Identification of Roles for *Arabidopsis thaliana* RING-Type Ubiquitin Ligase XBAT35.2 and its Substrate Accelerated Cell Death11 (ACD11) in Abiotic Stress Tolerance

by

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I dedicate this work to my parents, for their unconditional love and open-mindedness.

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ABSTRACT

To survive adverse environmental conditions plants utilize various mechanisms including the production of the hormone abscisic acid, which promote cellular changes to ameliorate the negative effects of abiotic stress. The Ubiquitination Proteasome System is used to regulate stress signaling to properly initiate and terminate responses. Ubiquitination involves E3s that govern substrate selection. Here, we demonstrate that the E3 XBAT35.2 and its substrate ACD11 are involved in the abiotic stress tolerance. XBAT35.2 levels, and levels of ACD11 increase in response to stress. Surprisingly, prolonged exposure to stress leads to a decrease in ACD11 abundance. *xbat35-1* mutants and plants overexpressing ACD11 display enhanced stress tolerance. Also, XBAT35.2 promotes proteasome-dependent degradation of ACD11 under stress conditions. We propose that while ACD11 promotes tolerance, stress-stabilized XBAT35.2 functions to attenuate/terminate ACD11-mediated responses. This study improves our understanding of XBAT35.2 and ACD11 function and may contribute knowledge to the development of stress tolerant crops.

LIST OF ABBREVIATIONS USED

Arabidopsis	*Arabidopsis thaliana*
ABA	Abscisic Acid
ABD1	ABA-hypersensitive DCAF1
ABI1	Abscisic Acid Insensitive 1
ABI5	Abscisic Acid Insensitive 5
ABRC	Arabidopsis Biological Resource Center
ACD11	Accelerated Cell Death11
ACD5	Accelerated Cell Death5
ATP	Adenosine triphosphate
BiFC	Bimolecular fluorescence complementation
BPA1	Binding Partner of ACD11
BPL-like	BPA1-Like proteins
bZIP	Basic leucine zipper
C-terminus	Carboxyl-terminus
C1P	Ceramide-1-phosphate
cDNA	Complementary deoxyribonucleic acid
CHX	Cycloheximide
CHYR1	A ubiquitin ligase
CIPK26	Calcineurin B-like Interacting Protein Kinase 26
Col-0	Columbia ecotype
COP1	CONSTITUTIVE PHOTOMORPHOGENIC1
CSU1	COP1 SUPPRESSOR1
DGAT1	Diacylglycerol acyltransferase 1
DNA	Deoxyribonucleic acid
DTT	DL-Dithiothreitol
DWA1	DWD hypersensitive to ABA1
DWA2	DWD hypersensitive to ABA2
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin Ligase
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetra acetic acid
FLS2	FLAGELLIN SENSING 2
GFP	green fluorescence protein
GM	Growth medium
GST	Glutathione S-transferase
GUS	β-glucuronidase
h	Hour

HA	Hemagglutinin
HECT	Homologous to E6-AP carboxyl terminus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IP	Immunoprecipitation
K+	potassium
kDa	Kilo Daltons
KEG	Keep on Going
LAZ	*LAZARUS* proteins
Lys	Lysine
MG132	N-(benzyloxycarbonyl)leucinylleucinylleucinal Z-Leu-Leu-Leu-al
MIEL1	MYB30-Interacting E3 Ligase 1
min	minute
mM	millimolar
MOCA1	monocation-induced [Ca2+] increases 1
MS	Murashige and Skoog
MYB30	MYB DOMAIN PROTEIN 30
MYB96	MYB DOMAIN PROTEIN 96
Na+	Sodium
NaCl	Sodium Chloride
NahG	The mutant of Nucleotide sequence analysis of the *Pseudomonas putida* PpG7 salicylate hydroxylase gene
NLS	nuclear localization signal
PAGE	Polyacrylamide gel electrophoresis
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl fluoride
PP2Cs	group A Pprotein Pphosphatase type 2Cs
PR genes	*pathogen-related* genes
PRA1.F	Prenylated Rab Acceptor 1.F
PTMs	Post-translation modifications
PUB12	Plant U-box 12
PUB13	Plant U-box 13
PUB22	Plant U-box 22
PVDF	Polyvinylidene fluoride
PYL4	A ABA receptor
PYR/PYLs	(PYR1)/PYR1-like (PYLs)/Rregulatory Ccomponents of ABA Receptors (RCAR) family
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RAV1	Related to ABI3/VP1
RGLG1	RING DOMAIN LIGASE1

RING	Really Interesting New Gene
ROS	Reactive oxygen species
RPN10	A subunit of the 26S proteasome
SA	Salicylic acid
SDS	Sodium dodecyl sulfate
SE	Standard error
sid2-2	The mutant of SALICYLIC ACID INDUCTION DEFICIENT 2 gene
Skp1	S-phase kinase-associated protein 1
SNF	Sucrose non-fermenting
SnRK	Sucrose non-fermenting-related kinase
SnRK2s	Sucrose Non-Fermenting 1 (SNF1)-Related Protein Kinase 2s
T-BST	Tris-buffered saline-Tween 20
TAG	Triacylglycero
TFs	Transcription factors
Ub	Ubiquitin
UPS	Ubiquitin Proteasome System
VPS35	Vacuolar Protein Sorting35
XB3	XA21-binding protein 3
XBAT35.2^{AA}	The cDNA of XBT35.2 with a RING domain-encoding region containing C426A and H428A point mutations
XBAT	XB3 in *Arabidopsis thaliana*
YFP	Yellow fluorescent protein
μM	Micromolar

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CHAPTER ONE: INTRODUCTION

1.1 Environmental Stress

Stress is described as a sudden change of the environment that exceeds the organism's ability to maintain homeostasis, to which the organism must then acclimate (Foundation, 2010). As immobile organisms, plants are inevitably challenged by environmental stress. These external negative factors can be categorized into two types, biotic and abiotic stress (Bulgari et al., 2019). The former is stress originating from other living organisms, including insects, fungi, bacteria, as well as virus, which possibly reduces the biomass and quality, or even kill plants. To counter this type of stress, plants establish a range of immune responses, such as the production of antimicrobial compounds or the induction of Programmed Cell Death (PCD), which is capable of restricting the progression of pests (Bulgari et al., 2019). The other type of stress is imposed by the non-living organism's factors, namely, adverse environmental conditions, such as high soil salinity, drought and heat (Foundation, 2010). These unfavorable conditions not only limit the distribution of plant species, but also lead to the huge loss of yield (Pandey et al., 2017).

1.1.1 Abiotic stress

In the light of current climate change scenarios, plants are increasingly exposed to the extreme weather (Rahmstorf and Coumou, 2011). On the population level, these negative external factors largely decrease productivity and quality of plants. On the individual level, abiotic stress damages cellular components of the cell, such as nucleic acids, proteins or membrane lipids (Acosta-Motos et al., 2017). Abiotic stress may also result in the dysfunction of particular processes in plants, such as the decrease in seed germination rate and the stomatal closure (Acosta-Motos et al., 2017). Abiotic stress might reduce the production of metabolites, which may heavily affect cereal grains during the grain filling period (Ramakrishna and Ravishankar, 2011; Stagnari et al., 2016). It is estimated that up to 20% of total cultivated lands and approximately one thirds of irrigated agricultural lands are influenced by high salinity worldwide (Jamil et al., 2011). High salinity inhibits water uptake of the cell, causing the water deficit in plants (Yang and Guo, 2018). In addition, excessive accumulation of sodium (Na^+) results in the ion imbalance that hinders the absorption of potassium (K^+), which is critical for activation of certain

enzymes (Hasanuzzaman et al., 2018). Also, the production of reactive oxygen species (ROS) is a negative effect of salt stress, which damages the DNA, proteins or enzymes, ultimately leading to the cell death (Choudhury et al., 2017).

To acclimate and survive the continually changing environment, plants have evolved an array of effective and sophisticated regulatory networks to sense as well as adapt to stress (**Figure 1.1**) (Bohnert, 2007; Foundation, 2010; Zhu, 2016). Once abiotic stress is perceived, plants are capable of initiating several signaling cascades, especially the abscisic acid (ABA) pathway (Foundation, 2010). The activation of signaling networks involves a myriad of kinases or phosphatases that in turn switch on activity of downstream effectors, such as transcription factors (TFs), ROS scavengers (e.g. proline), as well as chaperon proteins (Lindemose et al., 2013). In general, these elements are responsible for the delay of seed germination, the closure of stomata, the arrest of early seedling growth, the elimination of ROS as well as limiting protein misfolding, which directly mitigates the damaging effects of abiotic stress (Foundation, 2010; Khan and Hakeem, 2014).

Phytohormones, including ABA, salicylic acid (SA), ethylene and gibberellin, are essential for plant development and stress tolerance (McSteen and Zhao, 2008; Vishwakarma et al., 2017). Among these hormones, ABA plays a fundamental role in plant tolerance of abiotic stress (Foundation, 2010; Sah et al., 2016). It is a small lipophilic sesquiterpeniod (C15), which has diverse functions in plants (Finkelstein, 2013). ABA finely tunes seed germination and dormancy, early seedling establishment, stomatal opening, leaf senescence, flowering time and pathogen resistance (Foundation, 2010; Finkelstein, 2013). Upon exposure to abiotic stress, plants accelerate ABA synthesis and then promote a range of responses, including the delay in germination, the arrest of early seedling growth and the induction of stomatal closure, in order to ameliorate negative impacts of abiotic stress (Cutler et al., 2010; Foundation, 2010). Emerging studies showed that ABA can also facilitate leaf senescence for nutrient reuse, which could promote drought tolerance (Rivero et al., 2007; Zhao et al., 2016; Liu et al., 2019). In addition to ABA, the role of SA in counteracting negative effects of abiotic stress has been demonstrated recently (Rivas-San Vicente and Plasencia, 2011; Khan et

al., 2015). SA is able to confer salinity stress tolerance in a variety of crops (e.g. *Brassica juncea Medicago sativa*) possibly through upregulating chlorophyll content or increasing activity of antioxidant enzymes (Lee et al., 2010; Palma et al., 2013). SA may also alleviate salt stress by impeding salt-induced membrane depolarization (Jayakannan et al., 2013). However, the SA signaling cascade in response to abiotic stress remains elusive. In contrast, the ABA signaling network in abiotic stress responses is well established.

1.1.2 ABA signaling

One of the most significant breakthrough for understanding ABA signaling was the characterization of the ABA-binding receptors, Pyrabactin Resistance 1 (PYR1)/PYR1-like (PYLs)/Regulatory Components of ABA Receptors (RCAR) family (hereafter referred to as PYR/PYLs) (Kline et al., 2010; Zhu, 2016). Other components of the core ABA signaling network include group A Protein Phosphatase type 2Cs (PP2Cs), Sucrose Non-Fermenting 1 (SNF1)-Related Protein Kinase 2s (SnRK2s), and downstream elements such as ABA-responsive transcription factors (Zhu, 2016). The PP2Cs are negative regulators in ABA signaling via their dephosphorylation activity towards the SnRK2s and other proteins (Zhu, 2016). The SnRK2s act as positive modulators by phosphorylating target proteins, such as transcription factors responsible for promoting expression of ABA-responsive genes (Hubbard et al., 2010; Zhu, 2016). In the absence of ABA, PP2Cs physically interact with SnRK2s to repress the phosphorylation activity of these kinases and to block ABA signal transduction (Zhu, 2016). In response to abiotic stress, ABA molecules are bound by PYR/PYLs. This interaction creates a binding surface for the PP2Cs on the receptors (Melcher et al., 2009; Zhu, 2016). PYR/PYLs binding of PP2C sequesters the phosphatase and makes it unable to dephosphorylate the SnRK2s, thus relieving the inhibition of kinase activity (Zhu, 2016). The released kinases are capable of auto-phosphorylation, which can then phosphorylate a group of downstream effectors, such as transcription factors, allowing for the ABA signaling transduction and stress responses (Soon et al., 2012). There have been multiple transcription factors elucidated to be associated with the ABA signaling. They serve as "molecular switches", to activate or repress expression of a wide range of downstream stress responsive genes (Khan et al., 2018). For instance, ABA Insensitive 5 (ABI5),

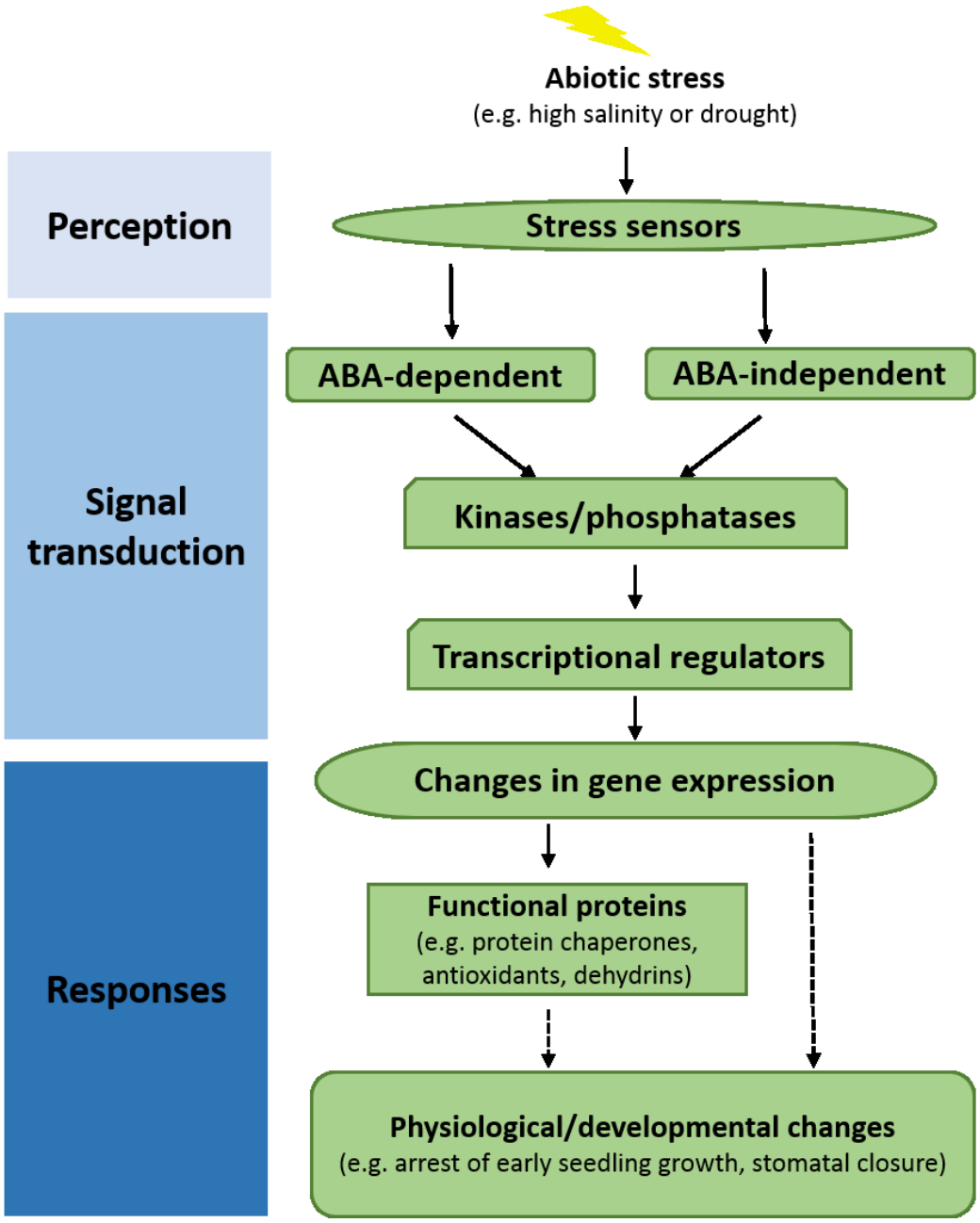


Figure 1.1. Simplified model for abiotic stress signaling in plants

The establishment of abiotic stress tolerance in plants has three stages; stress perception, signal transduction and responses. Following exposure to abiotic stress, stress sensors in plants are able to perceive the change and then trigger ABA-dependent and/or -independent pathways. The activated signaling networks lead to changes in gene expression that enable adaptions to adverse environmental conditions (Xiong et al., 2002; Huang et al., 2012; Cabello et al., 2014).

belonging to the basic leucine zipper (bZIP) transcription factor family, is a positive regulator of ABA responses. (Khan et al., 2018). In the presence of ABA, *ABI5* expression is induced, which leads to the upregulation of *diacylglycerol acyltransferase 1* (*DGAT1*) that is responsible for catalyzing the biosynthesis of Triacylglycerol (TAG). The accumulation of TAG in vegetative tissues confers plant with tolerance to drought stress (Kong et al., 2013; Lee et al., 2019).

To mount or terminate ABA responses in an efficient and timely manner, plants employ a selection of molecular regulators at transcriptional and post-translational levels (Fujita et al., 2011; Yang et al., 2017a; Stone, 2019). One well-studied point of transcriptional regulation is mediated through transcription factors. For example, upon exposure to ABA, plants increase transcript levels of *ABI5* through repressing activity of the negative regulator, Related to ABI3/VP1 (RAV1) transcription factor (Feng et al., 2014). Another principal layer of regulation in ABA signaling is post-translation modifications (PTMs), which controls the activity or abundance of primary components of the pathway (Stone, 2019). Phosphorylation/dephosphorylation and ubiquitination are two of the most prevalent PTMs used to modulate ABA signaling components. Phosphorylation/dephosphorylation directed by the core components, PP2Cs or SnRK2s, functions to repress or to activate signal transduction (Yang et al., 2017a). Likewise, ubiquitination plays critical roles in ABA signaling by regulating endomembrane trafficking and proteolysis of core components. (Yu et al., 2016; Yu and Xie, 2017). Endocytic trafficking is responsible for sorting and transferring of plasma membrane proteins, in turn modulating their abundance or compartmentalization (Paez Valencia et al., 2016; Yu and Xie, 2017). Several novel studies showed that the endomembrane trafficking finely tuned the turnover of ABA receptor, PYL4, leading to the attenuation of ABA responses (Belda-Palazon et al., 2016). The Ubiquitin-Proteasome System (UPS) is also found to regulate the abundance of several ABA elements from the point of hormone perception to output (Liu and Stone, 2011; Yu et al., 2016). For example, in the absence of stress, ABI5 is ubiquitinated to keep levels low (Liu and Stone, 2010; Liu and Stone, 2013). In response to abiotic stress, increase in ABA levels prohibits the ubiquitination of ABI5, which leads to accumulation of the transcription factor (Liu and Stone, 2010; Liu

and Stone, 2013). In fact, ABI5 proteins can be also again ubiquitinated to dampen or terminate ABI5-mediated responses (Lee et al., 2010a; Seo et al., 2014; Stone, 2019).

1.2 Ubiquitin-Proteasome System (UPS)

The UPS is composed of two distinct processes: first, the ubiquitination of protein substrates and, second the turnover of modified targets by the 26S proteasome (Callis, 2014). Ubiquitination is a highly conserved pathway in eukaryotes that was discovered by Aaron Ciechanover, Irwin Rose, and Avram Hershko in the early 1980s, for which they received the 2004 Nobel prize in chemistry (Wilkinson, 2005). Proteins with certain ubiquitination signatures will be sent to the 26S proteasome in both the cytoplasm and the nucleus for degradation, and the resulting amino acids and ubiquitin molecules are recycled (Vierstra, 2009a). In plants, the UPS is extensively involved in growth, development and responding to the environment. Previous reports have shown that loss of components of the UPS results in abnormal development or failures to establish stress responses (Moon et al., 2004; Craig et al., 2009). For instance, RPN10, an important component of the 26S proteasome plays a significant role in ABA-dependent stress responses, because *rpn10-1* mutants are hypersensitive to ABA and are less tolerant to salt in both seed germination and early seedling growth (Smalle et al., 2003).

1.2.1 Ubiquitination

Ubiquitination is a multistep enzymatic reaction that results in the covalent conjugation of ubiquitin molecules to a selected protein (**Figure 1.2**) (Stone, 2014). The attaching cascade begins with the E1 (ubiquitin activating enzyme; UBA) that activates the ubiquitin moiety and forms an E1-ubiquitin intermediate. The ubiquitin is then transferred from the E1 to the E2 (ubiquitin-conjugating enzyme), also forming a thioester linked E2-ubiquitin intermediate. The substrate-recruiting E3 ligase interacts the E2-ubiquitin intermediate, leading to the transfer of ubiquitin to a lysine residue on the bound target (monoubiquitination) (Stone, 2014). The conjugation process can be repeated to generate a polyubiquitin chain on a single lysine of the substrate (polyubiquitination) or to attach additional ubiquitin molecules to other lysine residues on the substrate (multimonoubiquitination). As for the outcome of each type of ubiquitination, monoubiquitination is reported to regulate chromatin structures, protein

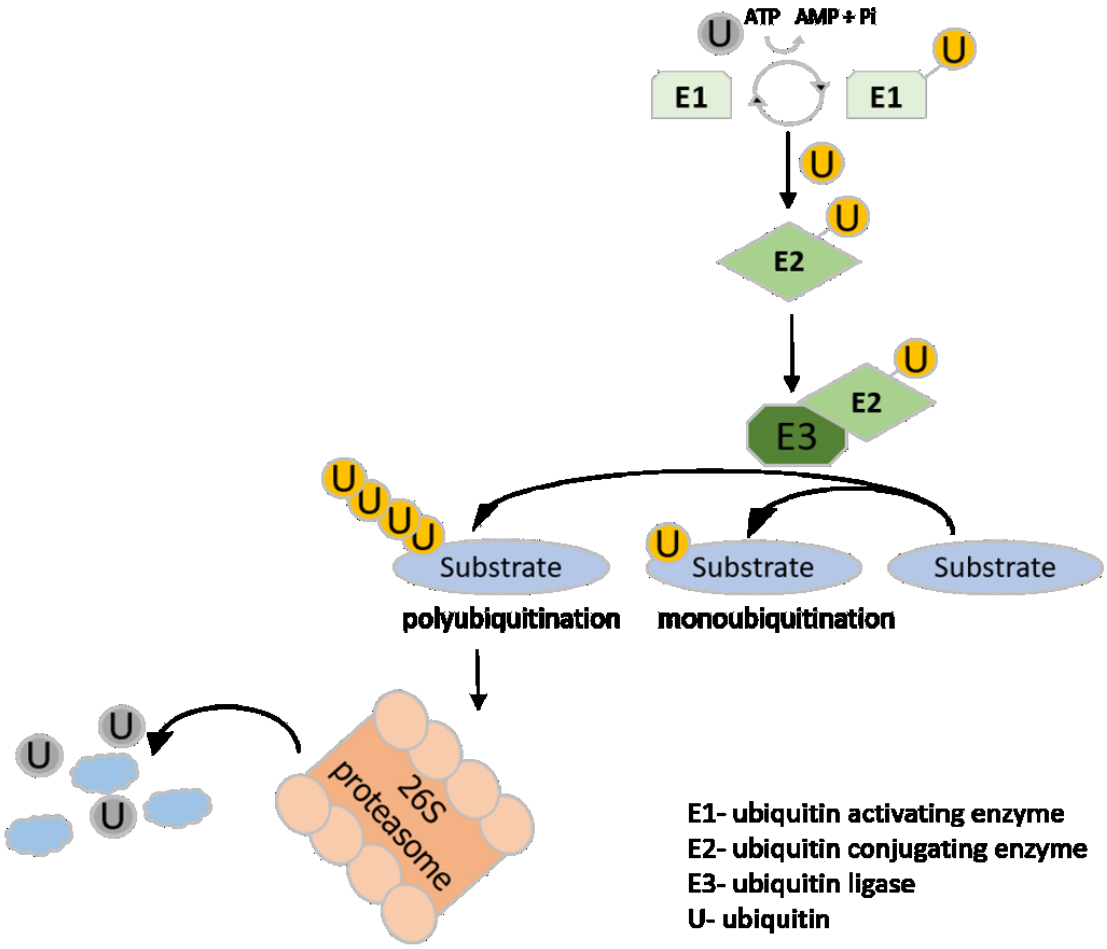


Figure 1.2. The Ubiquitin-Proteasome System (UPS)

The UPS involves the attachment of ubiquitin molecule(s) to a selected protein followed by degradation of the substrate via the 26S proteasome. The attachment of ubiquitin to a substrate involves three enzymes; the E1 (ubiquitin activating enzyme), which activates ubiquitin with the consumption of ATP; the E2 (ubiquitin conjugating enzyme) that binds ubiquitin to form an E2-intermediate and the E3 (ubiquitin ligase) who facilitates the transfer of ubiquitin from E2 to the selected substrate (monoubiquitination). The conjugation process is repeated to generate a polyubiquitin chain on the substrate (polyubiquitination). The polyubiquitinated protein is recognized and degraded by the 26S proteasome. Released ubiquitin molecules and peptides are recycled by the cell (Callis, 2014).

sorting and trafficking as well as proteasome-dependent turnover (Barberon et al., 2011; Feng and Shen, 2014; Braten et al., 2016). However, polyubiquitinated proteins could be removed from the cell through proteasomal or autophagic degradation (Callis, 2014; Ronai, 2016).

