SELECTIVE PROTEOLYSIS IN *ARABIDOPSIS THALIANA*: DISSECTING THE TRANSCRIPTIONAL REGULATION OF THE UBIQUITIN/26S PROTEASOME SYSTEM

BY

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ABSTRACT

Protein degradation by the ubiquitin (Ub)/26S proteasome system (UPS) is an essential eukaryotic pathway that removes mis-folded/aberrant polypeptides and short-lived regulatory proteins from the cell. Proteolysis through the UPS is mediated by the post-translational modification of target proteins with a covalently bonded poly-Ub chain. Proteins tagged with Ub chains are then recognized by the 26S proteasome, an ATP-dependent protease that cleaves the target into smaller polypeptides that will then be degraded further into individual amino acid residues by other cellular proteases. The 26S proteasome is a 2.5-MDa particle composed of at least 33 unique subunits that form two subcomplexes: the 20S core protease (CP) that houses the proteolytic active sites and the 19S regulatory particle (RP) that binds to the CP and bestows a variety of functions onto the 26S holoprotease. Proteasome activity is modulated by subunit expression and composition, various post-translational modifications, associated factors, and other activating particles. My thesis is focused on understanding UPS function and regulation in response to stress in *Arabidopsis thaliana*.

Previous small-scale transcriptomic and translational studies in eukaryotes showed that proteasome-specific stress induces a concerted transcriptional up-regulation of numerous UPS genes, especially those that encode 26S proteasome subunit genes. I have performed the first RNA-sequencing (RNAseq) analysis of *Arabidopsis* seedlings during conditions that challenge cellular proteasome capacity and identified a conserved suite of up-regulated genes, which I term the proteasome stress-induced regulon (PSIR). I also characterized the DNA-binding motif landscape in the upstream regions of PSIR and other UPS genes and identified a collection of dedicated *cis*-acting regulatory motifs that can be flexibly recognized by numerous DNA-binding proteins.

Finally, I demonstrated that two plant-specific NAM-ATAF1,2-CUC2 (NAC) family transcription factors, NAC53 and NAC78, are activators of the PSIR during proteasome-specific stress. *Arabidopsis* seedlings overexpressing NAC78 robustly activates the regulon, whereas

double mutants lacking both factors are incapable of properly up-regulating proteasome gene transcripts, resulting in a strong hypersensitivity of seedlings to proteasome inhibition. NAC53 and NAC78 are closely related over dicot evolution, and are likely part of a conserved feedback mechanism that is responsible for regulating UPS activity across multiple plant lineages.

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TABLE OF CONTENTS

Section **Page**

ABSTRACT……...i

ACKNOWLEDGMENETS…….iii

TABLE OF CONTENTS……v

LIST OF TABLES……..vii

LIST OF FIGURES……...viii

ABBREVIATIONS……...x

CHAPTER 1: THE UBIQUITIN/26S PROTEASOME SYSTEM……1

Introduction……2

Cited Literature……43

CHAPTER 2: THE *ARABIDOPSIS* PROTEASOME-STRESS REGULON……..54

Abstract…….....55

Introduction……...56

Results…….....57

Discussion……96

Materials and Methods……98

Cited Literature…….....102

CHAPTER 3: NAC53 AND NAC78 ARE ACTIVATORS OF UPS GENE EXPRESSION DURING PROTEASOME STRESS……...…106

Abstract…….....107

Introduction……108

Results……..109

Discussion……..135

Materials and Methods……138

Cited Literature……142

CHAPTER 4: MOLECULAR CHARACTERIZATION OF THE *ARABIDOPSIS* CP ACTIVATOR PA200……145

Abstract……146

Introduction……147

Results……148

Discussion……149

Materials and Methods……154

Cited Literature……156

CHAPTER 5: FUTURE DIRECTIONS AND CLOSING REMARKS……157

Abstract……158

Introduction……147

Future Directions……159

Closing Remarks……166

Cited Literature……167

LIST OF TABLES

Number		Page
TABLE 1-1	UPS Pathway Composition in the *Arabidopsis* Genome	8
TABLE 1-2	Subunits of the Eukaryotic 26S Proteasome	12
TABLE 2-1	All genes in the Proteasome Stress-Induced Regulon (PSIR)	64
TABLE 2-2	Motif Occurrence in the 26S Proteasome Subunit Genes	86
TABLE 2-3	Motif Occurrence in the Proteasome-Stress Response Regulon	86
TABLE 2-4	Motif Occurrence in the 3 hr MG132 Up-regulated Gene Set	86
TABLE 2-5	Proteasome DNA-binding Proteins as Predicted by a Directed-Y1H Screen	91

LIST OF FIGURES

Number **Page**

FIGURE 1-1 The Ubiquitin 26S Proteasome System……6

FIGURE 1-2 26S Proteasome Structure and Function……11

FIGURE 1-3 19S Regulatory Particle Organization……16

FIGURE 1-4 The Alternate Proteasome Activator PA200……..22

FIGURE 1-5 Other Proteasome Activating Particles……...25

FIGURE 1-6 The 26S Proteasome Assembly Pathway……..28

FIGURE 1-7 The *Arabidopsis* Proteasome Stress Response……34

FIGURE 1-8 Eukaryotic Proteasome-Stress Response Transcriptional Feedback Loops……36

