive genes have uncovered several E3 ligases with potential roles in regulating ABA signalling. E3 ligases with mRNA levels affected by ABA and genes encoding E3 ligases have surfaced in screens for mutants with aberrant ABA-related phenotypes. Interaction screens used to isolate signalling components that modulate ABA-responsive gene expression have also identified E3 ligases. This section highlights some of the E3 ligases with defined roles in ABA signalling. A comprehensive list of E3 ligases with known and potential roles in ABA signalling as well as ABA-independent stress responses can be found in **Table 2**.

Keep on Going (KEG)

KEG is a large multi-domain protein that contains functional RING-type E3 ligase and kinase domains, followed by a series of ankyrin repeats and previously unidentified HERC2-like repeats (Stone *et al*. 2006). Both the ankyrin and HERC2-like repeats facilitate interactions with substrate proteins (Stone *et al.* 2006; Gu and Innes 2011). KEG is a negative regulator of ABA signalling required for maintaining low levels of ABI5 in the absence of ABA (Stone *et al*. 2006). Gene disruption of *KEG* due to T-DNA insertions results in ABA hypersensitivity, an accumulation of extremely high levels of ABI5 and seedling growth arrest shortly after germination. In the absence of ABA, KEG is thought to target ABI5 for ubiquitination leading to its degradation and suppression of ABI5-dependent post-germinative growth arrest. Loss of *ABI5* in the *KEG* mutant background only partially rescues the growth-arrest phenotype of *keg* seedlings suggesting that KEG regulates the stability of a number of proteins including other ABA-responsive transcription factors.

Recent studies have begun to shed light on the mechanism by which ABA protects ABI5 from degradation by KEG. In the presence of ABA, the turnover of KEG protein increases significantly (Liu and Stone 2010). The ABA-induced degradation is dependent on KEG's own E3 ligase domain and on the activity of the 26S proteasome. These results suggest that KEG protein levels are reduced via ABA-induced self-ubiquitination and subsequent degradation by the 26S proteasome, thus allowing ABI5 level to rise. ABA signalling may also modify ABI5 to prevent KEG-mediated ubiquitination. This is supported by several studies that demonstrate that in the presence of ABA, ABI5 exist in multiple migrating isoforms (Lopez-Molina *et al.* 2001,

Table 2 E3 ubiquitin ligases with known or predicted roles in ABA signalling, ABA-dependent or independent stress responses.

E3	Type	Species*	Function	References
AIP2	RING	*At*	Negative regulator of ABA signalling	Zhang *et al*. 2005
AIRP1	RING	*At*	ABA-dependent drought response	Ryu *et al.* 2010
ARIA	CRL	*At*	Positive regulator of ABA signalling	Kim *et al*. 2004
BIRF1	RING	*Os*	Response to drought and oxidative stress possibly through reduced ABA sensitivity	Liu *et al*. 2008
CHIP	RING	*At*	Response to temperature fluctuations	Yan *et al*. 2003; Luo *et al*. 2006
CNI1/ATL31	RING	*At*	Response to carbon and nitrogen levels during growth phase transition in seedlings	Sato *et al*. 2009
COP1	RING	*At*	Regulation of ABA signalling via HY5	Chen *et al*. 2008
DDB1	CRL	*At*	Maintains genome integrity under UV stress	Molinier *et al*. 2008
DOR	CRL	*At*	Response to drought stress by inhibiting ABA-induced stomatal closure	Zhang *et al*. 2008
DRIP1/2	RING	*At*	Response to dehydration stress	Qin *et al*. 2008
DSG1	RING	*Os*	Regulator of ABA signaling	Park *et al.* 2010
DWA1/1	CRL	*At*	ABA signalling	Lee *et al.* 2010
FBP7	CRL	*At*	Cold temperature tolerance	Calderón-Villalobos *et al*. 2007
GMPOZ	CRL	*Hv*	Negative regulator of ABA signalling, activator of gibberellin signalling	Woodger *et al*. 2004
HOS1	RING	*At*	Negatively regulates cold responses	Dong *et al*. 2006
KEG	RING	*At*	Negative regulator of ABA signalling	Stone *et al*. 2006; Liu and Stone 2009
NLA	RING	*At*	Response to nitrogen stress	Peng *et al*. 2007
PUB1	U-box	*Ca*	Drought and salinity stress tolerance	Cho *et al*. 2006
PUB15	U-box	*Os*	Response to oxidative stress	Park *et al.* 2011
PUB22/23	U-box	*At*	Drought and salinity stress tolerance	Cho *et al*. 2008
PUB9	U-box	*At*	ABA signaling	Samuels *et al.* 2008
RFP1	RING	*Ca*	ABA dependent response to osmotic stress	Hong *et al.* 2007
RFP1	RING	*Gm*	Cold, salt and drought stress tolerance	Du *et al*. 2009a
RHA2a	RING	*At*	Positive regulator of ABA signalling	Bu *et al*. 2009
RING-1	RING	*Os*	Drought and heat tolerance	Meng *et al*. 2006
Rma1/2/3	RING	*At*	Response to drought stress	Lee *et al*. 2009
Rma1H1	RING	*Ca*	Response to drought stress	Lee *et al*. 2009
SAP5	RING	*At*	Salt and dehydration stress	Kang *et al.* 2011
SDIR1	RING	*At*	Response to drought and salt, positive regulator of ABA signalling	Zhang *et al*. 2007
SDIR1	RING	*Os*	Drought tolerance	Gao *et al*. 2011
XERICO	RING	*At*	Response to drought stress, increase ABA biosynthesis	Ko *et al*. 2006
ZF1	RING	*Zm*	Drought and salinity tolerance	Huai *et al*. 2009
ZFP1	RING	*Ad*	Drought tolerance, possible role in ABA signalling	Yang *et al*. 2008b