Ubiquitin (Ub) is a 76-amino-acid polypeptide highly conserved across eukaryotic cells (Callis, 2014). Ub has seven lysine (K) residues (K6, K11, K27, K29, K33, K48, and K63) as well as the amino-terminal methionine (M1), which can be used in ubiquitin-ubiquitin linkages for generating a polyubiquitin chain (Zheng and Shabek, 2017). K48- or K11-linked polyubiquitin chains are the most common forms found in cells (Jacobson et al., 2009; Zheng and Shabek, 2017). Substrates modified with a K48-linked polyubiquitin chain containing at least four ubiquitin molecules are targeted to the proteasome for degradation (Thrower, 2000).

1.2.2 Ubiquitin Ligases

Ubiquitin ligases or E3s are central to the ubiquitination process because they govern substrate specificity (Sun et al., 2004; Vierstra, 2009b; Callis, 2014). The genome of *Arabidopsis thaliana* (Arabidopsis) predictably expresses 2 E1s, at least 37 E2s and more than 1300 E3s or components of E3 complexes (Smalle and Vierstra, 2004; Kraft et al., 2005; Stone et al., 2005). Based on the presence of E2-binding domains, ubiquitin ligases are classified into three groups: Homologous to E6-AP carboxyl terminus (HECT), U-box, or Really Interesting New Gene (RING) domain (Callis, 2014). Among these, the RING-type E3s are the most abundant with around 500 proteins encoded by the Arabidopsis genome (Stone et al., 2005). The RING domain is defined by a 40-60 amino acid sequence that contains an octet of conserved cysteine and histidine residues that coordinate two zinc ions in a cross brace structure (Callis, 2014).

Generally, the RING domain functions as the E2-interacting region, which could facilitate the transfer of ubiquitin from the E2-ubiquitin intermediate to protein substrates (Callis, 2014). RING-type ubiquitin ligases can be further grouped into two categories, monomeric or multimeric E3s (Chen and Hellmann, 2013). The monomeric RING-type E3s interact with both the E2 and substrates simultaneously in order to mediate

ubiquitination, whereas multimeric ones rely on different proteins within the complex for recruiting the E2 and substrates to promote ubiquitination (Chen and Hellmann, 2013).

Functions for ubiquitin ligases in plant development and stress responses have been well-characterized over the past two decades. Regarding biotic stress, there are more than 30 E3s that can regulate plant resistance ranging from modulating pathogen detection to regulating transcriptional activation of defense genes (Casey et al., 2017; Yin et al., 2019). For example, the pattern-recognition receptors, Flagellin Sensing 2 (FLS2), serving to trigger plant immune responses, is reported to be polyubiquitinated by Plant U-box 12 (PUB12) and PUB13, which subsequently leads to its turnover via the 26S proteasome (Lu et al., 2011).Such degradation is proposed to attenuate the immune signaling (Lu et al., 2011). Roles for over 35 ubiquitin ligases have been revealed in abiotic stress responses, including targeting core transcription factors for degradation and directing the breakdown of hormone receptors (Stone, 2019). For instance, in the presence of ABA, E3 ligases PUB12/13 ubiquitinate one of PP2Cs, ABI1, to promote its proteasome-dependent degradation, enhancing ABA responses. (Kong et al., 2015). In fact, PUB12/13 are among an emerging suite of ubiquitin ligases, such as KEG and MYB30-Interacting E3 Ligase 1 (MIEL1), with dual function in both biotic and abiotic stress signaling (Wawrzynska et al., 2008; Liu and Stone, 2010; Hong et al., 2011; Kong et al., 2015; Lee and Seo, 2016). During the immune signaling, MIEL1 targets the positive defense modulator MYB Domain Protein 30 (MYB30) for breakdown via the 26S proteasome to attenuate defense responses (Marino et al., 2013). However, in the context of abiotic stress, MIEL1 is responsible for the ubiquitination and subsequent proteasome-dependent degradation of MYB96 (Lee and Seo, 2016). As a consequence, it is proposed that MIEL1 is able to coordinate ABA-dependent stress responses and immune responses in Arabidopsis (Lee and Seo, 2016).

1.2.3 Modulation of ubiquitin ligase activity

Due to essential roles of ubiquitin ligase in the cell, their activity is tightly controlled via several mechanisms. One unique feature of most ubiquitin ligases is that they can mediate self-ubiquitination to modulate their own abundance or activity (Liu and Stone, 2010; Lyzenga et al., 2012a; Furlan et al., 2017). For instance, it is reported that ABA-mediated

the proteasome-dependent turnover of KEG is facilitated by self-ubiquitination, allowing for the accumulation of ABA-related substrate proteins (Liu and Stone, 2010; Chen et al., 2013). Alternatively, ubiquitination of E3s could be mediated by other ubiquitin ligases. For instance, the RING-type E3 Constitutive Photomorphogenic1 (COP1), a repressor of photomorphogenesis, is targeted by another RING-type ubiquitin ligase COP1 Suppressor1 (CSU1) for ubiquitination and subsequent turnover via the 26S proteasome, in order to control the abundance of COP1 in the dark (Xu et al., 2014). Phosphorylation is also a common mechanism used to modulate the ubiquitin ligase activity in plants. For example, phosphorylation of E3 Plant U-box 22 (PUB22) blocks its oligomerization, which in turn dampens self-ubiquitination so that the E3 is then able to negatively regulate immune responses (Furlan et al., 2017). Moreover, myristoylation of RING Domain Ligase1 (RGLG1) is involved in controlling translocation of the E3 from the nuclear to the plasma membrane (Furlan et al., 2017). ABA hinders the myristoylation of RGLG1 to promote its nuclear translocation, possibly allowing interactions of RGLG1 and its nuclear-localized partners PP2Cs (Furlan et al., 2017).

1.2.4 XBAT35

Monomeric RING-type E3 subfamily XBAT (for XB3 in *Arabidopsis thaliana*), is named due to its structural similarity to the rice (*Oryza sativa*) XB3 protein (Nodzon et al., 2004). There are five members in this subgroup (XBAT31-XBAT35), each characterized by the presence of a carboxyl-terminal RING domain and a series of amino-terminal Ankyrin repeats (**Figure 1.3**) (Yuan et al., 2013). Ankyrin repeats mediate protein-protein interaction and may function to recruit substrates for ubiquitination (Yuan et al., 2013). Three members, XBAT31, XBAT32 and XBAT35 have been assigned physiological functions. XBAT31 has been found to mediate iron deficiency responses in plants (Schiavi et al., unpublished results). XBAT32 has been identified as a negative regulator of ethylene biosynthesis and is also involved in lateral root development (Prasad et al., 2010; Lyzenga et al., 2012b). XBAT35 has been implicated in apical hook formation and pathogen defense (Carvalho et al., 2012; Liu et al., 2017).

XBAT35 transcript is alternatively spliced to produce two isoforms; the nuclear localized XBAT35.1, and the cytosol and Golgi localized XBAT35.2 (**Figure 1.4**)

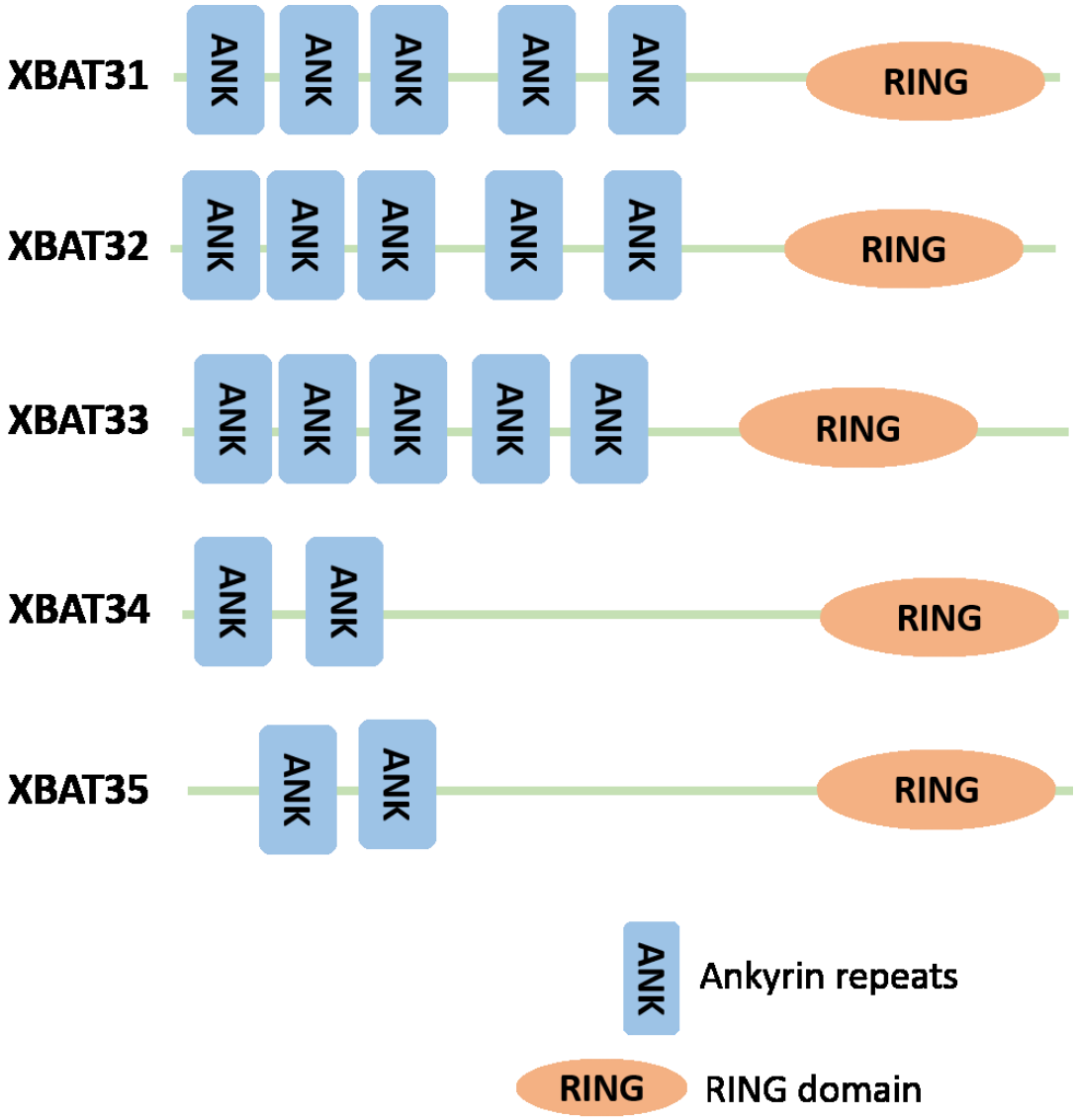


Figure 1.3. The RING-type E3 XBAT subgroup in Arabidopsis

XBAT subfamily includes five members and each has a carboxyl terminal E2-binding RING domain and a series of Ankyrin repeats (Yuan et al., 2013).

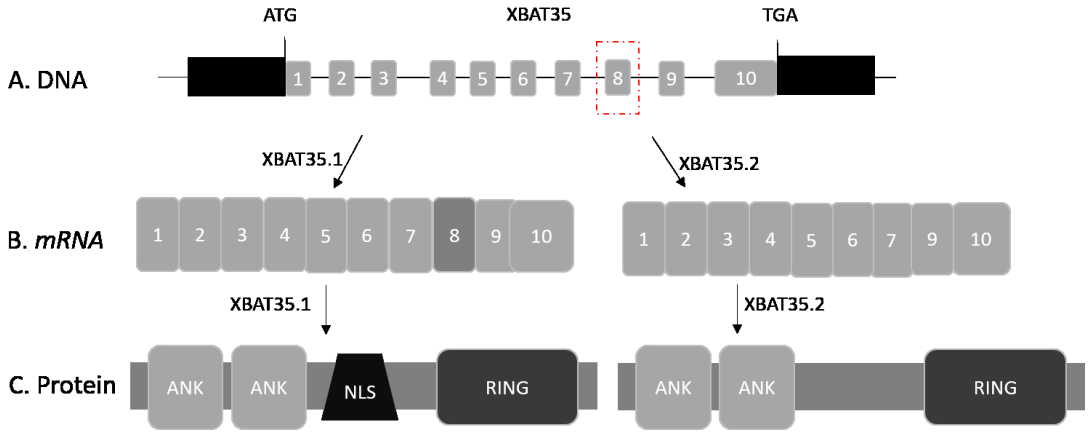


Figure 1.4. The alternative splicing event of XBAT35

Schematic of XBAT35 gene structure (A; boxes represent exons and untranslated regions in gray and black, respectively, and lines between boxes indicate introns), the coding region of the transcripts for the two splice variants, *XBAT35.1* and *XBAT35.2*, produced by the alternative splicing (B; exon 8 is skipped), and the predicted domain structure of the two protein isoforms (C) (Carvalho et al., 2012).

(Carvalho et al., 2012; Liu et al., 2017). Exon skipping allows for the exclusion of exon 8 from the XBAT35 mRNA, leading to the generation of a slightly smaller transcript, *XBAT35.2*, which lacks a nuclear localization signal (NLS) (Carvalho et al., 2012). Both *XBAT35* transcripts are ubiquitously expressed in Arabidopsis (Carvalho et al., 2012). In addition, both XBAT35.1 and XBAT35.2 proteins are functional ubiquitin ligases (Carvalho et al., 2012). It is notable that XBAT35.2 undergoes self-ubiquitination to modulate its own degradation through the 26S proteasome (Liu et al., 2017). As for biological roles, both isoforms are biologically active in the ethylene signaling network and serve as negative regulators of hook curvature (Carvalho et al., 2012). In addition, XBAT35.2, but not XBAT35.1, was found to promote cell death (Liu et al., 2017). Most recently, XBAT35.2 was reported to interact with Accelerated Cell Death11 (ACD11) in plant cells by using the Bimolecular fluorescence complementation (BiFC) assays (Liu et al., 2017). Besides, immunoprecipitation (IP) assays also indicated that XBAT35.2 is capable of interacting with and pulling down XBAT35.2 (Liu et al., 2017). Moreover, Glutathione S-transferase (GST) pull-down assays showed that XBAT35.2 can directly interact with ACD11 (Liu et al., 2017). Furthermore, results of colocalization between XBAT35.2 and ACD11 also supported the conclusion that XBAT35.2 truly interacts with ACD11 (Liu et al., 2017).

Using in vitro cell free degradation experiments, it was demonstrated that a functional XBAT35.2 is required for the proteasome-dependent turnover of ACD11 (Liu et al., 2017). Secondly, following exposure to pathogen infection, the abundance of XBAT35.2 elevates, which correlates with the decrease in polyubiquitination of the E3 (Liu et al., 2017). On the other hand, upon pathogen infections, the protein levels of ACD11 decrease, which is consistent with the increased ubiquitination levels of ACD11 (Liu et al., 2017). In addition, xbat35-1 are vulnerable to pathogen infection relative to wild type plants, whereas transgenic plants overexpressing XBAT35.2 exhibit less susceptibility to such infection, suggesting the positive role for the E3 in mounting pathogen defense responses (Liu et al., 2017). A model for XBAT35.2 function proposes that in absence of pathogen, XBAT35.2 is unstable due to self-ubiquitination (**Figure 1.5**). Therefore, ACD11 is accumulated and can prohibit the cell death. In contrast, in the presence of

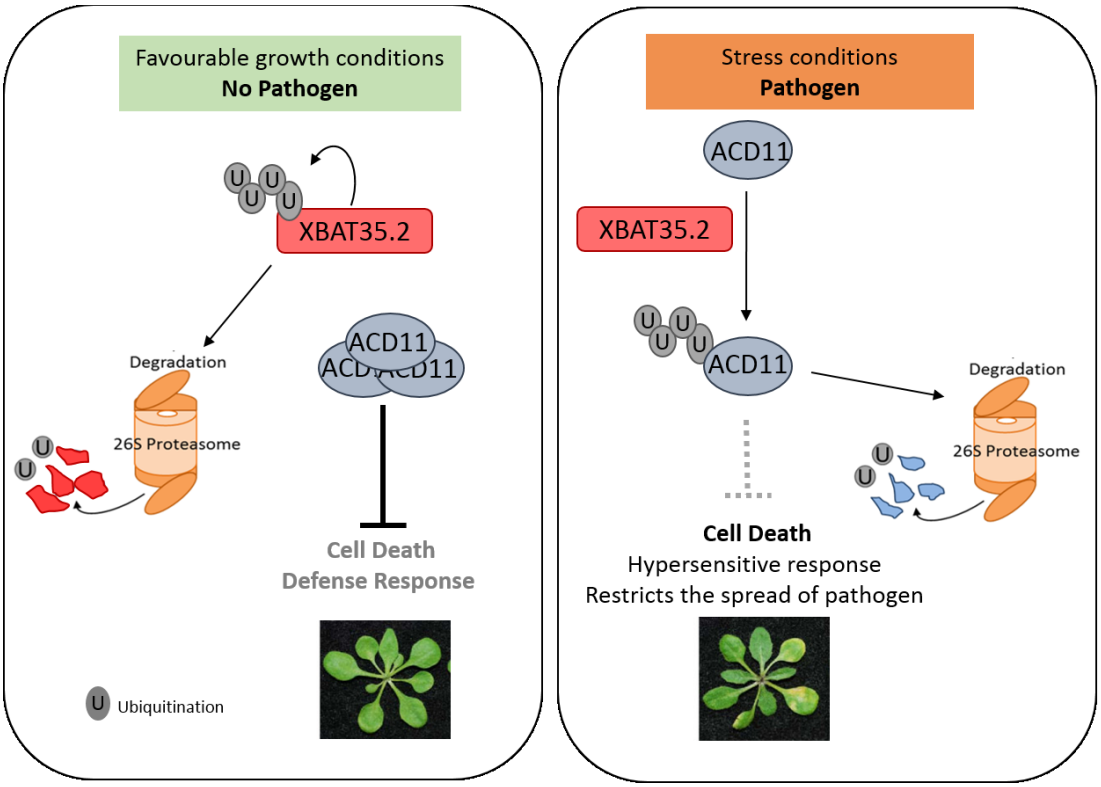


Figure 1.5. Model for XBAT35.2 regulating ACD11 abundance during biotic stress

Under non-stress conditions, XBAT35.2 is subjected to self-ubiquitination and degradation, which maintains low levels of the E3, allowing ACD11 to accumulate to a particular level and inhibit the cell death. In the presence of pathogen, XBAT35.2 becomes stable and promotes the 26S proteasome-dependent turnover of ACD11, which leads to cell death as part of hypersensitive responses (Liu et al., 2017).

pathogen, XBAT35.2 becomes stable, promoting the proteasome-dependent degradation of ACD11, which leads to cell death that restricts the spread of pathogen as part of defense responses.

1.2.5 Accelerated Cell Death 11 (ACD11)

The *acd11* mutant displays PCD at the four leaf stage and also constitutively expresses a number of pathogen-related genes (Brodersen et al., 2002). The activation of PCD and defense pathways in *acd11* requires SA signaling (Brodersen et al., 2002). In addition, *acd11* accumulates SA, and its cell death phenotype can be fully suppressed in the SA-deficient mutants, *NahG* and *sid2-2* (Brodersen, 2005). A group of suppression mutations in the *LAZARUS* (*LAZ*) genes were isolated based on their ability to suppress the cell death phenotype in *acd11*. *LAZ1* is a potential regulator of PCD related to the hypersensitive response and pathogen defense, (Malinovsky et al., 2010). LAZ2 encodes a histone lysine methyltransferase (Palma et al., 2010). LAZ4 encodes for Vacuolar Protein Sorting35 (VPS35), which plays a role endomembrane trafficking (Munch et al., 2015). LAZ5 is a member of immune receptor associated with the detection of pathogens and following cell death (Palma et al., 2010). Furthermore, there were several proteins identified as the interactors of ACD11. XBAT35.2 is one of such proteins that regulates the abundance of ACD11 with exposure to pathogen infection (Liu et al., 2017). Binding Partner of ACD11 (BPA1) and all six BPA1-Like proteins (BPL-like) also interact with ACD11, which affect the proteasome-dependent degradation of ACD11 following inoculation to *Phytophthora capsici* (Petersen et al., 2009; Li et al., 2019). Additionally, the Prenylated Rab GTPase Receptor PRA1.F proteins were also reported to be interactors of ACD11 (Petersen et al., 2009).

ACD11 was first characterized as a putative sphingosine transfer protein, and subsequently Simanshu et al (2014) further described it also as the ceramide-1-phosphate (C1P) transfer protein (Brodersen et al., 2002). Both sphingosine and C1P are important elements of sphingolipid signaling in plants (**Figure 1.6**) (Ali et al., 2018). Sphingolipids are critical for plants, acting as structural components of membranes (Ali et al., 2018; Breslow and Weissman, 2010). Therefore, they are capable of regulating the proper functioning of lipid bilayers (Weissman, 2010). In addition, sphingolipids are reported to

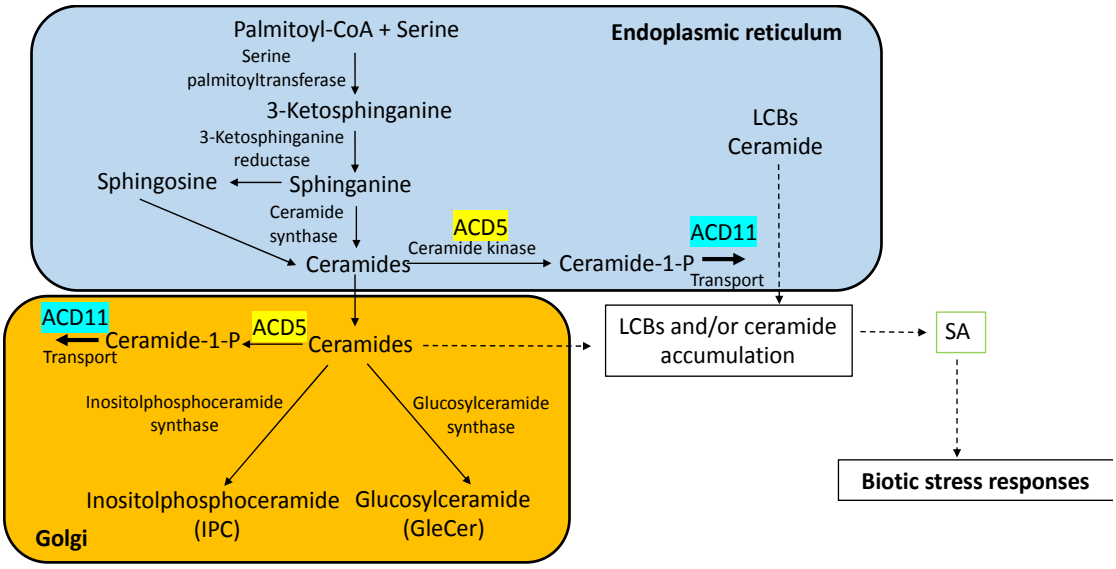


Figure 1.6. Simplified model of sphingolipid metabolism, and its link with SA signaling in plants.

The biosynthesis of sphingolipid begins in the endoplasmic reticulum and ends in the Golgi. ACD5, encoding for the ceramide kinase, can convert ceramide to ceramide-1-P. ACD11 is responsible for the intermembrane transfer of ceramide-1-P. The abundance of Long-chain Bases (LCBs), which are the precursors for ceramides, and ceramide can influence the synthesis of SA that is required for biotic stress resistance (Ali et al., 2018).

have functions in response to biotic and abiotic stresses by acting as regulatory elements (Ali et al., 2018). During immune signaling, sphingolipids, severving as the elicitors in plants following invision of pathogens, which can impact production of SA to modulate biotic stress response (Ali et al., 2018). On the other hand, sphingolipids have been linked to control the stomatal closure upon exposure to abiotic stress (Ali et al., 2018).

In *acd11* there is a rise in the level of normally low ceramide-1-phosphate (C1P) and an acute increase in the cell death inducer phytoceramide (Simanshu et al., 2014). Another ACD protein Accelerated Cell Death5 (ACD5) was identified as the ceramide kinase who can convert ceramide to be C1P (**Figure 1.6**) (Liang et al., 2003; Ali et al., 2018). Similar to ACD11, loss of ACD5 leads to spontaneous cell death and shows ceramide accumulation during the cell death stage (Bi et al., 2014). ACD5 resides in the endoplasmic reticulum and plasma membrane (Bi et al., 2014). Similar to the SA accumulation in *acd11*, *acd5* also accumulates SA at the cell death stage (Bi et al., 2014). A recent study further demonstrated a role for ACD5 in abiotic stress (Dutilleul et al., 2015; Yang et al., 2019). Exposure to salt stress is able to delay the cell death phenotype in *acd5* and to boost pathogen resistance in *acd5* (Yang et al., 2019). Also, the transcript level of *ACD5* is upregulated in response to salt treatments. Moreover, it is noted that the *acd5* mutant exhibits higher ABA contents relative to wild type under salt stress (Yang et al., 2019). Unlike ACD5, roles for ACD11 in abiotic stress have not been identified.

1.3 Purpose and Significance of Study

Accumulating evidence showed that ubiquitin ligases have dual functions in plant responses to biotic and abiotic stress. A role for the XBAT35.2 has been described in pathogen defense, so we are interested in determining whether XBAT35.2 also functions in abiotic stress tolerance in this study. Sphingolipid metabolism has been linked to both biotic and abiotic stress responses in plants (Ali et al., 2018). As a critical element of sphingolipid signaling, the role of ACD11 in abiotic stress is possible but remains unexplored. Therefore, we are also interested in characterizing the role of ACD11 in abiotic stress responses. The objectives of this research are to: 1) conduct transgenic and mutant analysis to determine if XBAT35.2 and ACD11 are involved in plant response to abiotic stresses such as drought and high salinity; 2) determine if ABA and exposure to

abiotic stress (e.g. high salinity) affect the transcript levels and protein stability of XBAT35.2 and ACD11; and 3) examine whether XBAT35.2 targets ACD11 for turnover by the 26S proteasome upon exposure to abiotic stress.

Due to global climate change, crops are increasingly challenged by adverse growth conditions. It is projected that by 2050 the world's population will exceed 9 billion, approximately 30% higher than today (FAO, 2009). Hence, it is necessary to improve our knowledge of the mechanism underlying stress responses, which would assist in developing crops with enhanced ability to survive and to maintain or increase yield during exposure to adverse environmental conditions. The outcomes of this study characterize the biological function of XBAT35.2 and ACD11. Also, the acquired knowledge will improve our understanding of the role of the UPS in abiotic stress tolerance. Moreover, the results may provide insights into possible connections between abiotic stress tolerance, sphingolipid metabolism and cell death in plants. Overall, knowledge gained may contribute to generating crops with enhanced abiotic stress tolerance.

CHAPTER TWO: MANUSCRIPT

This chapter is structured as a manuscript for submission to a scientific journal. References used in this chapter are included in the Chapter 4. All data presented and the initial draft of this manuscript was generated by Qiaomu Li, except the RT-qPCR results which was generated by Renata Serio.

2.1 ABSTRACT

To adapt to constantly fluctuating environment, plants employ the phytohormone, abscisic acid (ABA), as the master regulator to mediate abiotic stress responses and tolerance. The Ubiquitin Proteasome System (UPS) is reported to be the major pathway for the turnover of cellular proteins. We previously reported that the ubiquitin ligase XBAT35.2 interacted with ACD11 and directed its breakdown via the 26S proteasome. Here we found that salt/ABA treatments increased transcript levels and stability of XBAT35.2. Additionally, *xbat35-1* mutants displayed enhanced drought stress tolerance, suggesting E3's negative role in stress tolerance. Moreover, lower concentrations of ABA/salt treatments upregulated *ACD11* expression and protein stability, while higher concentrations of ABA/salt triggered ACD11 26S proteasomal degradation. Overexpression of ACD11 leads to improved salt and drought stress tolerance, indicating the positive function of ACD11 in stress tolerance. And in the in vivo degradation assay, the turnover rate of ACD11 slowed down in the absence of XBAT35.2 following exposure to ABA/salt treatments. Overall, we conclude that upon abiotic stress, the self-ubiquitinated XBAT35.2 is stabilized to regulate the degradation of ACD11 via the 26S proteasome, in turn manipulating the stress tolerance.

2.2 INTRODUCTION

Plants are continually exposed to a multitude of adverse environmental conditions such as drought, high salinity, nutrient deprivation and temperature extremes (high heat and cold/chilling). These unfavorable factors are harmful to cellular components and may result in the dysfunction of metabolic activities, thereby inhibiting plant growth and limiting yield (Colebrook et al., 2014; Mickelbart et al., 2015; Zhu, 2016). To cope with this constantly changing environment, plants have evolved a number of effective and sophisticated regulatory networks to sense as well as adapt to stress (Bohnert, 2007; Zhu, 2016). Phytohormones, including abscisic acid (ABA), salicylic acid (SA), ethylene and gibberellin, are essential for plant growth and development, and also for plants to respond to environmental stress (McSteen and Zhao, 2008; Vishwakarma et al., 2017). Among these hormones, ABA plays a critical role in plant tolerance of abiotic stress (Pereira, 2016). Following exposure to abiotic stress, such as high salinity or drought, plants elevate the production of ABA, which then promotes a range of responses, including delay in germination, early seedling growth arrest and induction of stomatal closure, in order to ameliorate negative impacts of stress (Tuteja, 2007;Nambara and Marion-Poll, 2005). To appropriately modulate ABA responses plants recruit a selection of molecular mechanisms, such as post-translational modification of major components of its signaling transduction pathway (Stone, 2019; Yang et al., 2017)

Ubiquitination plays diverse roles in plant development and stress responses ranging from targeting specific proteins for turnover via the 26S proteasome to activating signaling transduction (Sun et al., 2004; Vierstra, 2009b; Callis, 2014). This post-translational modification involves the attachment of one or more ubiquitin molecule(s) to the lysine residue(s) of substrates. Ubiquitin conjugation is achieved by three key enzymes: the E1 (ubiquitin activating enzyme), which activates the ubiquitin; the E2 (ubiquitin conjugating enzyme), which binds the activated ubiquitin to form an E2-ubiquitin intermediate; and the E3 (ubiquitin ligase), which promotes the transfer of ubiquitin from E2 to the substrate (Callis, 2014; Stone, 2014). Within the Ubiquitin Proteasome System (UPS), the conjugation process is repeated to generate a poly-ubiquitin chain, which would target the modified substrate for proteolysis by the 26S

proteasome. Central to the UPS are the large and diverse families of ubiquitin ligases who govern substrate selection (Chen and Hellmann, 2013).

The *Arabidopsis thaliana* (Arabidopsis) genome is predicted to contain more than 1300 genes that encode for E3s or components of E3 complexes. In addition, the genome is predicted to encode for 2 E1 isoforms and 37 E2s (Smalle and Vierstra, 2004; Kraft et al., 2005; Stone et al., 2005). Based on the presence of E2 binding domain, E3s are categorized into three types, the homology to E6-associated carboxyl-terminus (HECT), U-box, or Really Interesting New Gene (RING) type (Callis, 2014). Among these, the RING group is predicted to be the largest group, which includes both monomeric and multimeric ubiquitin ligases (Stone et al., 2005). A growing number of RING-type E3s have been reported to be critical regulators in ABA production, signaling and responses. For example, the RING-type E3 Keep on Going (KEG) suppresses ABA signaling by mediating the proteasome-dependent degradation of several components of the ABA signaling network, including protein kinases (e.g. Calcineurin B-like Interacting Protein Kinase 26 [CIPK26]) and transcription factors (e.g. ABA Insensitive 5 [ABI5]) (Liu and Stone, 2010; Chen et al., 2013; Lyzenga et al., 2013; McNeilly et al., 2018).

XBAT35 (for XB3 ortholog 5 in *Arabidopsis thaliana*), a member of the XBAT RING-type E3 subfamily, is characterized by a series of Ankyrin repeats used for substrate interaction, followed by a functional RING domain (Stone et al., 2005). *XBAT35* transcript is alternatively spliced producing two isoforms, nuclear-localized XBAT35.1 and Golgi- and cytosol-localized XBAT35.2 (Liu et al., 2017). Both isoforms were shown to be involved in ethylene signaling as negative regulators of apical hook curvature (Carvalho et al., 2012). More recently, XBAT35.2, but not XBAT35.1, was found to promote cell death to positively modulate defense responses (Liu et al., 2017). The *xbat35-1* mutant is more susceptible to pathogen infection, whereas plants overexpressing the E3 are less susceptible compared to wild type (Liu et al., 2017). Interestingly, XBAT35.2 was found to self-regulate, promoting its own proteasome-dependent degradation. Challenging plants with pathogen resulted in an increase in XBAT35.2 abundance and a concomitant decrease in the polyubiquitinated forms of this E3 (Liu et al., 2017).

Accelerated Cell Death11 (ACD11) was shown to interact with XBAT35.2 in plant cells (Liu et al., 2017). ACD11, a putative sphingosine transfer protein, is a known negative regulator of plant defense responses (Brodersen et al., 2002). Loss of ACD11 results in Programmed Cell Death (PCD) and the constitutive expression of defense-related genes (Brodersen et al., 2002). The activation of PCD and defense pathways in *acd11* requires SA signaling (Brodersen et al., 2002). Previous results showed that XBAT35.2 promotes the proteasome-dependent degradation of ACD11 and the abundance of ACD11 decreases following pathogen infection which correlates with the increased ubiquitination of ACD11. The suggestion is that to promote pathogen defense, stabilized XBAT35.2 directs ACD11 for degradation via 26S proteasome. In addition, the reduced colonization of the pathogen on ACD11-silenced Arabidopsis following inoculation of *Phytophthora capsici* further suggests a negative role of ACD11 in defense responses (Li et al., 2019).

In this study, we demonstrate a role for XBAT35.2 and ACD11 in plant response to abiotic stress. XBAT35.2 transcript levels and protein stability increased with duration and concentration of ABA and salt. In contrast, ACD11 transcript and protein abundance increased significantly shortly after exposure to ABA and salt. However, longer duration of exposure or higher concentrations of ABA and salt induced the proteasome-dependent degradation of ACD11. The ABA- and salt-induced degradation of ACD11 slowed down in *xbat35-1* mutants, suggesting the involvement of the E3 in proteasome-dependent degradation of ACD11. *xbat35-1* mutants and transgenic plants overexpressing *ACD11* are less to sensitive high salt and drought conditions than wild type, suggesting a positive role for ACD11 in abiotic stress tolerance. In contrast to biotic stress, stabilized XBAT35.2 might promote the proteasome-dependent degradation of ACD11 to dampen/terminate responses upon abiotic stress.

2.3 RESULTS

XBAT35.2 and *ACD11* are abscisic acid (ABA)-responsive genes

To begin to characterize the roles of XBAT35.2 and ACD11 in abiotic stress tolerance, we first used quantitative polymerase chain reaction (qPCR) to assess changes in transcript abundance in response to ABA. As shown in the **Figure 2.1A**, the expression of *XBAT35.2* increased in Arabidopsis seedlings treated with ABA, while no significant increase in transcript abundance was observed in treated *xbat35-1* seedlings. A similar increase in ACD11 transcript abundance was observed in ABA treated Arabidopsis seedlings (**Figure 2.1A**). To further investigate the responsiveness of *XBAT35.2* and *ACD11* to the stress hormone, transcript abundance was again assessed following exposure to increasing concentrations of ABA over time. For *XBAT35.2*, transcript abundance increased with duration of exposure, with higher levels observed after 4 hours compared to 2 hours of treatment for all concentrations tested (**Figure 2.1B**). In addition, in the 4-hour treatment, *XBAT35.2* transcript levels accumulated along with the increase in ABA concentration. These results suggest that the concentration and duration of exposure to ABA influences the expression of XBAT35.2. We also tested how differences in duration of exposure and concentrations of ABA affect *ACD11* expression. Similar to XBAT35.2 no significant changes were observed following two hours of exposure to different concentrations of ABA (**Figure 2.1C**). However, a significant gradual increase in *ACD11* transcript abundance was observed following four hours of treatment with lower concentrations of ABA (10 μM and 25 μM), while transcript abundance did not show any significant change, compared to untreated set (control, 0 μM), following treatment with the higher concentrations of ABA (50 μM and 100 μM) used in the assay (**Figure 2.1C**). Similar to XBAT35.2, these results indicate that the length of treatment and the concentration of ABA affects the expression of ACD11; however, changes in expression are observable following exposure to lower concentrations of the hormone.

Exposure to ABA and salt stress increases XBAT35.2 protein levels and stability.

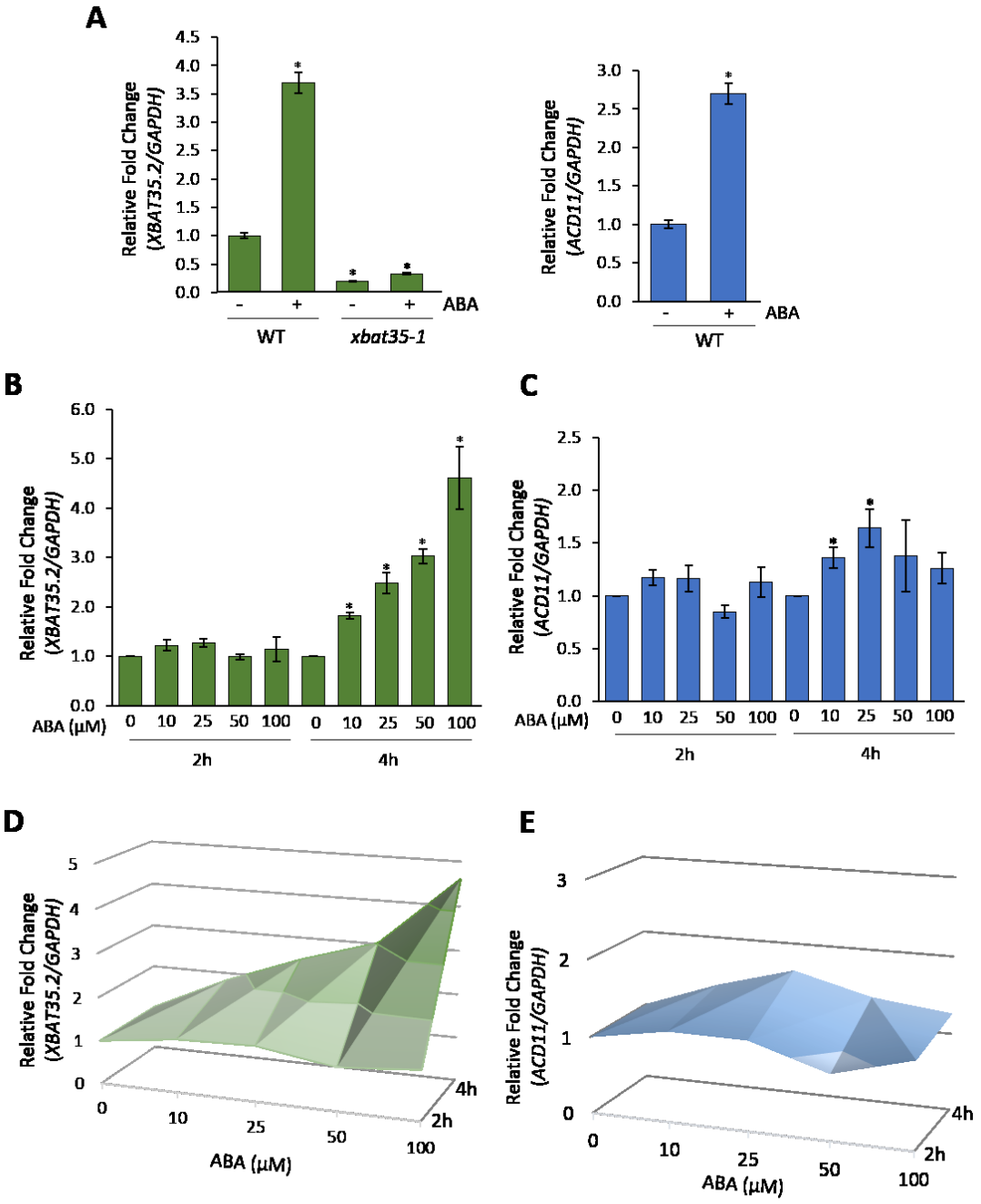

A
Relative Fold Change (XBAT35.2/GAPDH)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
- + - + ABA
WT
xbat35-1
Relative Fold Change (ACD11/GAPDH)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
- + ABA
WT
B
Relative Fold Change (XBAT35.2/GAPDH)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ABA (μM) 0 10 25 50 100 0 10 25 50 100
2h
4h
C
Relative Fold Change (ACD11/GAPDH)
2.5
2.0
1.5
1.0
0.5
0.0
ABA (μM) 0 10 25 50 100 0 10 25 50 100
2h
4h
D
Relative Fold Change (XBAT35.2/GAPDH)
5
4
3
2
1
0
0 10 25 50 100
ABA (μM)
4h
2h
E
Relative Fold Change (ACD11/GAPDH)
3
2
1
0
0 10 25 50 100
ABA (μM)
4h
2h

Figure 2.1. Changes in *XBAT35.2* and *ACD11* expression in response to ABA.

A) RT-qPCR analysis using mRNA isolated from seven-day-old Arabidopsis wild-type (WT) and *xbat35-1* seedlings treated without or with 50 μM ABA for 5 hours (h). GAPDH served as an internal control. All results were normalized to untreated WT. "*" indicates a statistically significant ($p \leq 0.05$) difference based on a paired t-test within each plant line. n=6 (3 biological replicates). Error bars represent ±SE.

B to E) RT-qPCR analysis using mRNA isolated from seven-day-old WT seedlings treated with the indicated concentrations of ABA (μM) for 2h or 4h. B) and C) Graphs show the relative expression of XBAT35.2 (B) and ACD11 (C). D) and E) Surface graphs showing information about peaks of the relative expression of XBAT35.2 (D) and ACD11 (E). GAPDH served as the internal control. All results were normalized to untreated WT. "*" indicates a statistically significance ($p \leq 0.05$) difference based on a paired t-test within each plant line. n=6 (3 biological replicates). Error bars represent ±SE.

We previously reported that the ubiquitin ligase XBAT35.2 mediates its own proteasome-dependent degradation (Liu et al., 2017). In addition, it was also demonstrated that exposure to pathogen leads to the stabilization of XBAT35.2, as determined by a drastic decrease in the level of ubiquitination and an increase in protein abundance (Liu et al., 2017). We therefore investigated if abiotic stress would also result in increased XBAT35.2 stability. An increase in E3 protein abundance would correlate with the observed abiotic stress-induced accumulation of *XBAT35.2* transcript (**Figure 1A and B**). To address this question, previously characterized transgenic Arabidopsis seedlings overexpressing hemagglutinin (HA) tagged XBAT35.2 (*35S:HA-XBAT35.2*) were treated with increasing concentrations of ABA (Liu et al 2017). **Figure 2.2A** showed that HA-XBAT35.2 abundance increased with the concentrations of ABA used in treatments. Likewise, the abundance of HA-XBAT35.2 was found to gradually increase following treatment with increasing concentrations of sodium chloride (NaCl) (**Figure 2.2B**). Similar results were obtained when treatment assays were repeated using transgenic Arabidopsis seedlings overexpressing yellow fluorescence protein (YFP)-tagged XBAT35.2 (*35S:YFP-XBAT35*.2) (**Supplemental Figure S2.1**).