* Species: *Ad - Artemisia desertorum*; *At - Arabidopsis thaliana*; *Ca - Capsicum annuum* (hot pepper); *Gm - Glycine max* (soybean); *Hv - Hordeum vulgare* (barley); *Os - Oryza sativa* (rice); *Zm - Zea mays* (maize).

2002; Smalle and Vierstra 2004). Conjugation of Small Ubiquitin-like Modifier (SUMO) to ABI5 by SUMO E3 ligase SIZ1 (for SAP [scaffold attachment factor, acinus, protein inhibitor of activated signal transducer and activator of transcription] and Miz1 [Msx2-interacting zinc finger] domain), inhibits ABI5 degradation by the proteasome (Miura *et al*. 2009). This suggests that sumoylated ABI5 is not a suitable substrate for KEG E3 ligase activity. Sumoylation of ABI5 adds another layer of regulation to ABA signalling. Miura *et al.* (2009) suggests that sumoylation results in the accumulation of an inactive form of ABI5, upon ABA signalling this pool of ABI5 is desumoylation and become activate. The active ABI5 can then mediate ABA-dependent responses.

DWD hypersensitive to ABA 1 (DWA1) and DWA2

DWA1 and DWA2 are DDB1 binding WD40 (DWD) proteins that function together as the substrate recruiting component of a CUL4 based CRL (Lee *et al.* 2008) (see **Fig. 2**). Similar to KEG, the DWA1/2 containing CRL has been implicated in regulating ABI5 protein levels (Lee *et al.* 2010). Compared to wild type plants, *dwa1*, *dwa2* and *dwa1 dwa2* seedlings accumulate higher levels of ABI5 protein following ABA treatment and exhibit ABA hypersensitive phenotypes. The *DWA* mutants differ from *keg* in one significant aspect, in the absence of ABA, ABI5 in undetectable in *dwa1*, *dwa2* and *dwa1 dwa2*, whereas all *KEG* mutants accumulate extremely high levels of ABI5. Multiple E3 ligases targeting a single substrate has been well documented in other eukaryotic systems. For example, the mammalian transcription factor p53 is targeted by RING-type E3 ligases, Mdm2, COP1 and PirH2 and HECT-type E3 ligase, ARF-BP1 (see review Brooks and Gu 2006). Each E3 ligase may regulate substrate abundance under certain conditions, for example stressed versus unstressed, or within specific cellular compartments, for example cytoplasmic versus nuclear. KEG and DWA1/2 E3 ligases may function together to maintain ABI5 abundance under different circumstances. KEG may function to repress ABI5 in unstressed cells (low ABA), while DWA1 and DWA2 may be required to down-regulate ABI5 in stressed cells (high ABA).