As XBAT35.2 regulates its own proteasome-dependent degradation in the absence of stress, we next aimed to determine if the increase in its abundance was due to enhanced stability. XBAT35.2 stability in the presence and absence of ABA was assessed using cycloheximide (CHX) chase assays (Ramos et al., 2001; Gilkerson et al., 2016). CHX blocks de novo protein synthesis, which enables the investigation of the effects ABA on the pool of previously synthesized protein. Prior to the addition of CHX, seedlings were treated with (25 μM or 100 μM) or without ABA. Seedlings were collected for immunoblot analysis to determine HA-XBAT35.2 protein levels at the time of CHX addition (0 hour [h]) and 6 h later. As expected, in the absence of ABA, the abundance of HA-XBAT35.2 decreased considerably over time (**Figure 2.2C**; compare lanes 1 and 2). In contrast, the decrease in HA-XBAT35.2 abundance over time was significantly reduced in the presence of ABA (**Figure 2.2C**; compare lanes 4 and 6 to lane 2), suggesting a decrease in degradation rate with an addition of the hormone. To provide further evidence for ABA-mediated reduction in ubiquitin-dependent proteasomal

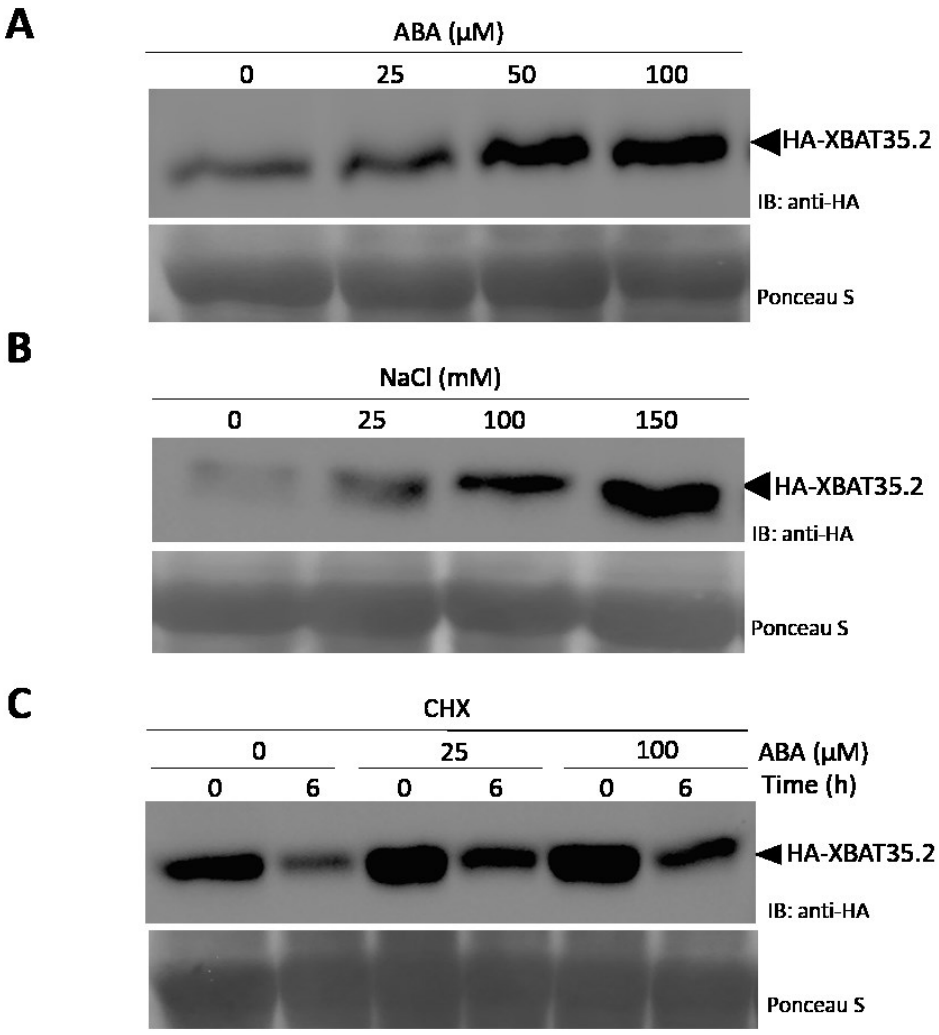

A
ABA (μM)
0
25
50
100
HA-XBAT35.2
IB: anti-HA
Ponceau S
B
NaCl (mM)
0
25
100
150
HA-XBAT35.2
IB: anti-HA
Ponceau S
C
CHX
0
25
100
ABA (μM)
0
6
0
6
0
6
Time (h)
HA-XBAT35.2
IB: anti-HA
Ponceau S
D
100 μM ABA
50 μM MG132
IP: anti-HA agarose
IB: anti-Ub
HA-XBAT35.2-(Ub)n
IP: anti-HA agarose
IB: anti-HA
HA-XBAT35.2

Figure 2.2. Accumulation and stabilization of XBAT35.2 in response to ABA and salt.

A and B) Increase in XBAT35.2 abundance in response to increasing concentrations of ABA (A) and NaCl (B). Seven-day-old transgenic seedlings overexpressing HA-XBAT35.2 (*35S:HA-XBAT35.2*) were incubated with ABA or NaCl for 12h and 3h, respectively. The levels of HA-XBAT35.2 were analyzed by immunoblot (IB) analysis with HA antibodies. Ponceau S staining was used to visualize protein loading.

C) CHX assay showing ABA-mediated increase in XBAT35.2 stability. Seven-day-old *35S:HA-XBAT35.2* transgenic seedlings were treated with the indicated concentration of ABA for 3h prior to the addition of 500 μM CHX. Samples were collected for analysis immediately after the addition of CHX (0h) and 6h later. The levels of HA-XBAT35.2 were analyzed by IB analysis with HA antibodies. Ponceau S staining was used to visualize protein loading.

D) Decrease in the polyubiquitinated form of XBAT35.2 in the presence of ABA. Seven-day-old *35S:HA-XBAT35.2* transgenic seedlings were treated with 50 μM MG132 for 4h prior to the addition of 100 μM ABA for 12h. HA-XBAT35.2 was isolated via immunoprecipitation (IP) using anti-HA agarose beads, followed by IB analysis with anti-Ub (top panel) and anti-HA (bottom panel). The high-molecular-weight isolates (top panel) are indicative of polyubiquitinated XBAT35.2 (HA-XBAT35.2-$(Ub)^n$).

degradation, the level of HA-XBAT35.2 ubiquitination was assessed in treated and untreated seedlings. HA-XBAT35.2 was isolated from protein extracts prepared from transgenic seedlings treated with or without 100 μM ABA and the proteasome inhibitor MG132, which allows for the accumulation of the ubiquitinated form of the E3. Isolates were prepared with immunoprecipitation assays and subjected to immunoblot analysis using HA and ubiquitin antibodies to detect, HA-XBAT35.2 and ubiquitinated forms of HA-XBAT35.2, respectively.

In the absence of ABA, a high level of polyubiquitinated forms of HA-XBAT35.2 (HA-XBAT35.2-$(Ub)^n$) was detected (**Figure 2.2D**). However, in the presence of ABA, the level of polyubiquitinated HA-XBAT35.2 detected was significantly reduced **(Figure 2.2D**). Similar results were observed when transgenic seedlings *35S:HA-XBAT35.2* were only treated by 100 μM ABA (**Supplemental Figure S2.2**). Above results suggest a decrease in ubiquitination of XBAT35.2 which would impact its subsequent degradation by the 26S proteasome.

The abundance and stability of ACD11 changes with the duration of exposure and concentration of ABA.

Our previous reports showed a decrease in ACD11 levels following pathogen infection, which correlated with increased XBAT35.2 abundance, suggesting that the stabilized E3 targets ACD11 for degradation to facilitate defense responses. Here our aim is to determine if a similar scenario occurs following exposure to abiotic stress. To investigate the effects of ABA on ACD11 abundance, previously characterized transgenic Arabidopsis seedlings overexpressing green fluorescence protein (GFP)-tagged ACD11 (*Ubq10:GFP-ACD11*) were treated with increasing concentrations of ABA (Munch et al., 2015). As shown in **Figure 2.3A** all concentrations of ABA (25, 50 and 100 μM) utilized resulted in the accumulation of GFP-ACD11 compared to the control (0 μM ABA). The drastic increase in ACD11 abundance maybe due to the use of the *ubiquitin10* promoter (Grefen et al., 2010; Munch et al., 2015). However, the accumulation may conceal changes in protein stability. Therefore, we investigated whether and how varying

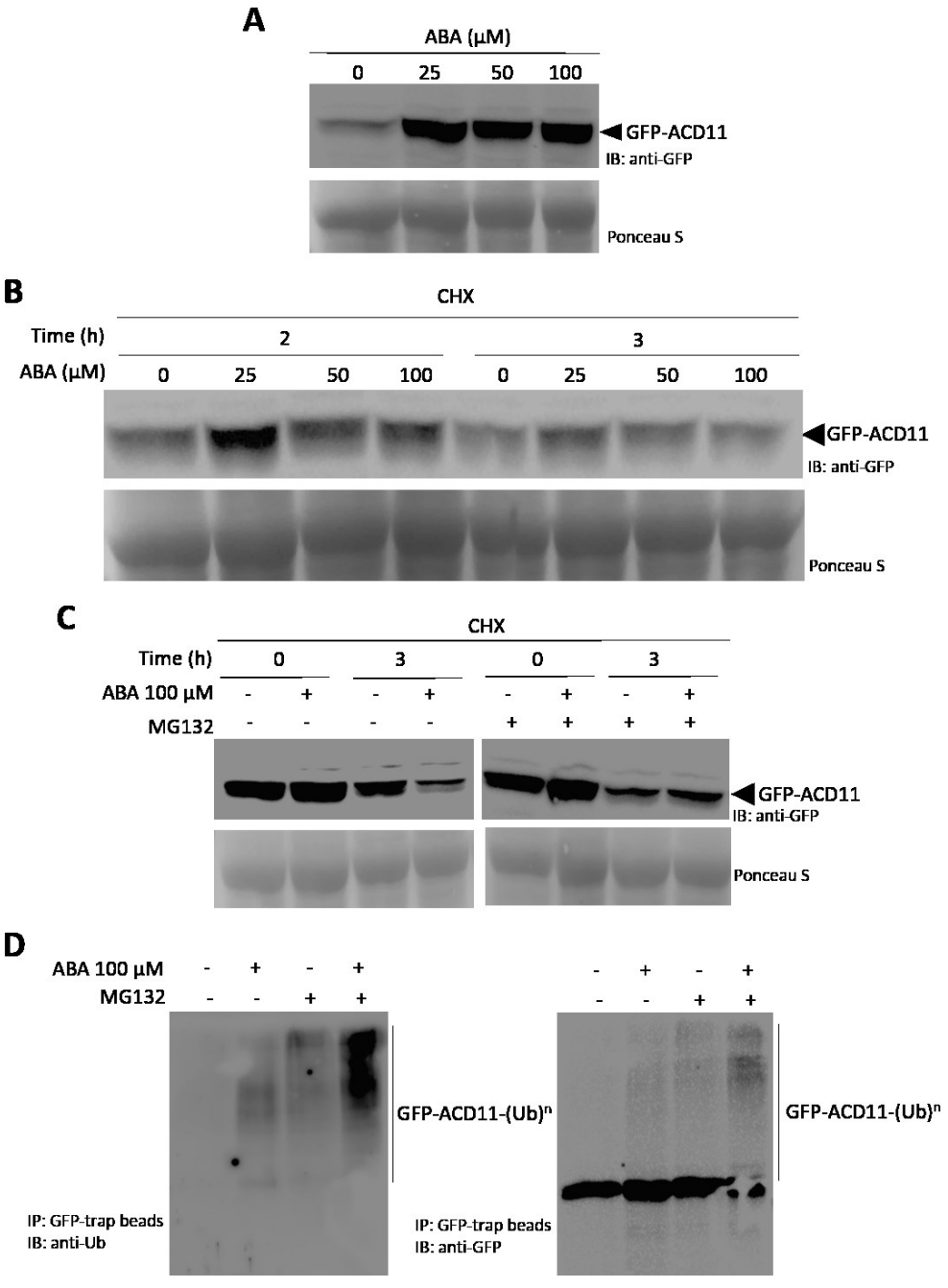

A
ABA (μM)
0
25
50
100
GFP-ACD11
IB: anti-GFP
Ponceau S
B
CHX
Time (h)
2
3
ABA (μM)
0
25
50
100
0
25
50
100
GFP-ACD11
IB: anti-GFP
Ponceau S
C
CHX
Time (h)
0
3
0
3
ABA 100 μM
MG132
GFP-ACD11
IB: anti-GFP
Ponceau S
D
ABA 100 μM
MG132
GFP-ACD11-(Ub)n
IP: GFP-trap beads
IB: anti-Ub
GFP-ACD11-(Ub)n
IP: GFP-trap beads
IB: anti-GFP

Figure 2.3. Changes in ACD11 abundance and stability in response to ABA.

A) Increased ACD11 abundance in ABA treated seedlings. Seven-day-old transgenic Arabidopsis seedlings overexpressing GFP-ACD11 (*Ubq10:GFP-ACD11*) were treated with the indicated concentration of ABA for 12h. The abundance of GFP-ACD11 was determined by immunoblot (IB) analysis using GFP antibodies. Ponceau S staining was used to visualize protein loading.

B) Increase in ACD11 stability at lower ABA concentrations. Seven-day-old transgenic seedlings overexpressing GFP-ACD11 (*Ubq10:GFP-ACD11*) were treated with 300 μM CHX and the indicated concentration of ABA, and samples collected after 2h and 3h. The abundance of GFP-ACD11 was determined by IB analysis using GFP antibodies. Ponceau S staining was used to visualize protein loading.

C) Decrease in ACD11 stability at higher concentrations of ABA. Seven-day-old transgenic overexpressing GFP-ACD11 seedlings (*Ubq10:GFP-ACD11*) were treated with (+) or without (-) 50 μM MG132 for 16h, prior to the addition of 300 μM CHX supplemented with or without 100 μM ABA. Samples were collected at the time of CHX and ABA addition (0h) and 3h later. The abundance of GFP-ACD11 was determined by IB analysis by using GFP antibodies. Ponceau S staining was used to visualize protein loading.

D) Increase in polyubiquitinated form of ACD11 in the presence of 100 μM ABA. Seven-day-old transgenic seedlings overexpressing GFP-ACD11 (*Ubq10:GFP-ACD11*) were treated with (+) or without (-) 50 μM MG132 for 16h, prior to the addition of 100 μM ABA for an additional 3h. GFP-ACD11 was isolated by IP with GFP-trap beads and then isolates were analyzed by IB using anti-Ub (left panel) and anti-GFP (right panel) antibodies. The high-molecular-mass smear observed using ubiquitin antibodies indicates the polyubiquitinated form of GFP-ACD11 (GFP-ACD11-$(Ub)^n$).

concentrations of ABA affect the stability of ACD11 over time. CHX assays were carried out without (0 μM, control) or with increasing concentrations of ABA (25 μM, 50 μM or 100 μM) for the indicated times (**Figure 2.3B**). First, regardless of ABA concentration, the abundance of GFP-ACD11 decreased over time. This result corresponds with our previous report, which shows that ACD11 is unstable. Second, at a specific time point, compared to the control, the abundance of GFP-ACD11 was significantly greater following treatment with 25 μM ABA, suggesting a decrease in protein turnover (**Figure 2.3B**). This increase in GFP-ACD11 abundance was not as apparent following treatment with 50 μM or 100 μM ABA, which indicates that higher concentrations of ABA do not promote the stabilization of ACD11 (**Figure 2.3B**). Subsequently, we further investigated the stability of ACD11 with the addition of 100 μM ABA and proteasome inhibitor (MG132) to confirm that higher concentrations of the hormone promote proteasome-dependent degradation of ACD11. Transgenic seedlings overexpressing *GFP-ACD11* were treated without or with MG132 prior to the addition of CHX in the absence or presence of ABA. The abundance of GFP-ACD11 was determined immediately after the addition of ABA (0 h) or 3 h later. In the absence of MG132, after 3h treatment the level of GFP-ACD11 was lower in seedlings treated with 100 μM ABA compared to untreated (**Figure 2.3C**). In contrast, the difference in GFP-ACD11 abundance between ABA treated and untreated seedlings was not observed when MG132 was included in the assays, suggesting that higher concentrations of ABA promote the proteasome-dependent turnover of ACD11 (**Figure2.3C**). To further verify that higher concentrations of ABA promote ubiquitin-dependent degradation of ACD11, we examined the levels of ubiquitination by isolating of GFP-ACD11 from seedlings treated without or with 100 μM ABA in the absence or presence of MG132. **Figure 2.3D** showed that treatment with 100 μM ABA or MG132 similarly increased the abundance of ubiquitinated GFP-ACD11. The level of ubiquitinated GFP-ACD11 was further increased when seedlings were treated with both ABA and MG132 (**Figure 2.3D**). Taken together, the results indicate that lower concentrations of ABA (e.g. 25 μM) stabilize ACD11, while higher concentrations of ABA (e.g. 100 μM) trigger its proteasome-dependent degradation. The changes in protein stability correlate with the observed ABA-induced changes in transcript abundance, where highest levels are detected following exposure to lower

concentrations of the hormone.

Concentration and duration of exposure to high salinity modulate ACD11 abundance and stability.

Salt stress can activate the biosynthesis of ABA and then utilize ABA signaling to induce downstream changes as part of stress responses (Xiong et al., 2002). Since concentrations of salt promote the accumulation of XBAT35.2 and ABA regulates the abundance of XBAT35.2 and ACD11, we hypothesize that the severity of salt stress modulates ACD11 protein levels. To examine this, transgenic seedlings expressing GFP-ACD11 were treated without (0 mM, control) or with increasing concentrations of salt (150 mM, 250 mM or 350 mM) for up to 36h, with samples collected at the indicated time points. Compared to the control, at the first time point examined (3h), the abundance of GFP-ACD11 was higher following treatment with all concentration of salt used in the assays compared to the control set (**Figure 2.4A**). However, GFP-ACD11 abundance was highest following treatment with 150 mM NaCl, the lowest concentration utilized. For each treatment, the abundance of GFP-ACD11 decreased overtime, suggesting degradation of this protein (**Figure 2.4A**). The decrease in GFP-ACD11 abundance was most drastic in the presence of 350 mM NaCl, with little to no protein detected after 12h. In contrast, while GFP-ACD11 abundance did decrease over time, GFP-ACD11 abundance remained significantly higher in the presence of 150 mM NaCl compared the control for up to 36 h (**Figure 2.4A**). We also tested above high salt treatment assays over a longer period, which showed the same trend (**Supplemental figure S2.3**).These results also suggest that, similar to ABA, exposure to lower levels or shorter duration of salt stress promotes the accumulation of ACD11, while more severe levels or longer exposure results in its degradation.

We subsequently asked whether the lower levels of GFP-ACD11 observed following treatment with the higher concentrations of salt or following longer exposure of salt stress were due to proteasome-dependent degradation. Transgenic seedlings overexpressing GFP-ACD11 were treated without (0 mM) or with (150 mM or 250 mM) NaCl in the

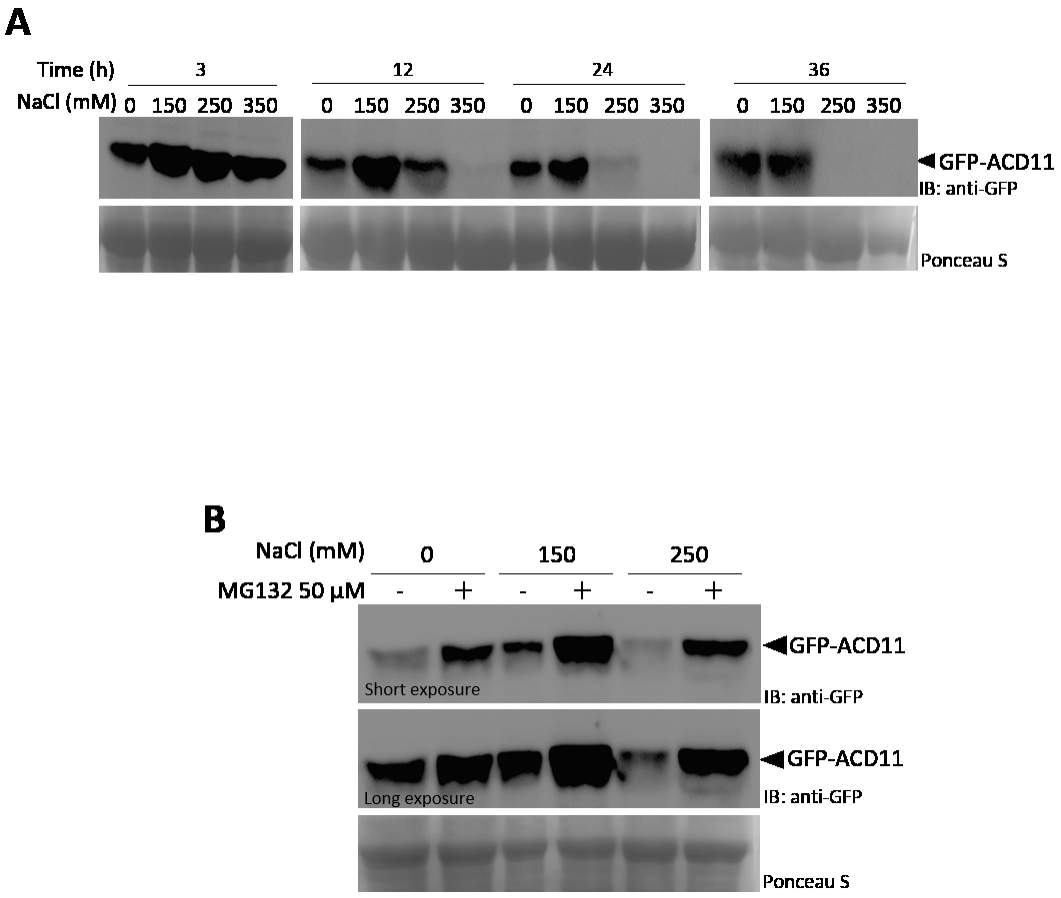


Figure 2.4. Changes in ACD11 abundance in response to salt.

A) Duration of exposure and severity of salt treatments modulate the abundance of ACD11. Seven-day-old transgenic seedlings overexpressing GFP-ACD11 (*Ubq10:GFP-ACD11*) were treated with the indicated concentration of NaCl. Samples were collected at the indicated time points and the abundance of GFP-ACD11 was determined by IB using anti-GFP antibodies. Ponceau S staining was used to visualize protein loading.

B) High salinity induced decrease in ACD11 abundance is prohibited by proteasome inhibitor. Seven-day-old transgenic seedlings overexpressing GFP-ACD11 (*Ubq10:GFP-ACD11*) were treated with (+) or without (-) 50 µM MG132 for 3h, prior to the addition of the indicated concentrations of salt and samples were collected after 21h. The abundance of GFP-ACD11 was determined by IB analysis using anti-GFP antibodies. Ponceau S staining was used to visualize protein loading.

presence or absence of the proteasome inhibitor, MG132, for 24 h. As observed previously (**Figure 2.4A**), compared to the control (0 mM NaCl), 150 mM NaCl promoted the accumulation of GFP-ACD11, while considerably less GFP-ACD11 was observed following treatment with 250 mM NaCl (**Figure 2.4B**). The inclusion of MG132 in the assays promoted the accumulation of GFP-ACD11 regardless of the concentration of NaCl (**Figure 2.4B**), providing evidence for the proteasome-dependent degradation of ACD11. However, the effect of inhibiting proteasome activity on increasing GFP-ACD11 abundance was most notable in assays utilizing 250 mM NaCl. These findings suggest that the decrease in GFP-ACD11 abundance observed with exposure to the higher concentrations of salt or longer exposure of salt stress relied on the 26S proteasome.

XBAT35.2 is involved in ABA- and salt-induced turnover of ACD11.

Previous report suggests that XBAT35.2 promotes the ubiquitin-dependent degradation of ACD11 upon pathogen infection (Liu et al., 2017). We have shown that, in CHX assays with higher concentrations of ABA, ACD11 is subjected to proteasomal degradation (**Figure 2.3**), while an increase in XBAT35.2 abundance and stability is observed (**Figure 2.2**), suggesting that under these conditions XBAT35.2 may be involved in ubiquitinating ACD11 and targeting the protein for proteasomal degradation. To investigate the potential role of XBAT35.2 in regulating ACD11 abundance under abiotic stress, the stability of GFP-ACD11 was compared in transgenic seedlings containing *XBAT35* (wild-type background; *GFP-ACD11/WT*) and transgenic seedlings lacking expression of the E3 (*xbat35-1* background; *GFP-ACD11/xbat35-1*). CHX assays utilizing both plant lines were carried out in the absence and presence of ABA or salt (**Figure 2.5**, **Supplemental Figures 2.4**). Without ABA, there was little to no difference in the degradation rate of GFP-ACD11 in the presence or absence of the E3 over a three-hour period (**Figure 2.5A**), indicating that XBAT35.2 is not involved in ACD11 degradation under these conditions. However, over a longer period, the degradation of ACD11 was slightly slower in the absence of XBAT35.2 (**Supplemental Figure S2.4A**),

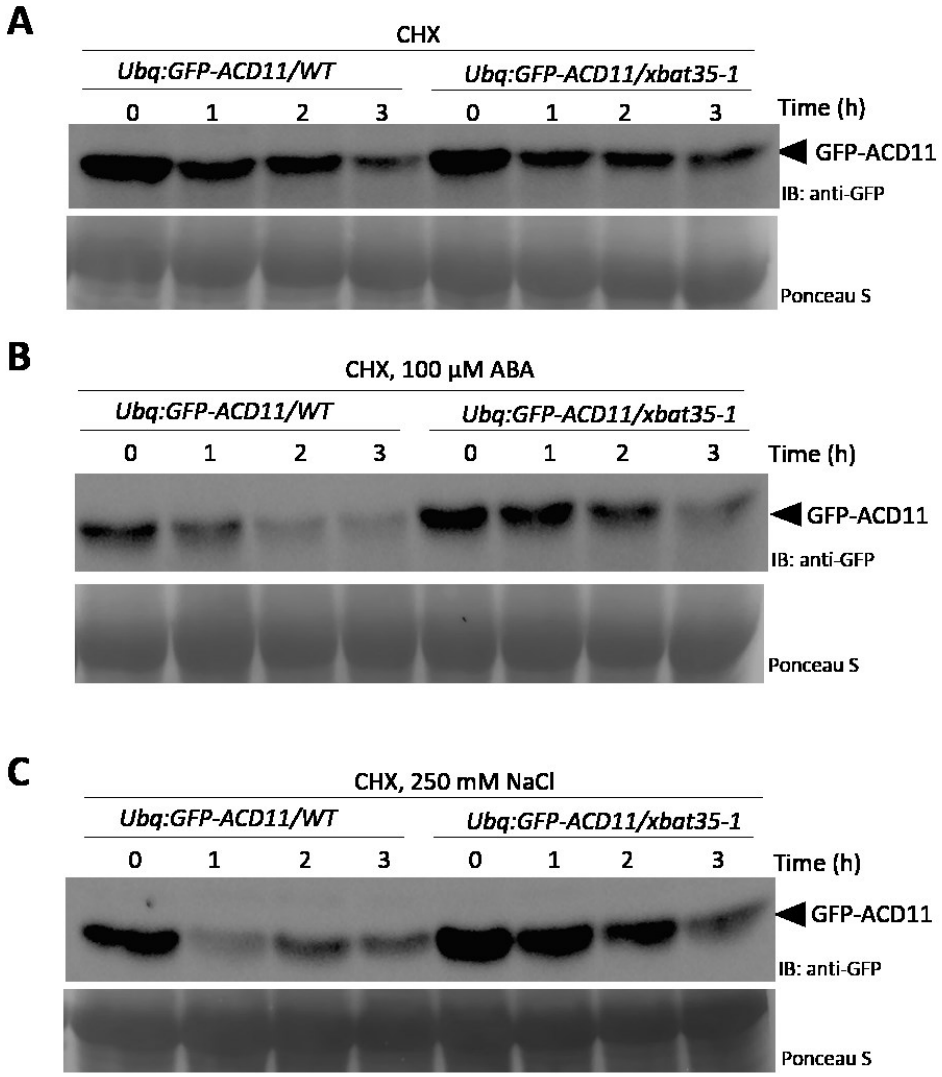


Figure 2.5. XBAT35.2 is involved in the degradation of ACD11 under stress conditions.

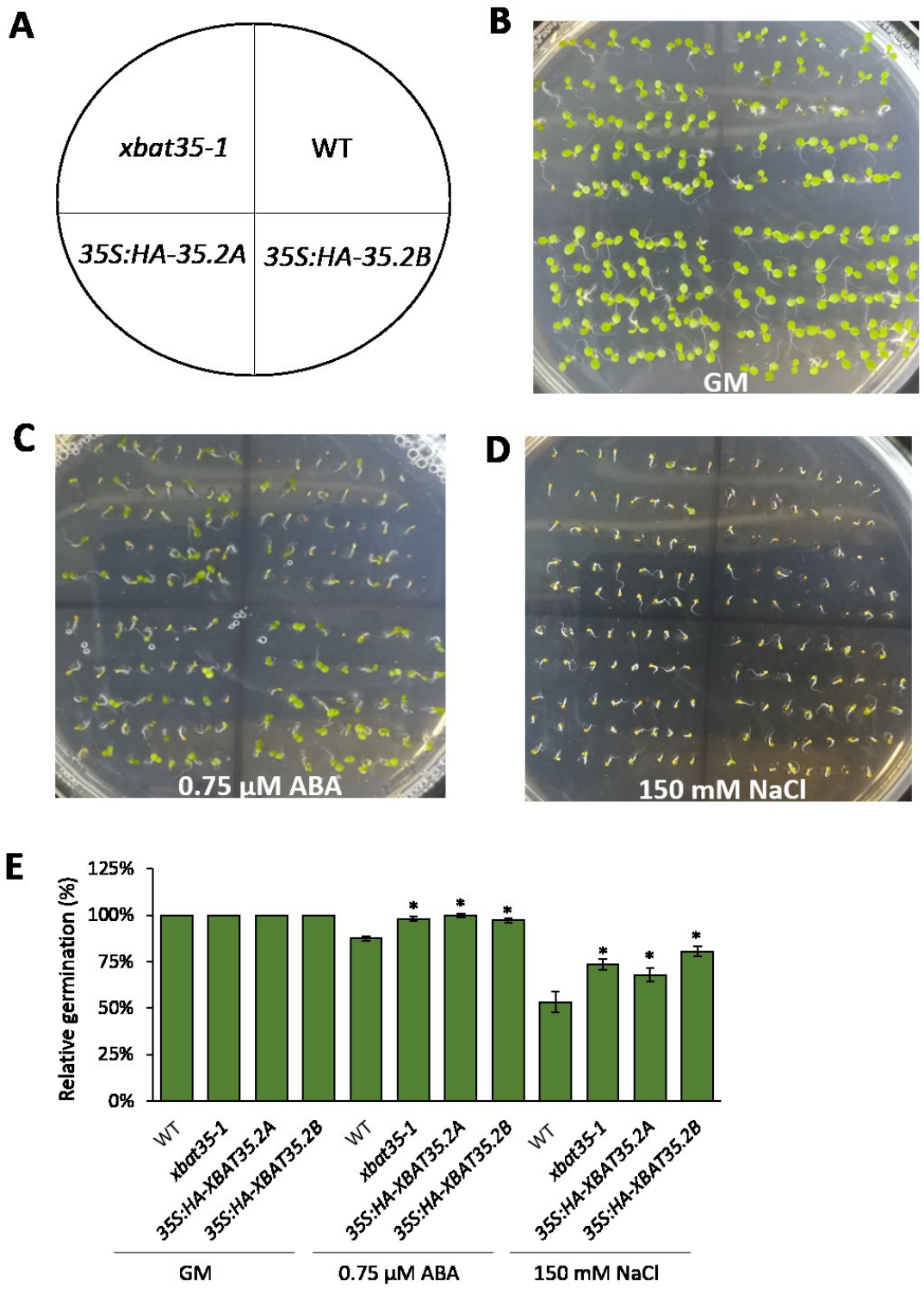
Seven-day-old transgenic seedlings overexpressing GFP-ACD11 in the presence (*Ubq10:GFP-ACD11/WT)* and absence (*Ubq10:GFP-ACD11/xbat35-1)* of XBAT35.2 were treated with 300 µM CHX supplemented without (A) or with 100 µM ABA (B) or 250 mM NaCl (C). Samples were collected at the indicated time points and the abundance of GFP-ACD11 was determined by IB using anti-GFP antibodies. Ponceau S staining was used to visualize protein loading.

which suggests the possibility of a minor role for the E3 in ACD11 turnover under non-stress conditions. In the presence of 100 μM ABA, the degradation of GFP-ACD11 was significantly slower in the absence of the E3, providing evidence for the involvement of XBAT35.2 in regulating ACD11 abundance (**Supplemental Figure S2.4B**). Assays were also carried out using salt, which was shown to promote the degradation of GFP-ACD11 at high concentrations (**Figure 2.5C**). Similar to ABA, in the presence of 250 mM NaCl, the degradation of GFP-ACD11 was considerably slower in the absence of the E3 compared to when XBAT35.2 was present (**Figure 2.5C)**. These results suggest that ABA and salt-induced stabilization of XBAT35.2 leads to the ubiquitin-mediated degradation of ACD11.

Changes in XBAT35 abundance and overexpression of ACD11 leads to hyposensitivity to ABA and salt.

We have so far provided evidence for a role for ABA and salt in regulating the abundance of XBAT35.2 and ACD11. Next, we aim to determine if either gene is involved in response to ABA/abiotic stress. To assess sensitivity to ABA and salt, germination assays were carried out using seeds from wild type (Col-), *xbat35-1*, transgenic plants overexpressing *35S:HA-XBAT35.2* as well as transgenic overexpressing *Ubq10:GFP-ACD11*. Seeds were geminated on solid growth medium without or with ABA (0.75 μM) or NaCl (150 mM) and percent germination determined by monitoring radicle emergence. The germination rate of WT seeds decreased significantly in the presence of ABA and NaCl (**Figure 2.6**).

Unexpectedly, compared to WT, *xbat35-1* and transgenic seeds overexpressing *35S:HA-XBAT35.2*, exhibited hyposensitivity to ABA and salt with significantly higher relative germination rates (**Figure 2.6**). Interestingly, the relative germination ratio of *ACD11* transgenic seeds was considerably higher than that of WT seeds in the presence of ABA and salt, indicating hyposensitivity (**Figure 2.6**). Overall, these results imply that altering the abundance of ACD11 via gene upregulation, overexpression or mutating XBAT35 abundance leads to the same outcome, insensitivity to the hormone or abiotic stress.


A
xbat35-1
WT
35S:HA-35.2A
35S:HA-35.2B
B
GM
C
0.75 µM ABA
D
150 mM NaCl
E
Relative germination (%)
125%
100%
75%
50%
25%
0%
WT
xbat35-1
35S:HA-XBAT35.2A
35S:HA-XBAT35.2B
GM
0.75 µM ABA
150 mM NaCl

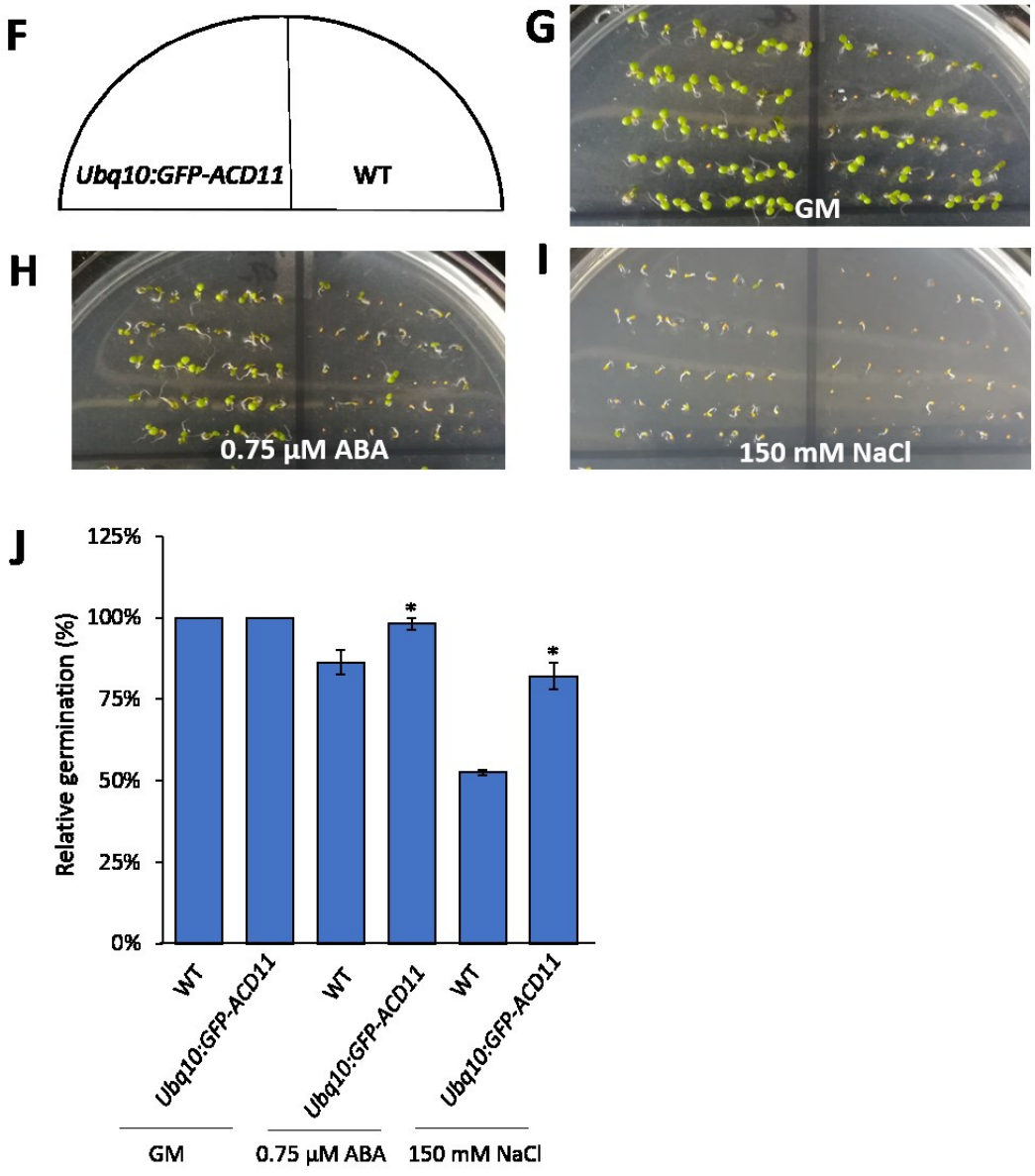

F
Ubq10:GFP-ACD11
WT
G
GM
H
0.75 µM ABA
I
150 mM NaCl
J
Relative germination (%)
125%
100%
75%
50%
25%
0%
*
*
WT
Ubq10:GFP-ACD11
WT
Ubq10:GFP-ACD11
WT
Ubq10:GFP-ACD11
GM
0.75 µM ABA
150 mM NaCl

Figure 2.6. Changes in XBAT35.2 and ACD11 abundance promote insensitivity to salt and ABA.

A) Schematic showing placement of genotypes, WT, *xbat35-1* and two transgenic lines overexpressing HA-XBAT35.2 (*35S:HA-35.2A* and *35S:HA-35.2B*), used in sensitivity growth assays.

B to D) Photographs showing four-day-old seedlings germinated and grown on growth medium (GM) without (B) or with 0.75 μM ABA (C) or 150 mM NaCl (D).

E) Graph showing germination rate at 3d for *xbat35-1*, *35S:HA-35.2A* and *35S:HA-35.2B* relative to WT. Germination was scored as radicle emergence. Bars represent average over 3 independent trials with 7 replicates (n>30 seeds per replicate). Error bars represent ±SE. "*" indicates a statistically significant ($p \leq 0.05$) difference compared to WT based on a Tukey's test.

F) Schematic showing placement of genotypes, WT and *Ubq10:GFP-ACD11* used in sensitivity growth assays shown in G-I.

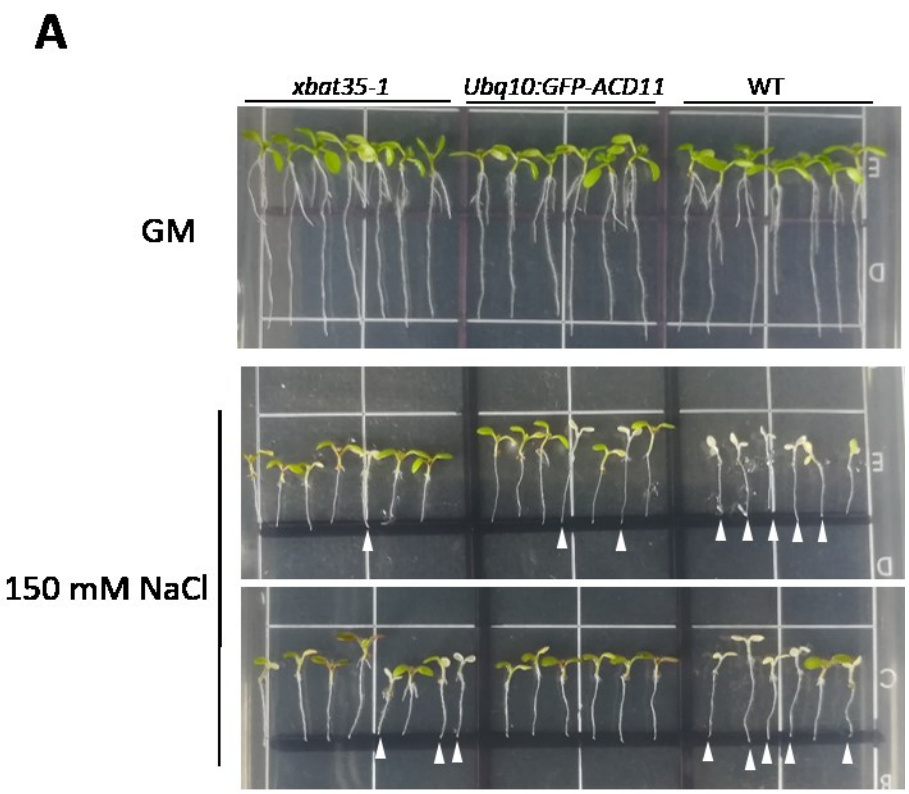
G to I) Photographs showing four-day-old seedlings germinated and grown on growth medium (GM) without (G) or with 0.75 μM ABA (H) or 150 mM NaCl (I).

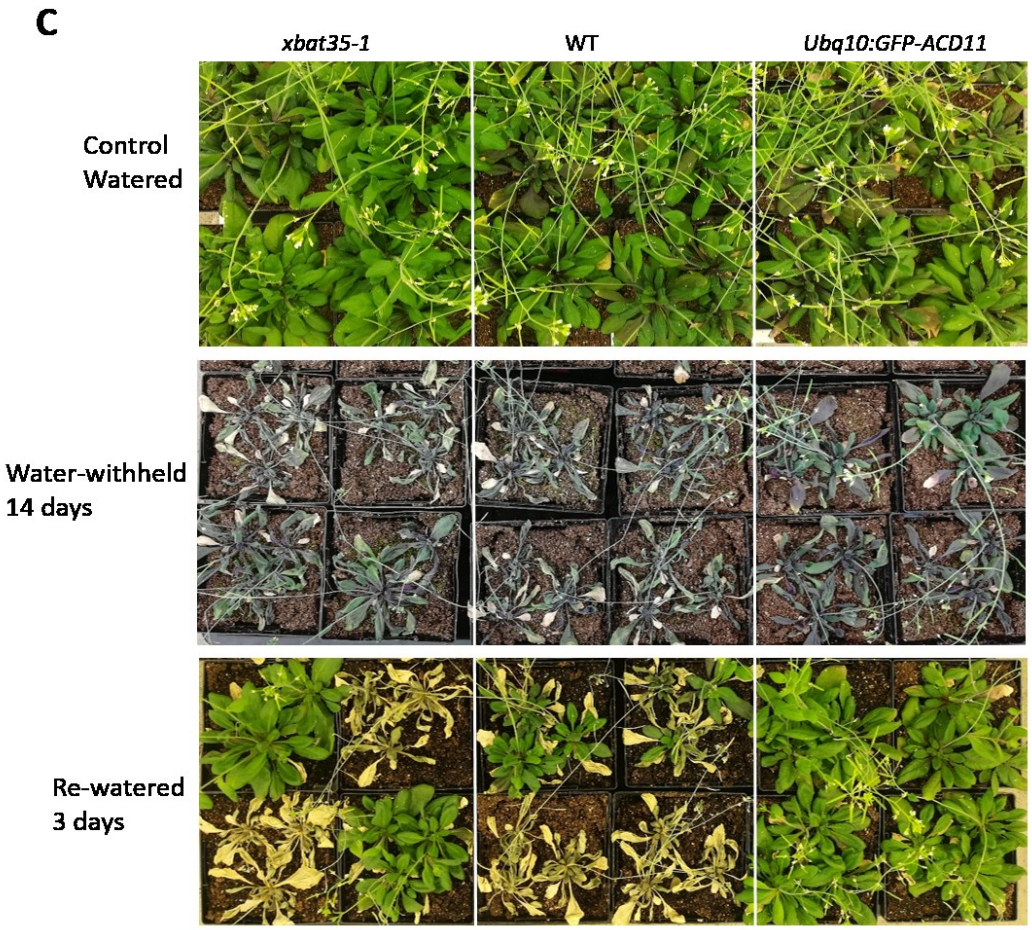
J) Graph showing germination rate at 3d for *Ubq10:GFP-ACD11* relative to WT. Germination was scored as radicle emergence. Bars represent average based on 3 independent trials with 7 replicates (n>30 seeds per replicate). Error bars represent ±SE. "*" indicates a statistically significant ($p \leq 0.05$) difference compared to WT based on a Tukey's test.

Increase in ACD11 abundance enhanced salinity and drought tolerance.

To confirm the involvement of XBAT35.2 and ACD11 in abiotic stress responses, we further evaluated the ability of *xbat35-1*, which should accumulate ACD11, and *ACD11* overexpressing seedlings to tolerate from high salinity conditions. Five-day-old seedlings were transferred to growth medium without or with 150 mM NaCl for an additional 3 days, after which seedling bleaching rate was assessed (**Figure 2.7A**). Compared to WT seedlings, *xbat35-1* and *ACD11* overexpressing seedlings had significantly higher survival rate, with fewer bleached (white cotyledons) seedlings (**Figure 2.7A and B**).

Drought tolerance of *xbat35-1* and *ACD11* overexpressing plants was also compared to WT plants. Three-week-old to four-week-old plants were either watered continually (control) or water was withheld for 14 days followed by rewatering for 3 days, after which plant survival was calculated. After rewatering, only 29.63% (n=54) of WT plants recovered (**Figure 2.8C and D**). The survival rate of *xbat35-1* plants was slightly higher (35.16%; n=52) than WT plants, but the difference was not significant (**Figure 2.7D**). It is noted that even though this difference was not significant with regard to survival rate, *xbat35-1* plants after rewatering had a noticeably better growth relative to WT plants (**Figure 2.7C**). Strikingly, the survival rate of *ACD11* overexpressing plants was significantly higher, with 95.37% of plants (n=52) recovering from the drought period (**Figure 2.7D**). The results from these assays indicate that increasing ACD11 abundance enhanced abiotic stress tolerance.