ABI3-Interacting Protein 2 (AIP2)

AIP2, a RING-type E3 ligase, was isolated as an interactor of ABI3 via a yeast two hybrid screen (Kurup *et al*. 2000). AIP2 is a negative regulator of ABA signalling involved in ubiquitinating and targeting ABI3 for degradation by the 26S proteasome (Zhang *et al*. 2005). *aip2-1* plants accumulate high levels ABI3 compared to wild-type and are hypersensitive to exogenous ABA. Overexpression of *AIP2* leads to reduced ABI3 protein levels, decrease in seed viability and a prolonged vegetative growth period (Zhang *et al*. 2005). *AIP2* is ubiquitously expressed and transcript abundance increases upon ABA application in seedlings (Zhang *et al*. 2005). The increase in *AIP2* transcript correlates with a decrease in ABI3 protein levels. These results suggest that AIP2 functions to keep ABI3 levels low (Lopez-Molina *et al*. 2002; Zhang *et al*. 2005).

Salt and Drought Induced RING Finger 1 (SDIR1)

SDIR1 encodes for a membrane bound RING-type E3 ligase that was first identified, via microarray analysis, as a salinity and drought stress-inducible gene (Zhang *et al*. 2007). Further research demonstrated that SDIR1 is a positive regulator of ABA signalling (Zhang *et al*. 2007). Transgenic plants overexpressing *SDIR1* are hypersensitive to

ABA and high salinity and display enhanced drought tolerance. The increased drought tolerance correlates with enhanced ABA-mediated stomatal closure. Opposite phenotypes are observed for *sdir1* plants, for example *SDIR1* mutants are less sensitive to salt stress compared to wild type. The phenotypes of the *SDIR1* overexpressors and *sdir1-1* mutants mirror those observed for *ABI5* overexpressing plants and *abi5-1* mutant plants, respectively (Lopez-Molina *et al*. 2001). Overexpression of ABI5 in the *sdir1-1* background is able to rescue the ABA insensitivity phenotype of *sdir1-1* plants whereas overexpression of *SDIR1* in an *abi5-1* background is unable to rescue the ABA insensitivity of *abi5-1* plants. This suggests that SDIR1 is acting upstream of ABI5 in the ABA signalling pathway (Zhang *et al*. 2007). SIDR1 is a functional E3 ligase *in vitro*, however like most E3 ligases substrates still remain to be identified. It is possible that SDIR1 targets negative regulators of ABA signalling for degradation. Another possibility mentioned by Zhang *et al* (2007) is that SDIR1 could activate a positive regulator via monoubiquitination which functions to enhance the ABA signalling cascade.

Arabidopsis thaliana Carboxyl Terminus of Hsc70-Interacting Protein (AtCHIP)

Mammalian CHIP proteins are chaperone-dependent U-box-type E3 ligases (Murata *et al*. 2001). CHIP E3s, via their interactions with molecular chaperones, target non-native and damaged proteins for degradation by the 26S proteasome (Meacham *et al*. 2001; Murata *et al*. 2001). In addition to the U-box domain, the Arabidopsis CHIP contains three tetratricopeptide (TRP) repeats (Yan *et al*. 2003). TRP repeat containing proteins have been implicated in stress response in a variety of organisms (Honoré *et al*. 1992; Hernandez Torres *et al*. 1995; Blatch *et al*. 1997). In *Arabidopsis*, cold, heat, and high salinity all induced expression of *AtCHIP* transcripts (Yan *et al*. 2003). Overexpression of *AtCHIP* renders plants sensitive to ABA and temperature fluctuations (Yan *et al*. 2003; Luo *et al*. 2006). *AtCHIP* overexpressors produce fewer seeds than wild type at high temperatures and growth is severely delayed at low temperatures.

AtCHIP interacts with and monoubiquitinates A3 and RCN1, subunits of Protein Phosphatase 2A (PP2A) (Luo *et al*. 2006; Farkas *et al*. 2007). The attachment of a single ubiquitin molecule suggests that the function of the modification is non-proteolytic. Analysis of the steady state levels of A3 and RCN1 provides support for this hypothesis. A3 and RCN1 protein levels are not altered in *AtCHIP* overexpressing plants, instead higher PP2A activity is observed under cold conditions suggesting that AtCHIP activates PP2A under cold stress which may lead to an altered ABA response. Cold-induced up-regulation of PP2A activity may account for the reduced growth phenotype observed for cold-treated *AtCHIP* overexpressing plants (Luo *et al*. 2006).