A
xbat35-1
Ubq10:GFP-ACD11
WT
GM
150 mM NaCl
B
100%
80%
60%
40%
20%
0%
Survival rate (%)
*
*
WT
xbat35-1
Ubq10:GFP-ACD11


C
xbat35-1
WT
Ubq10:GFP-ACD11
Control
Watered
Water-withheld
14 days
Re-watered
3 days

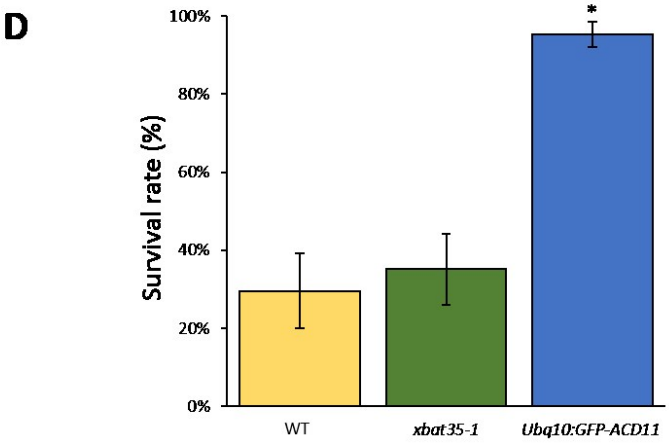

D
Survival rate (%)
100%
80%
60%
40%
20%
0%
*
WT
xbat35-1
Ubq10:GFP-ACD11

Figure 2.7. Increase in ACD11 abundance improves salt and drought stress tolerance.

A and B) Five-day-old wild type (WT), *xbat35-1*, and transgenic seedlings overexpressing GFP-ACD11 (*Ubq10:GFP-ACD11*) were transferred to growth medium (GM) supplemented without or with 150 mM NaCl and grown for 3 days. Pictures were then taken (A) and survival rate was determined (B). Arrow heads (A) indicate seedlings with bleached (white) cotyledons. The graph (B) represents percent green seedlings after growth on NaCl. Bars represent average based on 3 independent trials with n>14 per genotype per trial. Error bars represent ±SE. "*" indicates a statistically significant ($p \leq 0.05$) difference compared to WT based on a Tukey's test.

C and D) Three- to four-week-old WT, *xbat35-1*, and transgenic plants overexpressing GFP-ACD11 (*Ubq10:GFP-ACD11*) were used for drought-tolerance assays. C) Representative pictures of plants that were watered (control, top panel), and plants that had water withheld for 14 days (middle panel) and then rewatered for 3 days (bottom panel). D) Graph shows percent survival after rewatering. Bars represent average based on 3 independent trials, with n>16 per genotype for each trial. Error bars represent ±SE. "*" indicates a statistically significant ($p \leq 0.05$) difference compared to WT based on a Tukey's test.

2.4 DISCUSSION

We previously elucidated that the UPS modulates the abundance of the E3 XBAT35.2 and its substrate protein ACD11 to promote defense against pathogens (Liu et al., 2017). In this study, we provide evidence for roles for XBAT35.2 and ACD11 in abiotic stress tolerance. Our results demonstrated that the expression of *XBAT35.2* and *ACD11* was ABA-inducible. In addition, ABA and salt stress affected the stability of both proteins. At the lower concentrations used in the study, ABA was found to increase transcript levels and stabilized ACD11, leading to accumulation of this protein. In contrast, higher concentrations of ABA did not increase *ACD11* transcript levels, and was also found to promote the ubiquitin-mediated proteasome-dependent degradation of ACD11. For XBAT35.2, transcript levels and protein stability increased along with the concentration of ABA and salt. The results also support a role for XBAT35.2 in targeting ACD11 for degradation under stress conditions. In fact, in the absence of the E3, the turnover of ACD11 was slower in the presence of ABA and salt, suggesting the involvement of XBAT35.2 in stress-induced degradation of ACD11. Furthermore, increase in ACD11 abundance, via overexpression or loss of E3 function (*xbat35-1* mutant), improved plant tolerance of salt and drought stresses. Taken together, the results strongly support for a novel role for XBAT35.2 in abiotic stress response through modulating the degradation of ACD11.

An E3 enzyme has the potential to facilitate the ubiquitination of multiple substrates and may therefore regulate an array of cellular processes. An increasing body of work has provided evidence for ubiquitin ligases with dual or multiple roles. RING-type E3s, such as KEG and MYB30-INTERACTING E3 LIGASE 1 (MIEL1), have been shown to regulate responses to abiotic stresses and defense against pathogens (Wawrzynska et al., 2008; Liu and Stone, 2010; Lu et al., 2011; Marino et al., 2013; Kong et al., 2015; Lee and Seo, 2016). KEG is required to suppress ABA signaling in the absence of stress and is involved in response to fungal infection (Wawrzynska et al., 2008; Liu and Stone, 2010). MIEL1 regulation of two MYB transcription factors, MYB DOMAIN PROTEIN 30 (MYB30) and MYB96, influences pathogen defense, ABA signaling and cuticle wax biosynthesis (Marino et al., 2013; Lee and Seo, 2016; Gil et al., 2017). The

multifunctional Plant U-box 13 (PUB13) is involved in flower development, cell death, and pathogen defense, as well as modulating ABA and brassinosteroid signaling (Lu et al., 2011; Marino et al., 2013; Kong et al., 2015; Zhou et al., 2015; Zhou et al., 2018). Our findings identify XBAT35.2 as a dual functioning E3 with roles in biotic and abiotic stress responses. XBAT35.2 is unstable under non-stress conditions, and is thought to promote its own ubiquitination and subsequent proteasomal degradation, which maintains low levels of the E3 (Liu et al., 2017). Pathogen infection (Liu et al., 2017) and abiotic stress (salt/ABA, this study) promote the accumulation of XBAT35.2. The decrease in the levels of ubiquitinated XBAT35.2 following exposure to stress, suggest stabilization of the E3 perhaps due to decrease in the self-ubiquitination. The involvement of other E3s in regulating the abundance of XBAT35.2 under non-stress conditions remains a possibility. The stress-induced stabilization of XBAT35.2 would lead to the increased ubiquitination of its substrates, including ACD11. Interestingly, varying outcomes of XBAT35.2-mediated degradation of ACD11 were observed under different stresses. Following pathogen infection, stabilized XBAT35.2 facilitates ACD11 turnover to promote cell death, thus restricting the spread of pathogen. In contrast, in response to abiotic stimulus, stabilized XBAT35.2 promotes the degradation of ACD11, attenuating or terminating responses. E3 ligases targeting a positive regulator to attenuate or terminate stress responses are well documented. For example, in the presence of abiotic stress, ABA promote the expression and increase stability ABI5. However, ABA induces the proteasome-dependent degradation of the transcription factor via multiple E3s, including DWD hypersensitive to ABA1 (DWA1), DWA2, and ABA-hypersensitive DCAF1 (ABD1) CULLIN4-based E3 ligase complexes to dampen/stop stress response (Lee et al., 2010a; Liu and Stone, 2013; Seo et al., 2014). Overall, XBAT35.2 has a positive role in biotic stress resistance, while negatively regulating abiotic stress response.

A dual role of XBAT35.2 through the modulation of a single substrate maybe explained by the function of ACD11, which has been described as a sphingosine transfer protein involved in sphingolipid metabolism (Brodersen et al., 2002). Sphingolipids are involved in cell proliferation, migration, senescence, and differentiation, as well as PCD in eukaryotes (Breslow and Weissman, 2010; Hannich et al., 2011). In plants, sphingolipid

metabolism has been linked to PCD, biotic and abiotic stress tolerance. While roles for sphingolipid metabolism in pathogen defense is well understood, knowledge of sphingolipid signaling in abiotic stress responses is limited (Takahashi et al., 2009; Berkey et al., 2012; Ali et al., 2018; Huby et al., 2019). ACD11 is proposed to function in the transfer of the sphingosine molecule across membranes (Brodersen et al., 2002). Disruption of the *ACD11* gene expression leads to spontaneous cell death early in development, as well as the constitutive expression of *pathogen-related* (*PR*) genes, which depends on SA (Brodersen et al., 2002). ACD11 acting as an inhibitor of cell death and defense signaling correlates with previous findings where ubiquitin-dependent decrease in ACD11 abundance promotes pathogen resistance (Liu et al., 2017). Here, we show that ABA and salt treatments promote the accumulation of ACD11 and the increase in ACD11 abundance results in the enhanced tolerance of abiotic stresses. The link between sphingolipid metabolism, cell death and abiotic stress tolerance is illustrated by another lesion-mimic mutant *Accelerated Cell Death 5* (*ACD5*), which encodes for ceramide kinase that converts the sphingolipid molecule ceramide (sphingosine with a fatty acid) into ceramide-1-phosphate (Liang et al., 2003; Bi et al., 2014). *acd5* mutants accumulate ceramide substrate, displaying normal early development with spontaneous SA-dependent cell death occurring later, and are more susceptible to pathogens (Greenberg et al., 2000; Liang et al., 2003; Bi et al., 2014). A recent study showed that exposure to salt suppresses ceramide accumulation and cell death in *acd5* mutants, while ABA was found to not inhibit but delay the onset of or reduce cell death (Yang et al., 2019). An interesting postulation is that the concomitant salt- and ABA- induced accumulation of ACD11 may be involved in the observed reduction in ceramide and suppression or delay in cell death. Yang et al. also showed that exposure to salt enhanced pathogen resistance in wild type Arabidopsis and *acd5* mutants. The report examined pathogen resistance after treating plants with salt (300 mM) for 24 hours. Our results show that high concentrations or prolonged exposure to salt lead to the degradation of ACD11. Therefore, it would be interesting to determine if salt-induced reduction in ACD11 abundance may contribute to the enhanced pathogen resistance observed in the salt treated plants. Another sphingolipid signaling element, the monocation-induced $[Ca2+]_i$ increases 1 (MOCA1), encoding for the glucuronosyltransferase, was recently

shown to be involved in salt sensing in the plasma-membrane (Jiang et al., 2019). Therefore, sphingolipid signaling components play important and distinct roles in abiotic stress responses, and mechanisms of ACD11 modulating abiotic stress responses and tolerance require further studies.

The involvement of XBAT35.2 activity in the proteasome-dependent degradation of ACD11 was previously demonstrated in cell free degradation assay using proteins derived from a transient expression system in tobacco (*Nicotiana benthamiana*) leaves (Liu et al., 2017). In this study, we provide further confirmation for a role for XBAT35.2 in directing ACD11 turnover. The in vivo degradation of ACD11 was compared in transgenic plants carrying a wild type or mutated version of the *XBAT35* gene. The degradation of ACD11 slowed down considerably in the absence of XBAT35.2 but only in the presence of ABA/salt, suggesting that XBAT35.2 is predominantly responsible for ACD11 for degradation under stress conditions. This supports the previously stated hypothesis that XBAT35.2-mediated degradation of ACD11 occurs in response to an external stimulus, which may cause modification of the E3 and/or substrate, thus allowing the interaction between XBAT35.2 and ACD11 (Liu et al., 2017). Further study is needed to identify the molecular mechanism(s) that directs the interaction between XBAT35.2 and ACD11, providing useful insights into how plants regulate E3-substrate engagement under different contexts. Regardless of the nature of the signal(s), the outcome of XBAT35.2 mediating turnover of ACD11 differs in response to biotic and abiotic stress, leading to increased resistance and decreased tolerance, respectively.

Intriguingly, seed germination sensitivity analysis showed that altering ACD11 levels, via mutating and overexpressing *XBAT35.2* or overexpressing *ACD11*, led to ABA/salt hyposensitivity. The hyposensitivity to ABA/salt may be due to other identified ACD11 interacting partners, such as Golgi-localized Prenylated Rab Acceptor 1.F (PRA1.F) proteins (Petersen et al., 2009; Braun P et al., 2011). A recent study showed that both knockdown or overexpression of *PRA1.F4* resulted in the same phenotype, hypersensitivity to salt (Lee et al., 2017). It is possible that the interaction between Golgi-localized ACD11 and PRA1.F proteins is important for responses to abiotic stress and disrupting the ACD11 levels may in turn affect the functioning of PRA1.F proteins,

causing the abnormal sensitivity to abiotic stress. Alternatively, the hyposensitivity observed in seeds with reduced levels of ACD11 may be due to the cross-talk of SA and ABA/salt responses. As reported previously, *acd11* mutants accumulate a significant amount of SA and the hormone has been shown to promote seed germination under high salinity (Brodersen et al., 2002; Brodersen, 2005; Lee et al., 2010b). Therefore, the potentially increased SA levels in *XBAT35.2* overexpressing seeds may reduce the inhibitory effects of ABA and high salt on germination, leading to their insensitivity to ABA/salt treatments.

On the basis of these findings, we propose a model that the ubiquitin ligase XBAT35.2 plays a negative role in abiotic stress responses, while ACD11 has a positive role, to promote stress tolerance (**Figure 2.8**). Under non-stress conditions, XBAT35.2 undergoes autoubiquitination to promote its degradation, which allows ACD11 to accumulate and mediate fundamental cellular processes. Upon abiotic stress, the abundance of XBAT35.2 gradually increases, and ACD11 levels are also upregulated to promote stress tolerance. As the stress continues or the severity of stress increases, the abundance of ACD11 decreases, which largely requires the stabilized XBAT35.2, perhaps leading to the termination of stress responses or the onset of cell death. Therefore, maintaining high levels of ACD11, through overexpressing ACD11 or mutating XBAT35, would enhance stress tolerance.

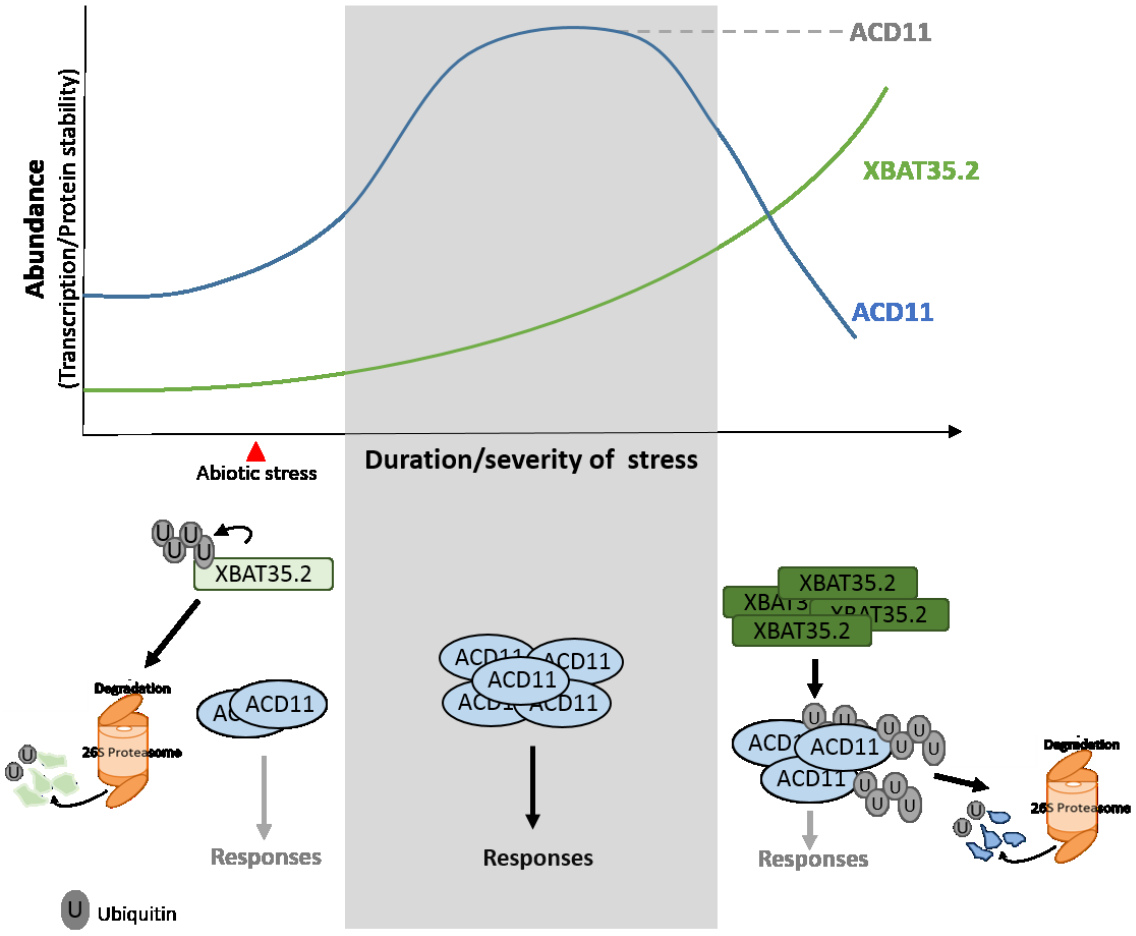


Figure 2.8. Model for XBAT35.2 regulating ACD11 abundance during abiotic stress.

Under non-stress conditions, the ubiquitin ligase, XBAT35.2, is subjected to self-ubiquitination and proteasome-dependent degradation, which allows low levels of the E3. Therefore, ACD11 proteins are maintained at the appropriate abundance to facilitate proper cellular functions. Upon exposure to stress (red arrow), ACD11 abundance increases, which promotes tolerance. Also, stabilized XBAT35.2 gradually accumulates, which then mediates the ubiquitination and proteasome-dependent degradation of ACD11 to terminate or attenuate ACD11-mediate responses. Alterations, such as loss of E3 activity (*xbat35-1*) or transgenic overexpression (*Ubq10:GFP-ACD11*), that result in higher or maintaining increased levels of ACD11 under stress conditions are thought to enhance stress tolerance (grey dashed line).

2.5 MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana ecotype Col-0 was used as wild type. The mutant *xbat35-1* (SALK_104813) was previously described (Liu et al., 2017). Generation of transgenic seeds, HA-XBAT35.2, YFP-XBAT35.2 were also described previously by Liu et al. (2017). Homozygous ACD11-GFP and ACD11-GFP/*xbat35-1* transgenic lines were kindly provided by Dr. Daniel Hofius. Expression of transgene in transgenic plants was confirmed by immunoblotting assays (**Appendix Figure 5.7**) and detailed information of seeds used in this study is in **Appendix Table A2.1 and Table A2.2**.

Seeds were surface-sterilized with 50% (v/v) bleach and 0.1% Triton X-100. After vernalization at 4 ^{o}C in darkness for 3 days, seeds were germinated and grown on half-strength MS medium containing 0.8% agar and 1% sucrose plates under continuous light at 22 ^{o}C. For plants grown in soil, seven- to ten-day-old plants were transferred from growth medium (GM) to soil in a growth chamber under a 16-h-light/ 8-h-dark photoperiod.

Seed germination sensitivity assay

Seeds were collected at the same time and used for assays. Different genotype seeds were placed on half MS plates with or without the indicated concentration of ABA or NaCl at 22 ^{o}C with continuous light for 3 days. Germination was scored as radical emergence.

Drought tolerance assay

3-week-old to 4-week-old plants were grown in soil under standard conditions were used. Water was withheld for 14 days and then plants were rewatered, and surviving plants were counted after 3 days. This experiment was repeated three times with at least 16 plants for each genotype.

Salt tolerance assay

5-day-old seedlings grown on half MS plates were transferred onto half MS plates without or with 150 mM NaCl. After 3 days, the surviving plants with non-bleached leaves, as the indicator of salt tolerance, were counted.

RNA extraction and RT-qPCR

RNA was extracted from seedlings using RNAzolTM reagent according to manufacturer's instructions (Sigma-Aldrich). Extracted RNA was then treated with DNase 1 (New England Biosystems). A Nanodrop machine (Nanophotometer P360, Implen) was used to determine RNA concentration and purity by measuring absorbance 260/280 and 260/230. For RT-qPCR, the Dnase-treated RNA was used for reverse transcription (RT) reaction using the iScriptTM Reverse Transcription Supermix (Biorad) as per manufacturer's instructions. No-RT enzyme controls were used to ensure that there was no DNA contamination. The cDNA produced was used to conduct a quantitative polymerase chain reaction (qPCR) analysis using the SsoAdvancedTM Universal SYBR® Green Supermix kit (BIO-RAD) as described by the manufacturer and the StepOneTM Real-Time PCR machine (Applied Biosystems). All primers (see **Appendix Table A2.3**) were validated before use for ideal annealing temperature and standard curve measurements to determine primer volume for the reaction. All primers annealed effectively at 60 ℃ and had an ideal concentration titter of 15 ng/microliter.

Cycloheximide treatments

Six-day-old Arabidopsis seedlings overexpressing *35S:HA-XBAT35.2*, *35S:YFP-XBAT35.2*, *Ubq10:GFP-ACD11*, *Ubq10:GFP-ACD11/WT* and *Ubq10:GFP-ACD11/xbat35-1* were transferred to liquid medium for 1 day. Then indicated amount of cycloheximide was added to the liquid medium with or without other chemicals. Treated seedlings were sampled at the indicated time points for immunoblotting assays.

Sample preparation and immunoblotting

Total protein was extracted from Arabidopsis seedlings by using extraction buffer (50 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM Na3VO4, 10 mM NaF, 50 mM b-glycerophosphate, 10 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5% glycerol, and protease inhibitor cocktail [Sigma-Aldrich]). The concentration of protein was determined by the Bradford reagent (BIO-RAD). Samples were mixed with sodium dodecyl sulfate (SDS) loading buffer and then boiled at 95 ^{o}C for 5 minutes.

For immunoblot analysis, prepared samples were separated by 7.5% SDS-PAGE gel and subsequently transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were blocked for 1 hour with 5% non-fat milk powder in T-BST (50 mM TRIS-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) at room temperature and then incubated with specific primary antibodies in T-BST buffer for overnight. After three 10-min washes with TBST, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG at 1:5000 dilution for 2 hours (Sigma-Aldrich). Following by three times washed, membranes were visualized using the enhanced Western Blotting Substrate kit (BIO-RAD) according to the manufacturer's instructions. Ponceau S staining was used to indicate equal protein loading.

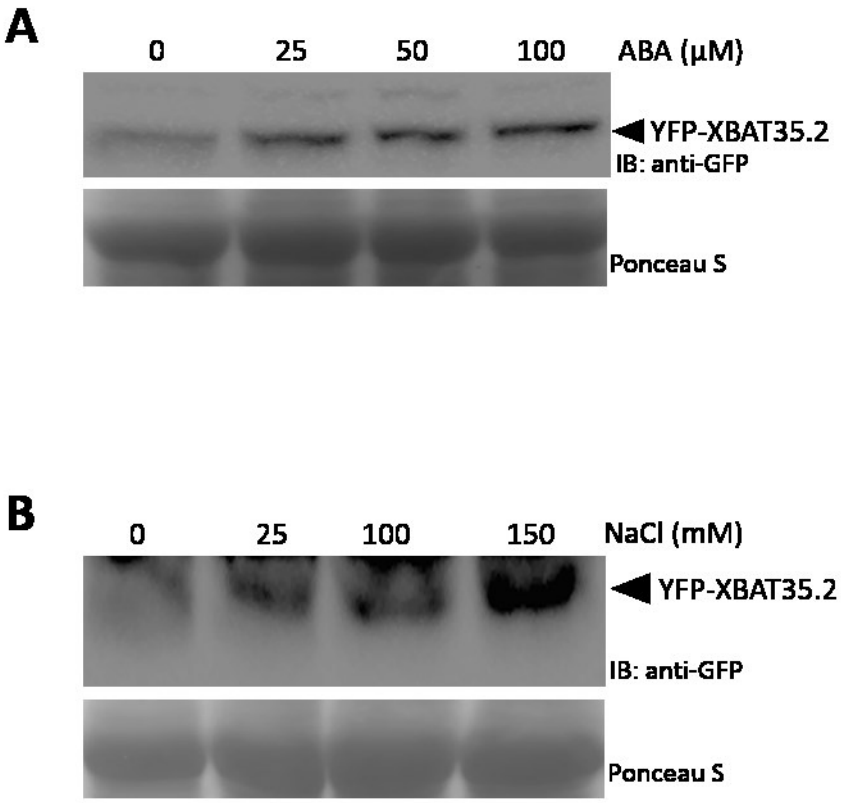
Immunoprecipitation (IP)

For IP assays with HA-XBAT35.2 overexpressing lines, protein extracts were incubated with anti-HA affinity beads (Sigma-Aldrich) at 4 ^{o}C for 2 hours after the Bradford protein quantification. After three 10-min washes with 20mM Tris-HCl and 150 mM NaCl, samples were boiled with SDS loading buffer for 10 minutes at 95 ^{o}C. Then supernatant was loaded to SDS-PAGE. Followed by transferred from the gel, PVDF membrane was detected by the anti-HA (Sigma-Aldrich) at 1:5000 or anti-ubiquitin (Boston Biochem) at 1:1250 as previously described. The target band, HA-XBAT35.2 after IP assays was verified (**Appendix Figure 5.8**).

For IP assays with ACD11-GFP transgenic plants, GFP-trap beads (Chromotek) were

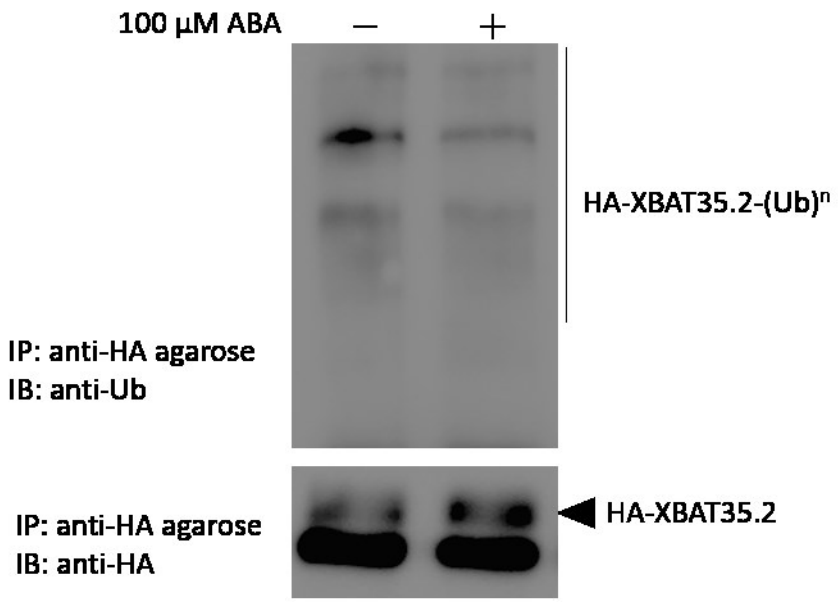
used following the manufacturer's instructions to isolate GFP-ACD11 from protein extracts prepared from *Ubq10:GFP-ACD11* seedlings with different treatments. Isolated proteins were then subjected to Western blot analysis with anti-GFP at 1:5000 or anti-ubiquitin antibodies as above described.

2.6 SUPPLEMENTAL FIGURES



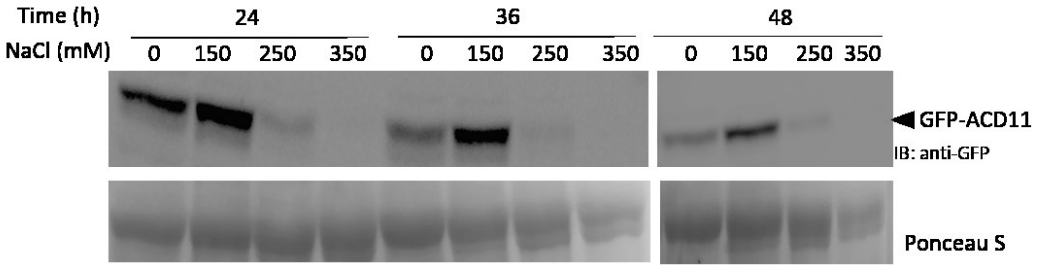
Supplemental Figure S2.1. Accumulation of XBAT35.2 in response to ABA and salt.

A and B) Increase in XBAT35.2 abundance in response to increasing concentrations of ABA (A) and NaCl (B). Seven-day-old transgenic seedlings overexpressing YFP-XBAT35.2 (*35S:YFP-XBAT35.2*) were incubated with ABA or NaCl for 12h. The levels of HA-XBAT35.2 were analyzed by immunoblot (IB) analysis with GFP antibodies. Ponceau S staining was used to visualize protein loading.



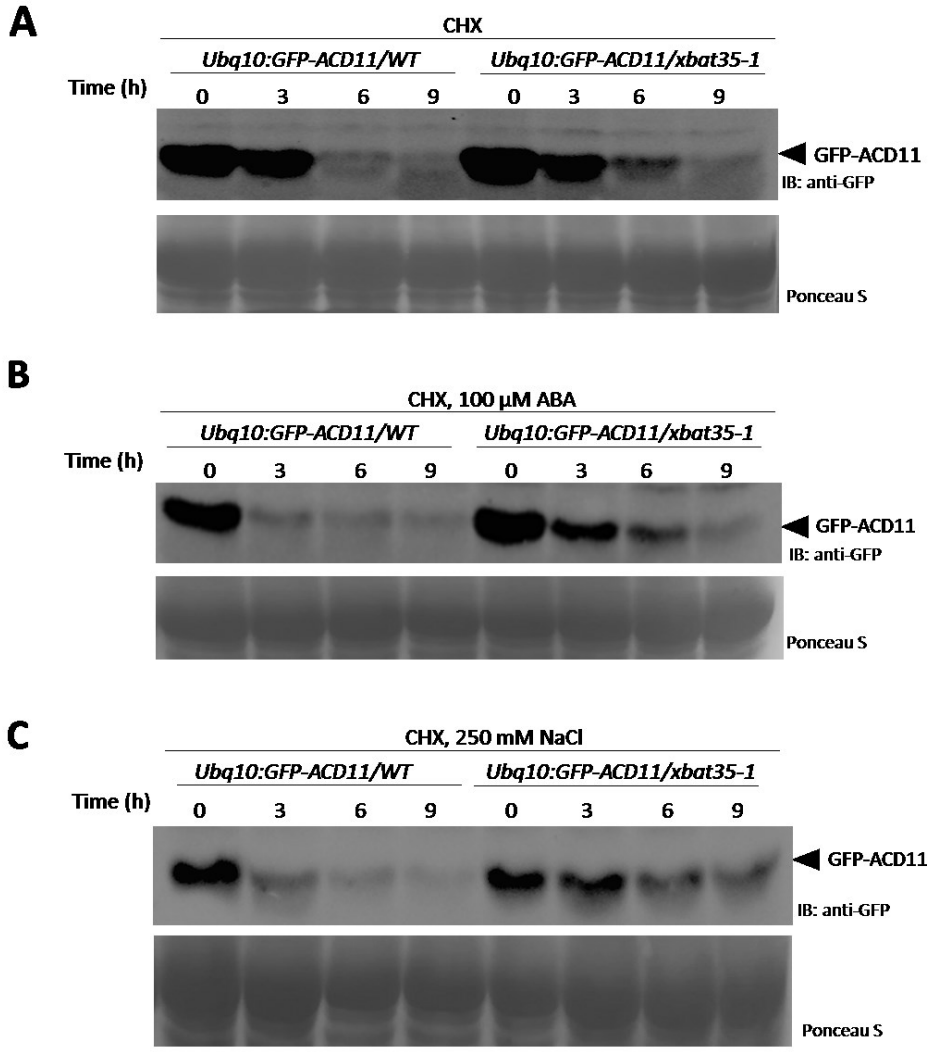
Supplemental Figure S2.2. Decrease in the polyubiquitinated form of XBAT35.2 following exposure to ABA.

Seven-day-old 35S:HA-XBAT35.2 transgenic seedlings were treated with (+) or without (-) 100 µM ABA for 12 hours. HA-XBAT35.2 was isolated by immunoprecipitation (IP) using anti-HA agarose beads, followed by immunoblot (IB) analysis with ubiquitin (Ub; top panel) and HA (bottom panel) antibodies. The high-molecular-weight isolates detected with ubiquitin antibodies (top panel) are indicative of polyubiquitinated HA-XBAT35.2 (HA-XBAT35.2-(Ub)n).



Supplemental Figure S2.3. Changes in ACD11 abundance in response to salt over a longer timeline.

Seven-day-old transgenic seedlings overexpressing GFP-ACD11 (*Ubq10:GFP-ACD11*) were treated the indicated concentration of NaCl. Samples were collected at the indicated time points and the abundance of GFP-ACD11 was determined by immunoblot (IB) analysis using GFP antibodies. Ponceau S staining was used to visualize protein loading.



Supplemental Figure S2.4. Degradation of ACD11 is slower in the absence of XBAT35.2 in the presence of ABA and salt.

Seven-day-old transgenic seedlings overexpressing GFP-ACD11 in the presence (*Ubq10:GFP-ACD11/WT*) and absence (*Ubq10:GFP-ACD11/xbat35-1*) of XBAT35.2 were treated with 300 µM CHX supplemented without (A) or with 100 µM ABA (B) or 250 mM NaCl (C). Samples were collected at the indicated time points and the abundance of GFP-ACD11 was determined by immunoblot (IB) analysis using GFP antibodies. Ponceau S staining was used to visualize protein loading.

CHAPTER THREE: CONCLUSIONS AND FUTURE WORK

Previously, we identified a role for ubiquitin ligase XBAT35.2 in biotic stress responses. Pathogen-stabilized XBAT35.2, acting as a positive regulator of defense responses via mediating the proteasomal degradation of ACD11, induces cell death. The dead plant cells promote plant resistance against pathogens by prohibiting the spread of pathogens (Liu et al., 2017).

In this study, we demonstrated that both XBAT35.2 and its known substrate ACD11 are involved in abiotic stress tolerance. Here ACD11 functions as a positive regulator to mount stress tolerance. However, stress-stabilized XBAT35.2 targets ACD11 for degradation via the 26S proteasome, perhaps leading to the attenuation of stress responses.

Based on these findings, we propose a model in which roles for XBAT35.2 differ during biotic and abiotic stress (**Figure 3.1**). Under non-stress conditions, XBAT35.2's self-ubiquitination promotes own proteasomal degradation of the E3, allowing for ACD11 abundance at a certain level required for proper regulation of cellular processes, including sphingolipid metabolism and cell death. Upon abiotic stress, ACD11 accumulates to induce downstream stress responses. In this context, XBAT35.2 is also induced, which triggers the ubiquitin-dependent degradation of ACD11. This turnover in ACD11 aims to avoid ACD11 accumulation out the control of plants or terminate signal transduction when abiotic stress relieves. During pathogen infection, XBAT35.2 also becomes stable and ubiquitinates ACD11, in turn sending ACD11 to the 26S proteasome for degradation. This decrease in ACD11 results in the cell death, as part of defense responses. Unlike its role upon exposure to abiotic stress, XBAT35.2 here acts as a positive regulator required for the activation of immune responses.

Due to the opposing roles of XBAT35.2 in biotic and abiotic stress, alterations in the E3 gene expression might benefit plants in some aspects, but these changes also incur several limitations. Loss-of-function analysis of XBAT35.2 provided evidence that downregulated expression levels of XBAT35.2, enhanced abiotic stress tolerance in plants. Nonetheless, such plants are more susceptible to pathogen infection. These results

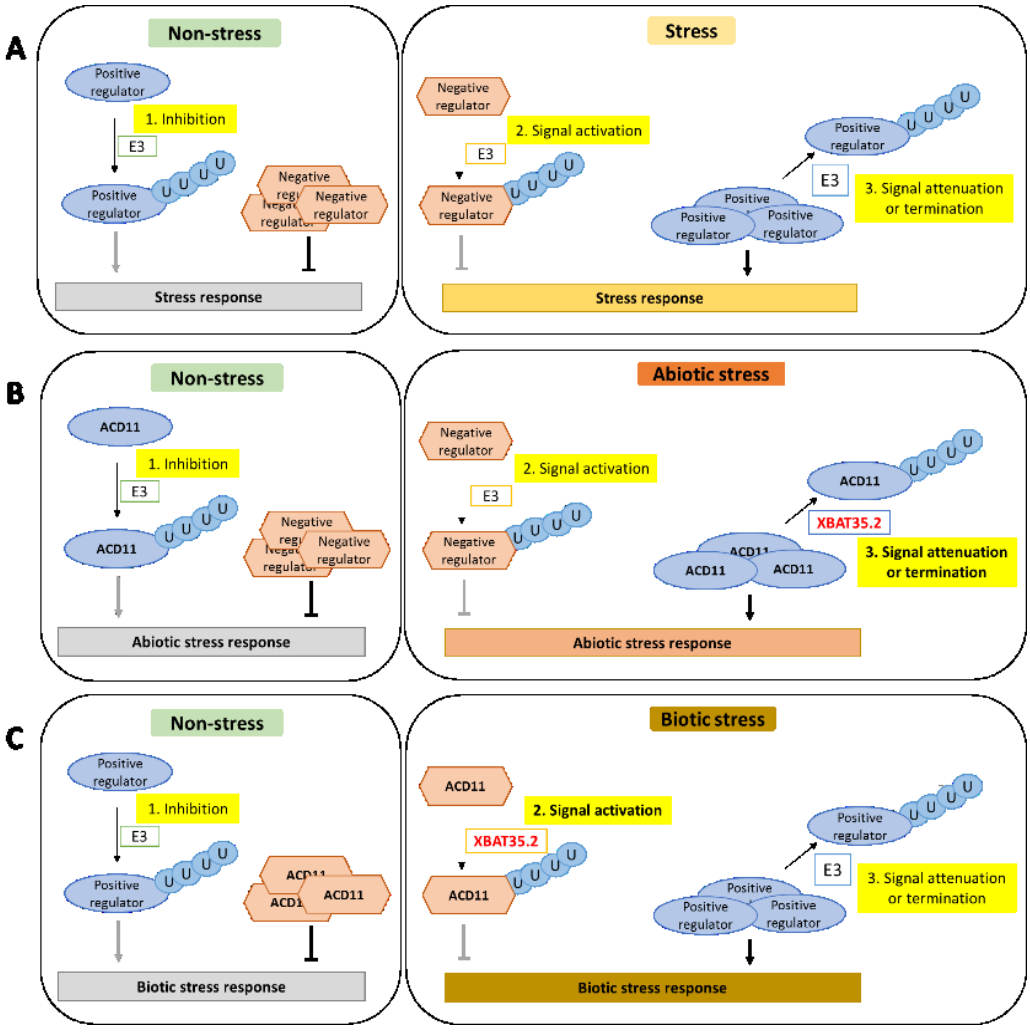


Figure 3.1. The different outcomes of the 26S proteasome-dependent degradation of ACD11 mediated by XBAT35.2 in response to biotic and abiotic stress.

A) The UPS regulation of stress signaling by targeting positive regulator for turnover to inhibit stress response in the absence of stress, or assisting in activating signaling by degrading negative regulators and attenuating signaling by destroying positive regulators in the presence of stress. B) In the case of abiotic stress, XBAT35.2 targets ACD11 for degradation to attenuate or terminate responses. C) In the context of biotic stress, XBAT35.2-mediated degradation of ACD11 serves to activate immune responses and promote resistance (Liu et al., 2017; Stone, 2014).

correlate with previous reports that some plants developed with improved tolerance to one particular stress are not resistant to the other type of stress (Kreye et al., 2009; Atkinson and Urwin, 2012; Cabello et al., 2014; Berens et al., 2019). These findings imply that perhaps XBAT35.2 has a role in balancing the trade-off between biotic and abiotic stress responses. As a result, manipulating expression of XBAT35.2 may not be an effective approach to engineer plants with both biotic and abiotic stress resistance.

Regarding future work, we have shown that in response to biotic and abiotic stress, the ubiquitination of XBAT35.2 is decreased. As previously mentioned, the decrease in self-ubiquitination of PUB22 relies on phosphorylation (Furlan et al., 2017). Consequently, it is feasible that phosphorylation of XBAT35.2 may also prohibit self-ubiquitination of the E3, allowing this protein to accumulate and target substrates for degradation. Besides, although we observed that the transgenic plants overexpressing ACD11 with enhanced abiotic stress, mechanisms by which ACD11 contributes to stress tolerance remain unknown. Given that ACD11 is a regulator of cell death, we postulate that plants upregulate levels of ACD11 to induce abiotic stress tolerance, perhaps aiming to delay progression of cell death. Therefore, future studies are required to investigate whether ACD11 promotes stress tolerance by manipulating cell death. Additionally, we have shown that under non-stress conditions, XBAT35.2 plays a minor role in targeting ACD11 for turnover. In fact, in our cycloheximide chase assay, we still observed noticeable 26S proteasome-dependent degradation and poly-ubiquitination of ACD11 under non-stress conditions, which implies that there are other ubiquitin ligases responsible for the turnover of ACD11. Identification of these ubiquitin ligases will help us tease out how ACD11 is regulated via ubiquitination in different conditions. Moreover, as mentioned, *acd5* mutants exhibited alterations in both ABA and SA production under salt treatment, which suggests a role for ACD5 in synthesis of ABA and SA upon abiotic stress (Yang et al., 2019). Consequently, it is possible that ACD11 might also be a modulator for the synthesis of both ABA and SA. Further analysis about the hormone composition of *acd11* mutants or ACD11 overexpressing plants under abiotic stress could tell us whether ACD11 coordinates ABA-dependent abiotic stress responses and SA-dependent immune signaling.

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APPENDIX

Appendix Table A2.1. Details of transgenic plant lines used

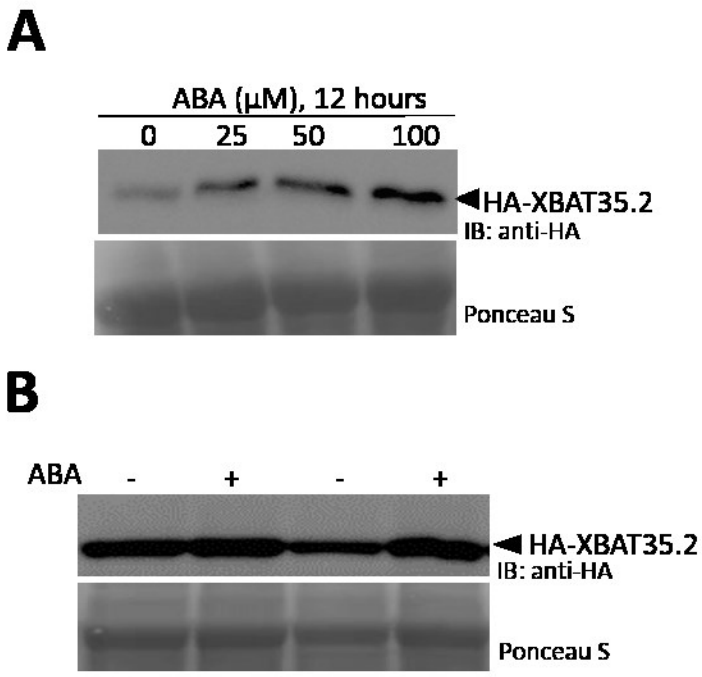
Plant name	Locus	Vector	Genotype	Background	Reference
35S:HA-XBAT35.2	AT3G23280.2	pEarleyGate 201-	Homozygous	WT(-Col)	Liu et al., 2017
35S:YFP-XBAT35.2	AT3G23280.2	pEarleyGate 104-	Homozygous	WT(-Col)	Liu et al., 2017
Ubq10:GFP-ACD11	AT2G34690	pUbiquitin10-ACD11	Homozygous	WT(-Col)	Grefen et al., 2010; Munch et al., 2015
Ubq10:GFP-ACD11/xbat35-1	\	pUbiquitin10-ACD11	Homozygous	*xbat35-1*	\

Appendix Table A2.2. Details of mutant lines used

Plant name	Locus	ABRC ID	Genotype	Background	Description
xbat35-1	AT3G23280	Salk_ 104813	Homozygous	WT(-Col)	Liu et al., 2017

Appendix Table A2.3. Details of primers used for qPCR

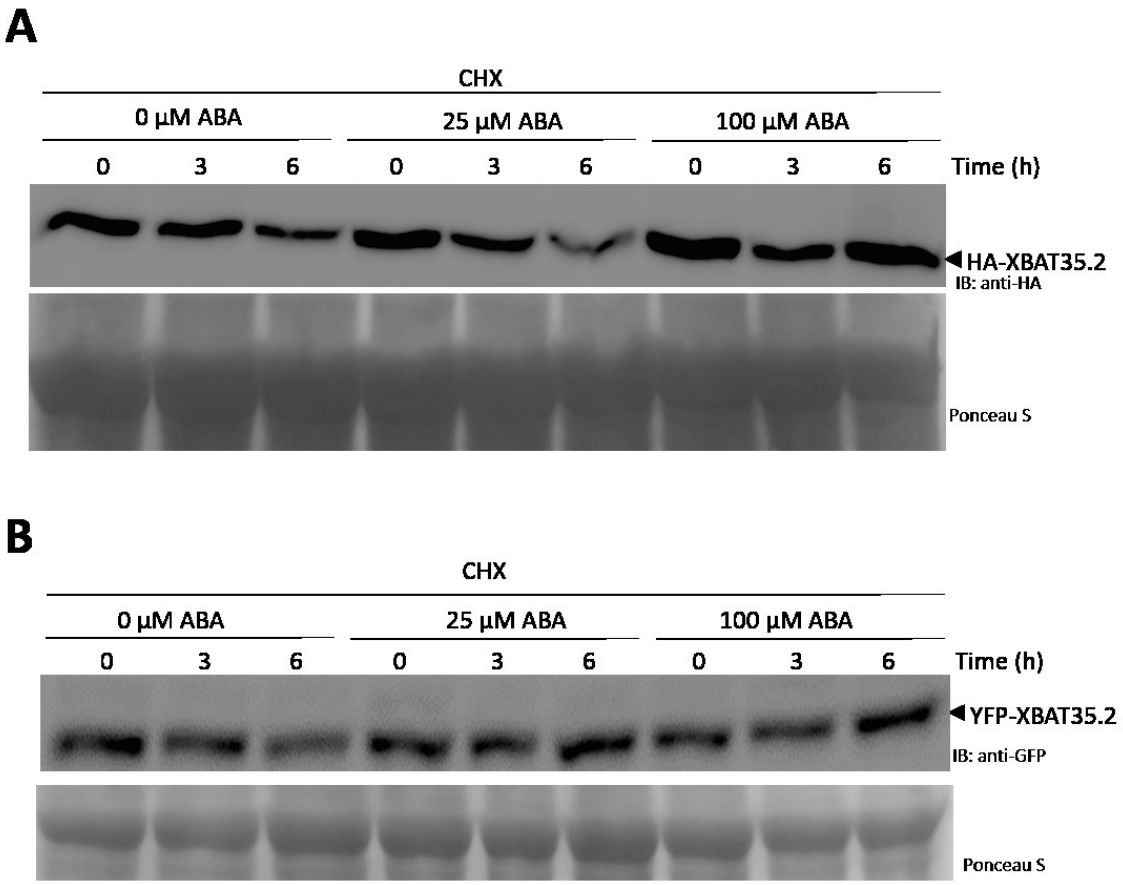
Gene/Primer Name	Sequence	
	Forward	**Reverse**
GAPDH	5'-TTGGTGACAACAGGTCAAGCA-3'	5'-AAACTTGTCGCTCAATGCAAT-3'
XBAT35.2	5'-ACTCCACAAAAACACGTC-3'	5'-TGTGGAATCCCTTTACATGC-3'
RD29A	5'-TATTCGCCGGAATCTGACGG-3'	5'-GATGCCTCACCGTATCCAGG-3'
ACD11	5'-ATGATCTTGTGAGGGCGTCG-3'	5'-ACCATGTCAAGACCACGCTT-3'



Appendix Figure A5.1. Assays showing the ABA-induced increase in XBAT35.2 protein abundance.