Constitutively Photomorphogenic 1 (COP1)

Light perceived by phytochromes and cryptochromes regulate photomorphogenesis via a set of transcription factors that mediate changes in expression of multiple downstream genes (Ma *et al*. 2001; Jiao *et al*. 2007). COP1, a RING-type E3 ligase, functions downstream of multiple photoreceptors to repress light mediated changes in development (Wei and Deng 1996). COP1 desensitizes light signalling by promoting the degradation of a variety of photomorphogenic-promoting factors (Osterlund *et al*. 2000; Saijo *et al*. 2003; Seo *et al*. 2004). One of the first targets identified for COP1 was Elongated Hypocotyl5 (HY5), a bZIP transcription factor which functions downstream of a number of photoreceptors (Koornneef *et al*. 1980; Oyama *et al*. 1997; Ang *et al*. 1998; Osterlund *et al*. 2000). In the dark, nuclear localized COP1 interacts with and promotes the degradation of HY5 and COP1 is depleted from the nucleus in the light, allowing HY5 proteins levels to increase (Osterlund *et al*. 2000).

Recent studies identified a role for HY5 in ABA signalling (Chen *et al*. 2008). Compared to wild type *hy5* seedlings are less sensitive to ABA-mediated inhibition of seed germination, seedling growth and lateral root production (Chen *et al*. 2008). Where stress responses are concerned, *hy5* seedlings are more susceptible to salt and osmotic stresses compared to wild type. HY5 regulates the expression of a subset of ABA-inducible genes, including ABI5, in dry seeds and young seedlings. The transcript levels of ABI5 were reduced in *hy5* seeds which correlated with the down-regulation of ABI5-regulated ABA-inducible late embryogenesis-abundant (LEA) genes (Carles *et al*. 2002; Chen *et al*. 2008). ABA does not influence the stability of HY5 but instead promotes the binding of HY5 to the ABI5 promoter which suggests a mechanism whereby ABA can induce the expression of ABI5. HY5 abundance is greatest during early seedling development, which is not only consistent with its role in promoting photomorphogenesis but also correlates with the developmental window within which ABI5 regulates growth under stress conditions (Hardtke *et al*. 2000; Lopez-Molina *et al*. 2001). The integration of light control of seedling development and ABA signalling may allow seeds and young seedlings to better sense and adapt to its environment.

E3 LIGASES IN ABIOTIC STRESS TOLERANCE

DREB2A-Interacting Protein 1 (DRIP1) and DRIP2

Numerous drought-inducible genes contain the dehydration responsive element (DRE) in their promoters (Baker *et al*. 1994; Yamaguchi-Shinozaki and Shinozaki 1994). Many of these genes are downstream targets of the transcription factor Dehydration-responsive Element Binding Protein 2A (DREB2A) which interacts with the *DRE* via an ERF/AP2 binding domain (Stockinger *et al*. 1997; Liu *et al*. 1998). The ability of DREB2A to regulate gene expression is influenced by its stability. Under favourable growth conditions, DREB2A protein is unstable due to the presence of the negative regulatory domain, a serine and threonine-rich 30–amino acid region (Sakuma *et al*. 2006a). Deletion of the negative regulatory domain increases DREB2A stability and overexpression of a DREB2A mutant lacking the negative regulatory domain (DREB2A-CA) renders plants more tolerant of drought and high temperature stresses (Sakuma *et al*. 2006a, 2006b). The negative regulatory domain may contain a degron, an amino acid sequence that serves as a signal for degradation. Temperature and hormone responsive degrons have been identified in plants and other eukaryotes (Dohmen *et al*. 1994; Dreher *et al*. 2006; Nishimura *et al*. 2009). In plants for example, binding of growth hormone auxin to its receptor, Transport Inhibitor Response 1 (TIR1), the substrate recruiting F-box subunit of SCF^{TIR} CRL, promotes the ubiquitination and rapid degradation of the Auxin/Indole-3-Acetic Acid (AUX/IAA) transcriptional repressor proteins. Auxin accelerates the degradation of AUX/IAAs and this relives its inhibitory effect on Auxin Response Factors (ARFs) which acts as transcriptional activators of auxin-responsive genes (Tiwari *et al*. 2004; Dharmasiri *et al*. 2005; Tan *et al*. 2007). Mutational analysis of the conserved domain II region, found in most AUX/IAA proteins, show that the domain regulates protein stability and contains a transferable auxin-inducible degron (Dreher *et al*. 2006; Nishimura *et al*. 2009). Similarly, the negative regulatory domain may function as a degron that facilitates the degradation of DREB2A under favourable growth condition. The DREB2A degron would be made unavailable to the degradation machinery under stress conditions, thus allowing DREB2A protein to accumulate and regulate the expression of stress-responsive genes.