A) Seven-day-old transgenic seedlings overexpressing 35S:HA-XBAT35.2 were treated with increasing concentrations of ABA for 12 hours. The levels of HA-XBAT35.2 were analyzed by immunoblot (IB) analysis with HA antibodies. Ponceau S staining was used to visualize protein loading.

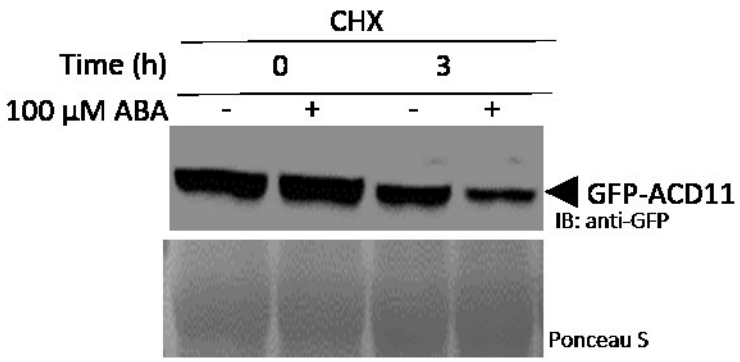
B) Seven-day-old transgenic seedlings overexpressing 35S:HA-XBAT35.2 were treated with or without 100 μM ABA for 12 hours. The levels of HA-XBAT35.2 were analyzed by immunoblot (IB) analysis with anti-HA antibodies. Ponceau S staining was used to visualize protein loading.



Appendix Figure A5.2. Assays showing the effect of ABA on XBAT35.2 stability.

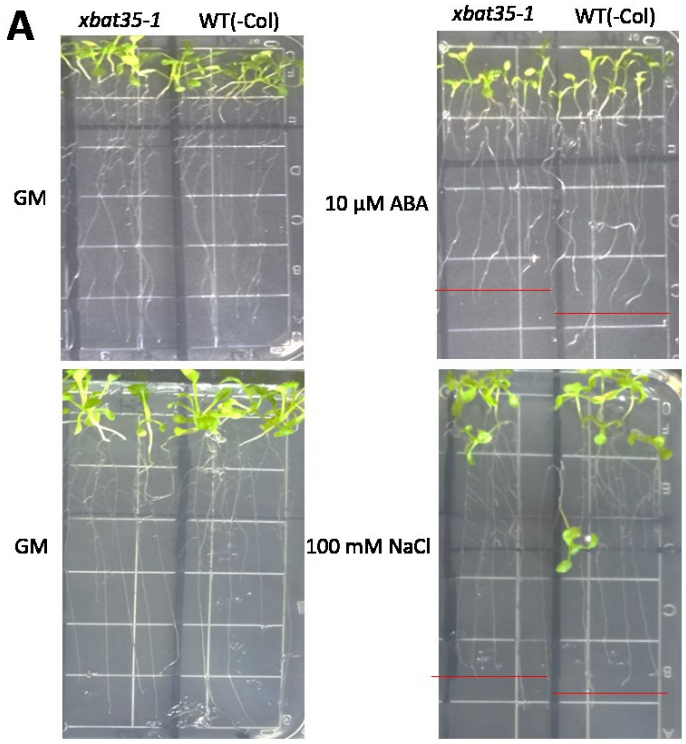
A) Seven-day-old *35S:HA-XBAT35.2* transgenic seedlings were treated with the indicated concentrations of ABA for 3 hours, and then with an addition of 500 μM CHX for indicated time. The levels of HA-XBAT35.2 were analyzed by immunoblot (IB) analysis with HA antibodies. Ponceau S staining was used to visualize protein loading.

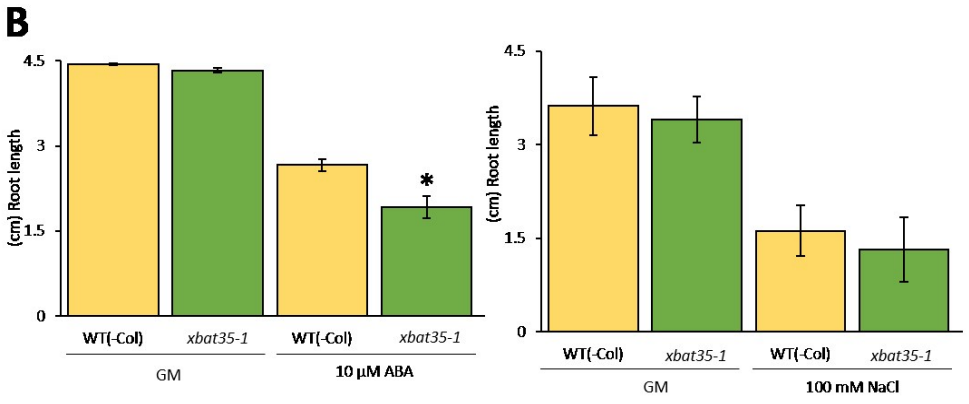
B) Seven-day-old *35S:YFP-XBAT35.2* transgenic seedlings were treated with the indicated concentrations of ABA for 3 hours, and then with an addition of 500 μM CHX for indicated time. The levels of YFP-XBAT35.2 were analyzed by immunoblot (IB) analysis with GFP antibodies. Ponceau S staining was used to visualize protein loading.



Appendix Figure A5.2. Assay showing decreased stability of ACD11 at higher concentrations of ABA.

Seven-day-old *Ubq10:GFP-ACD11* transgenic seedlings were treated with 300 µM CHX and with (+) or without (-) 100 µM ABA. The abundance of GFP-ACD11 was analyzed by immunoblot (IB) analysis with GFP antibodies. Ponceau S staining was used to visualize protein loading.

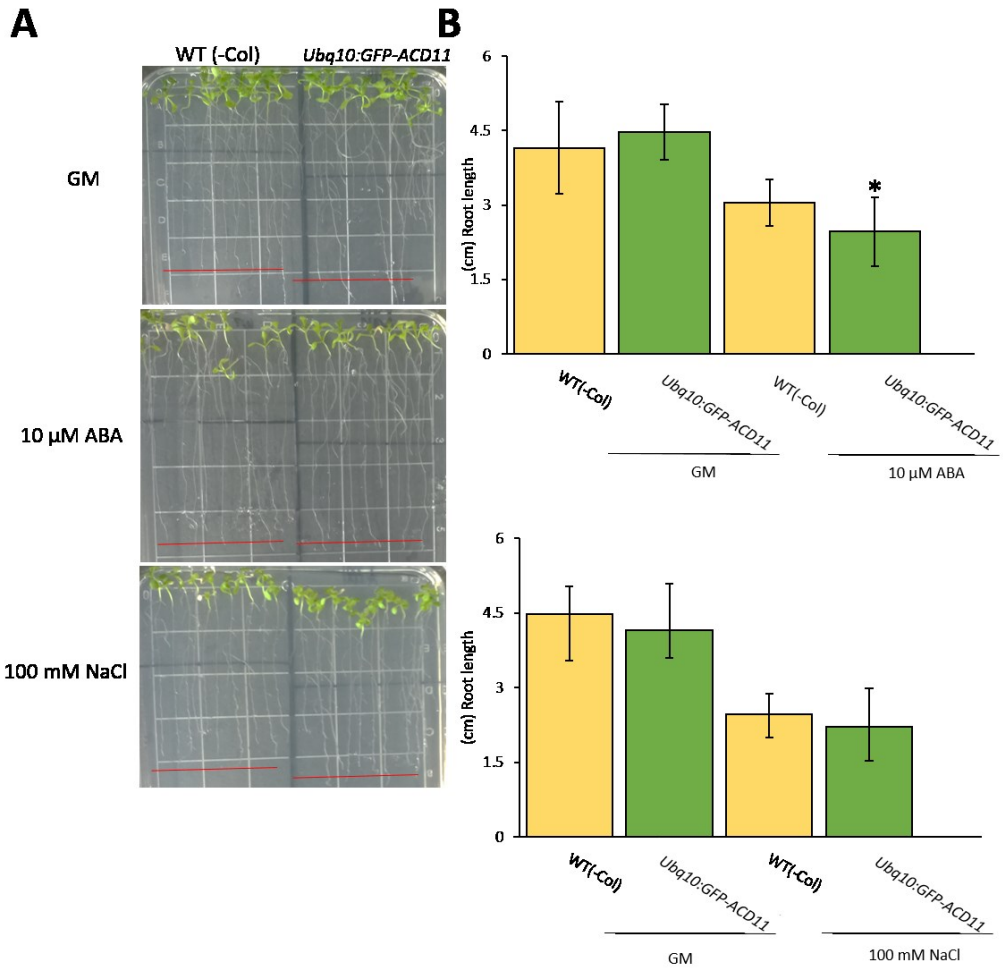

A
xbat35-1
WT(-Col)
xbat35-1
WT(-Col)
GM
10 μM ABA
GM
100 mM NaCl


B
4.5
3
1.5
0
(cm) Root length
WT(-Col)
xbat35-1
WT(-Col)
xbat35-1
GM
10 μM ABA
*
4.5
3
1.5
0
(cm) Root length
WT(-Col)
xbat35-1
WT(-Col)
xbat35-1
GM
100 mM NaCl

Appendix Figure A5.3. Growth of *xbat35-1* primary roots was sensitive to ABA, but not salt.

A) Five-day-old wild type (WT) and *xbat35-1* seedlings were transferred to growth medium supplemented with 0.5 μM ABA or 100 mM NaCl. Photographs were taken after 5 days.

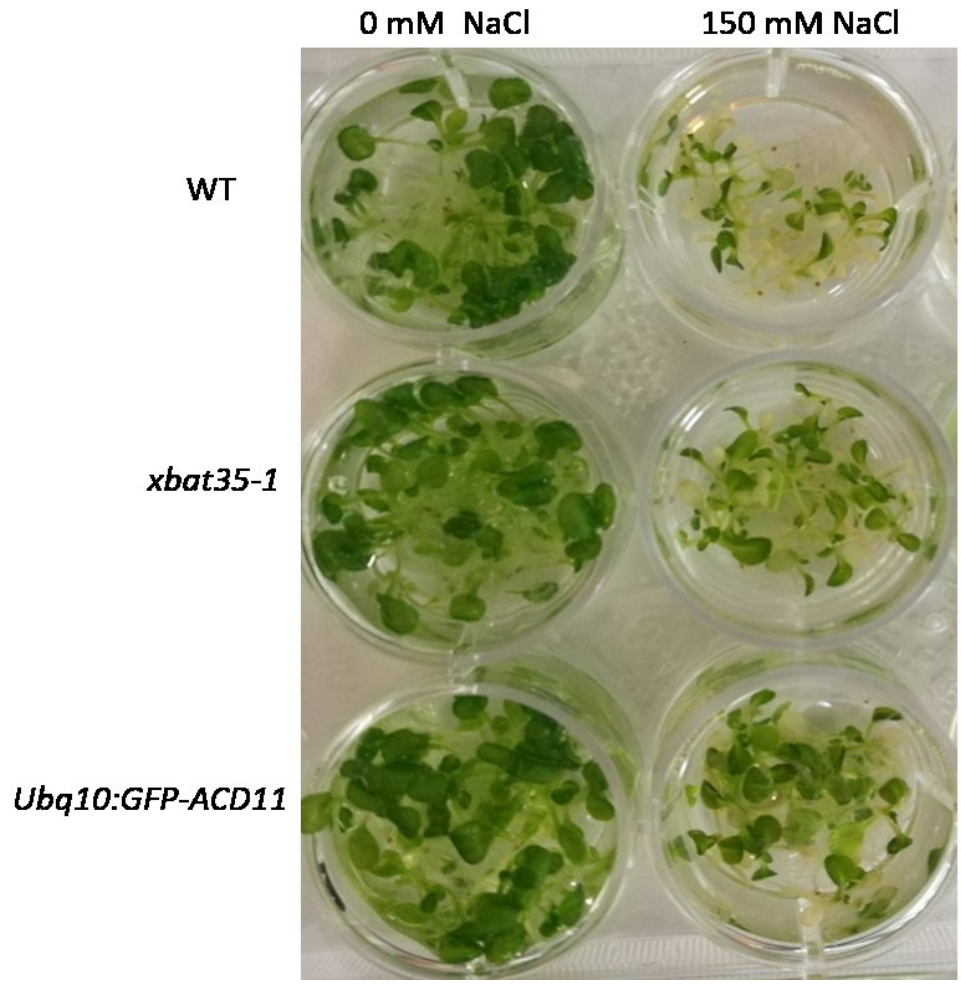
B) Graphs shows root length growth after transfer**.** Root length was measured using ImageJ (Abràmoff et al., 2004). Bars represent averages of root length based on 3 independent trials with 3 replicates each (n>5 per replicate). Error bars represent ±SE. "*" indicates a statistically significant ($p \leq 0.05$) difference compared to WT based on a Tukey's test.


A
WT (-Col)
Ubq10:GFP-ACD11
GM
10 µM ABA
100 mM NaCl
B
(cm) Root length
6
4.5
3
1.5
0
*
WT(-Col)
Ubq10:GFP-ACD11
WT(-Col)
Ubq10:GFP-ACD11
GM
10 µM ABA
(cm) Root length
6
4.5
3
1.5
0
WT(-Col)
Ubq10:GFP-ACD11
WT(-Col)
Ubq10:GFP-ACD11
GM
100 mM NaCl

Appendix Figure A5.4. Growth of *Ubq10:GFP-ACD11* primary roots was sensitive to ABA, but not salt.

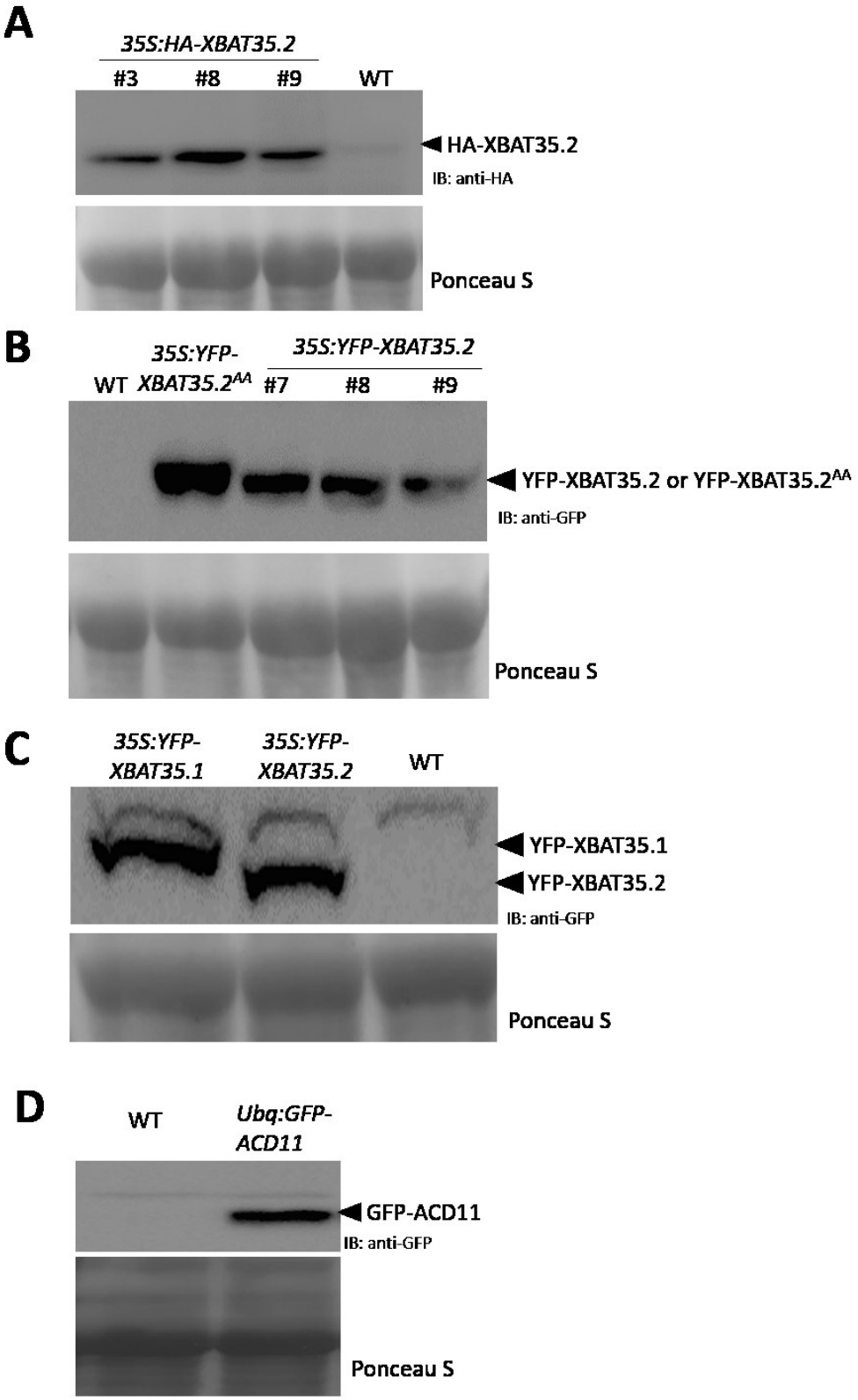
A) Five-day-old wild type (WT) and *Ubq10:GFP-ACD11* seedlings were transferred to growth medium supplemented with 0.5µM ABA or 100mM NaCl. Photographs were taken after 5 days.

B) Graphs shows root length growth after transfer. Root length was measured using ImageJ. Bars represent averages of root length based on 3 independent trials with 3 replicates each (n>5 per replicate). Error bars represent ±SE. "*" indicates a statistically significant ($p \leq 0.05$) difference compared to WT based on a Tukey's test.



Appendix Figure A5.5. Overexpression of ACD11 and mutation of XBAT35.2 leads to improved salt stress tolerance.

Seven-day-old wild type (WT), *xbat35-1* and *Ubq10:GFP-ACD11* seedlings grown on solid growth medium were transferred to liquid growth medium without and with 150 mM NaCl for 72 hours. Photograph shows the representative image of seedlings with indicated treatments.


A
35S:HA-XBAT35.2
#3
#8
#9
WT
HA-XBAT35.2
IB: anti-HA
Ponceau S
B
35S:YFP-XBAT35.2
WT
35S:YFP-XBAT35.2AA
#7
#8
#9
YFP-XBAT35.2 or YFP-XBAT35.2AA
IB: anti-GFP
Ponceau S
C
35S:YFP-XBAT35.1
35S:YFP-XBAT35.2
WT
YFP-XBAT35.1
YFP-XBAT35.2
IB: anti-GFP
Ponceau S
D
WT
Ubq:GFP-ACD11
GFP-ACD11
IB: anti-GFP
Ponceau S

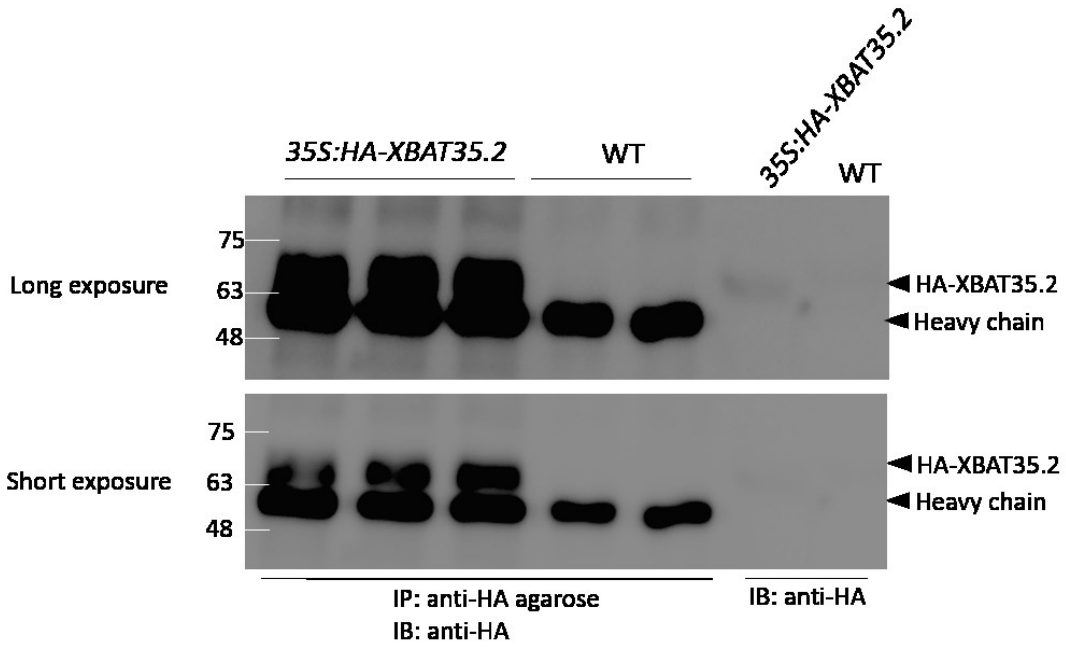
Appendix Figure A5.6. Verification of transgene expression.

A) Seven-day-old wild type (WT) and three different lines of transgenic seedlings overexpressing *35S:HA-XBAT35.2 (#3, #8, #9)* were sampled. The presence of HA-XBAT35.2 was analyzed by immunoblot analysis by using anti-HA antibodies. Ponceau S staining was served as the loading control.

B) Seven-day-old wild type (WT), three different lines of transgenic seedlings overexpressing *35S:YFP-XBAT35.2 (#7, #8, #9)* as well as transgenic seedlings overexpressing *35S:YFP-XBAT35.2*^{AA} were harvested. The existence of YFP-XBAT35.2 or YFP-XBAT35.2^{AA} was analyzed by IB analysis by using anti-GFP antibodies. Ponceau S staining was served as the loading control.

C) Seven-day-old wild type (WT), transgenic seedlings overexpressing *35S:YFP-XBAT35.1 or 35S:YFP-XBAT35.2* were collected. The existence of YFP-XBAT35.1 or YFP-XBAT35.2 was analyzed by IB analysis by using anti-GFP antibodies. Ponceau S staining was served as the loading control.

D) Seven-day-old wild type (WT), transgenic seedlings overexpressing *Ubq10:GFP-ACD11* were collected. The existence of *GFP-ACD11* was analyzed by IB analysis by using anti-GFP antibodies. Ponceau S staining was served as the loading control.



Appendix Figure A5.7. Detection of immunoprecipitated HA-XBAT35.2.

HA-XBAT35.2 is ~65 kDa, while the heavy chain of mouse IGg is ~50 kDa, which hinders the easy detection of HA-XBAT35.2 using mouse anti-HA following immunoprecipitation with HA antibody attached to agarose beads. Assays were carried out to confirm that immunoblot analysis can be used to detect HA-XBAT35.2 despite the presence of the heavy chain of IGg. Protein extracts prepared from wild type (WT) and *35S:HA-XBAT35.2* seedlings were subjected to immunoprecipitation (IP) using anti-HA agarose beads, followed by immunoblot (IB) analysis with HA antibody to detect HA-XBAT35.2.