The fluctuation in DREB2A abundance in response to growth conditions and the fact that DREB2A accumulates upon inhibition of the 26S proteasome proteolytic activity

provides evidence for regulation by the UPS (Qin *et al*. 2008). Two RING-type E3 ligases, DREB2A Interacting Protein 1 (DRIP1) and DRIP2, were identified via a yeast two hybrid screen as interactors of DREB2A (Qin *et al*. 2008). DRIP1 is capable of mediating DREB2A ubiquitination *in vitro* and DREB2A protein is more stable in *drip1-1* plants compared to wild-type. These results suggest that DREB2A protein is normally maintained at low levels through ubiquitination and subsequent degradation by the 26S proteasome. Disruption of either *DRIP1* or *DRIP2* alone did not produce any significant changes in stress tolerance or developmental phenotypes. However, *DRIP1 DRIP2* double mutants displayed enhanced drought tolerance which coincided with a significant increase in the expression of a number of drought stress-responsive genes, specifically genes regulated by DREB2A (Qin *et al*. 2008). Conversely, overexpression of *DRIP1* delayed the expression of DREB2A-regulated drought-responsive genes. These results suggest that DRIP1 and DRIP2 may function redundantly to maintain low levels of DREB2A under non-stressed conditions.

High Expression of Osmotically Responsive Gene 1 (HOS1)

HOS1 and *Inducer of CBF/DREB1 expression 1* (*ICE1*), were identified in a series of genetic screens aimed at isolating mutants that affect the expression of cold-inducible genes (Ishitani *et al*. 1998; Chinnusamy *et al*. 2003). ICE1, which encodes a MYC transcription factor, controls the expression of cold-responsive genes such as *C-Repeat (CRT) 3/dehydration responsive element (DRE) binding proteins 1A* (*CBF3/DREB1A*). The expression of *ICE1*, which is normally constitutive, is upregulated in response to cold temperatures. Overexpression of *ICE1* leads to increased expression of *CBF3* under cold but not warm temperatures and also enhances cold tolerance (Chinnusamy *et al*. 2003). HOS1, a RING-type E3 ligase, negatively regulates cold responses. Increase in *HOS1* expression results in a reduction in transcript accumulation of *CBF1*, *CBF2* and *CBF3* as well as several other stress-responsive genes such as *cold-regulated 15* (*COR15*), *COR47* and *RD29A* (Xiong *et al*. 2002; Dong *et al*. 2006). Accordingly, transgenic *HOS1* overexpressing plants were less tolerant of cold temperatures (Dong *et al*. 2006).

HOS1 has been shown to interact with and ubiquitinate ICE1 both *in vitro* and *in vivo* (Dong *et al*. 2006). Cold treatment promotes the reduction of ICE1 protein levels. The cold-induced reduction of ICE1 protein levels can be blocked by addition of proteasome inhibitors suggesting that cold promotes HOS1-mediated ubiquitination and degradation ICE1. The effect of ICE1 elimination is reduced expression of *CBF3* along with other cold stress-responsive genes. Dong *et al* (2006) proposes that ICE1 maybe post-translationally modified in response to cold (before ubiquitination) and this active form of ICE1 switches on target gene expression. HOS1 may recognize and ubiquitinate the activated form of ICE1 and attenuate the cold response signal. This suggestion is supported by the fact that cold responsive genes are only transiently induced in response to cold (Chinnusamy *et al*. 2003; Dong *et al*. 2006).

Plant U-box 22 (PUB22) and PUB23

U-box-type E3 ligases, PUB22 and PUB23, were initially identified as homologs of *Capsicum annuum* (hot pepper) PUB1 (CaPUB1) (Cho *et al*. 2006). Similar to *CaPUB1*, the expression of Arabidopsis *PUB22* and *PUB23* increases in response to cold, drought and salt stresses but not upon ABA treatment (Cho *et al*. 2008) Overexpression of either *PUB22* or *PUB23* render plants more sensitive to drought and salt stresses (Cho *et al*. 2008). The *pub22 pub23* double mutant, which is phenotypically similar to wild-type under favourable growth conditions, is highly resistant to drought and salt stress. Both PUB22 and PUB23 may function together to regulate ABA-independent stress signalling.

RPN12a, a non-ATPase subunit of the 19S regulatory particle, was identified as an interactor of PUB22 and PUB23 (Baumeister *et al*. 1998; Cho *et al*. 2008). Both PUB22 and PUB23 are able to ubiquitinate RPN12a *in vitro* and *in vivo*. Cytosolic gel filtration analysis show that RPN12a elutes in a protein complex with a molecular mass (800–900 KDa) consistent with the size of the 19S regulatory particle (Peng *et al*. 2001; Cho *et al*. 2008). However, RPN12a elutes with a wider range of protein complexes (200 to 900 KDa) in transgenic plants that overexpress *PUB22* or *PUB23*. Interestingly, in drought stressed plants RPN12a exhibits the same elution pattern as the *PUB22* or *PUB23* overexpressing plants (Cho *et al*. 2008). The non-ATPase subunit of the 19S regulatory particle is thought to direct specific proteins to the 26S proteasome complex for degradation (Smalle and Vierstra 2004). During drought stress, PUB22/23 ubiquitination of RPN12a may cause its dissociation from the 19S regulatory particle, which would affect the function of the proteasome and the degradation of specific proteins. Whether or not PUB22 or PUB23 mediated ubiquitination of RPN12a is stress dependant is not currently known.

Ring membrane-anchor 1 Homolog 1 (Rma1H1)

Hot pepper *Rma1H1* was identified as a dehydration-inducible gene, which encodes for an endoplasmic reticulum (ER)-membrane associated RING-type E3 ligase (Park *et al*. 2003; Lee *et al*. 2009). Overexpression of hot pepper *Ram1H1* in Arabidopsis enhances drought stress tolerance (Lee *et al*. 2009). To further evaluate the role of Ram1H1 in stress tolerance, Arabidopsis PIP2;1, a plasma membrane aquaporin that is down-regulated by drought stress, was selected as a potential target for Rma1H1 (Tyerman *et al*. 1999; Jang *et al*. 2004; Alexandersson *et al*. 2005). Transfection experiments using Arabidopsis protoplasts demonstrated that Rma1H1 modulates PIP2;1 protein levels (Lee *et al*. 2009). Co-transformation of *PIP2;1* and *Rma1H1* into protoplast resulted in lower PIP2;1 protein levels compared to when *PIP2;1* is introduced alone. The Rma1H1 dependent reduction of PIP2;1 can be inhibited by treatment with proteasome inhibitors. These results and the fact that Rma1H1 is able to ubiquitinate PIP2;1 *in vivo* indicate that PIP2;1 protein stability is regulated by the UPS. In addition to regulating PIP2;1 abundance, Rma1H1 also influences PIP2;1 localization. PIP2;1 is localized mainly at the plasma membrane, however in the presence of Rma1H1, PIP2;1 is mostly found at the ER membrane. Hot pepper *Rma1H1* has three *Arabidopsis* homologs, *Rma1*, *Rma2* and *Rma3* (Lee *et al*. 2009). Similar to Rma1H1, Rma1 overexpression reduces PIP2;1 levels and inhibits its trafficking from the ER to the plasma membrane in protoplasts. During drought stress, Rma1H1 and its *Arabidopsis* counterparts Rma1 may function to inhibit aquaporin trafficking and mediate proteasomal degradation of PIP2;1 to reduce water loss.

UTILIZING THE UPS TO GENERATE PLANTS WITH ENHANCED STRESS TOLERANCE

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 growth and production, 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 tolerance establishes a direct link between the UPS and various stress response mechanisms. The UPS may function downstream of perception of external stimuli to ensure fast, efficient and effective cellular responses to environmental stresses. The UPS enables plants to alter their proteome in order to ensure cellular adaptations essential for growth and survival.

Recent advances in our understanding of the role of the UPS in stress tolerance provide opportunities to exploit this important and versatile pathway to improve plant tolerance of abiotic stresses. Plant stress tolerance may be enhanced by manipulating components of the UPS, in particular the substrate-recruiting E3 ubiquitin ligases. The potential usefulness of this approach is illustrated by the RING-type E3 ligase SDIR1. Overexpression of *SDIR1*, a positive regulator of ABA signalling, in crop plants successfully increased drought stress tolerance (Zhang *et al*. 2008; Gao *et al*. 2011). After 28 days of exposure to drought conditions followed by 10 days of rewatering 60% of transgenic tobacco plants overexpressing Arabidopsis *SDIR1* survived, compared to only 30% of control plants (Zhang *et al*. 2008). Improved drought tolerance observed for the transgenic plants may be due to increased efficiency in ABA-mediated stomatal closure. The rice *SDIR1* gene, *OsSDIR1*, was recently identified and shown to function similarly to the Arabidopsis *SDIR1* (Gao *et al*. 2011). OsSDIR1 is also a functional membrane bound RING-type E3 ligase. Transgenic rice plants overexpressing *OsSDIR1* displayed enhanced drought tolerance. For example, at the seedling stage, the survival rate for transgenic rice plants, following six days of drought treatment and one day of rewatering, was reported to be over 90%, whereas none of the control plants survived (Gao *et al*. 2011). As observed with Arabidopsis, the increased drought tolerance correlated with an increase in stomata closure. Under favourable growth conditions transgenic overexpressing *OsSDIR1* rice plants grew slower that control plants at the seedling stage, exhibiting shorter aerial organs and roots. This growth delay is an unwanted effect; however growth and seed set of the transgenic rice plants was comparable to that of control plants once they were transferred to soil (Gao *et al*. 2011). As *SDIR1* can function as a drought tolerant gene in both dicotyledons and monocotyledons it may prove to be a useful candidate for engineering drought tolerant crops. As this area of research develops, additional ubiquitin ligases that regulate abiotic stress responses will be identified, expanding the list of suitable candidates that maybe used to generate plants with enhanced stress tolerance.

The utility of the UPS to enhance stress tolerance also hinges on the identification of target proteins of the E3 ligase in question. Manipulation of E3 ligases will have varying consequences on plant stress tolerance depending on the function of the target protein. For example, down-regulation of an E3 ligase would result in an accumulation of its substrate which would lead to increased stress tolerance if the substrate was a positive effector of the response pathway. If the substrate is a stress-responsive transcriptional activator, this would lead to increased expression of all downstream stress-responsive genes. An additional advantage would be if the target transcription factor is utilized by the promoter of genes that respond to different stresses, this may lead to enhanced tolerance to multiple stresses. Similar predictions can be made for other potential E3 ligase substrates that are components of stress response signalling pathways such as a kinase, phosphatases as well as hormone biosynthetic or catabolic enzymes. How target proteins function to alleviate the effects of stress will also influence whether or not a particular E3 ligase is a suitable candidate for manipulation. DREB2A, a target of DRIP1 and DRIP2 RING-type E3 ligases, is suggested to alleviate the effects of adverse growth conditions by slowing or delaying plant growth. Overexpression of a stable form of DREB2A or down-regulation of DRIP1 and DRIP2, which stabilizes DREB2A protein, significantly enhances drought tolerance (Sakuma *et al*. 2006a, 2006b; Qin *et al*. 2008). However, stabilization of DREB2A produces an undesired delayed growth phenotype under favourable growth conditions. The fact that DREB2A overexpression negatively affected plant growth and development under non-stressed conditions limits the potential of targeting DRIP1 and DRIP2 for enhancing plant stress tolerance. Lack of knowledge of target identity and function limits understanding the full effects of manipulating the ubiquitin ligase of interest. Thus, in addition to identifying E3 ligases with roles in regulating stress responses, substrate identification is critical to furthering our understanding of the role of the UPS in stress tolerance and to engineering plant tolerance to abiotic stresses.

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