FIGURE 1-9 Mechanism of Action and Chemical Structures for Common Proteasome Inhibitors……..……39

FIGURE 2-1 *Arabidopsis* Proteasome Genes are Up-Regulated in Response to Proteasome Stress..……60

FIGURE 2-2 *Arabidopsis* Proteasome Proteins Accumulate in Response to Proteasome Stress……62

FIGURE 2-3 The Proteasome Stress-Induced Regulon (PSIR)…….....71

FIGURE 2-4 Expression Heat Maps of PSIR and PSIR-related Genes……..75

FIGURE 2-5 “Housekeeping” Gene Expression During Proteasome Stress……..77

FIGURE 2-6 The 3 hr MG132 Up-regulated Gene Interaction and Co-expression Network…79

FIGURE 2-7 PRCE and MDM Occurrence in the Upstream Regions of 26S Proteasome Genes……...84

FIGURE 2-8 Occurrence of Enriched Motifs in Proteasome Genes…….....88

FIGURE 2-9 Occurrence of the SORLIP2, T-box, PRCE, and MDM Sequences in Proteasome Genes……...90

Number **Page**

FIGURE 3-1 *NAC78* Overexpression Induces Proteasome Gene Expression……112

FIGURE 3-2 ClustalW Pairwise Alignment of NAC53, NAC78, and NAC13……115

FIGURE 3-3 *NAC53* and *NAC78* Physically Interact and Activate Proteasome Gene Expression……117

FIGURE 3-4 NAC53 and NAC78 Physically Interact (BiFC MG132 Treatment and Experimental Controls)……..……..119

FIGURE 3-5 NAC53 and NAC78 Do Not Interact with All NAC Proteins *in planta*…….....121

FIGURE 3-6 NAC53 and NAC78 Dimers Localize to the Nucleus……..123

FIGURE 3-7 *nac53-1 x nac78-1* Double Mutants Are Deficient in Proteasome Gene Expression During Stress……128

FIGURE 3-8 Elevated Ub-conjugate Levels in *nac* Double Mutant Backgrounds….....130

FIGURE 3-9 The *nac53-1xnac78-1* Double Mutant is Hypersensitive to Proteasome-specific Inhibition……131

FIGURE 3-10 The *nac53-1xnac78-1* Double Mutant is Hypersensitive to Bortezomib…….....133

FIGURE 4-1 Genetic and Phenotypic Characterization of *Arabidopsis* PA200…….....151

FIGURE 4-2 PA200 Accumulates During Proteasome Stress……153

ABBREVIATIONS USED IN TEXT

ACT2	Actin 2
AGI	*Arabidopsis* gene identifier
AGRIS	*Arabidopsis* gene regulatory information server
AT	*Arabidopsis thaliana*
ATG	Autophagy gene
ATcisDB	AGRIS *cis*-regulatory element database
ATP	Adenosine triphosphate
AUX	Auxin
B-Est	β-estradiol
BiFC	Bimolecular complementation
BLM10	Yeast bleomycin sensitive 10, aka PA200
BME	β-mercaptoethanol
Bp	Base pair
C24	C24 *Arabidopsis thaliana* ecotype
CaMV	Cauliflower mosaic Virus
CDC	Cell division cycle
CDS	Coding sequence
ChIpSeq	Chromatin immunoprecipitation sequencing
CP	Core protease
Col-0	*Columbia*
COP9	Constituitive photomorphenesis 9
CRL	Cullin-Ring Ligase
Cys	Cystidine
DAG	Days after germination

DAPI	4',6-diamidino-2-phenylindole
DAVID	Database for Annotation, Visualization and Integrated Discovery
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DUB	De-ubiquitylating enzyme
E1	Ub activating enzyme
E2 (UBC)	Ub conjugating enzyme
E3	Ub ligase
ECM29	Extra-cellular matrix 29 protein
EM	Electron micrograph
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum-Associated Protein Degradation
EST	Expressed sequence tags
FDR	False discovery rate
GO	Gene ontology
GSTU	Glutathione-S-transferase
GUS	β-glucuronidase
H3	Histone 3
His	Histidine
hr	Hour(s)
HSF	Heat-shock transcription factor
HSP	Heat-shock protein
IAA	Indoleacetic acid
K	Lysine
Kb	Kilobase (pair)

KDa	Kilo-dalton
lacZ	β-galactosidase
Leu	Leucine
Lys	Lysine
MCODE	Molecular complex detection
MDa	Mega-dalton
MDM	Mitochondrial dysfunction motif
MES	2-(N-Morpholino)ethanesulfonic acid hydrate
$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid
MS	Murashige and Skoog media
NAC	**N**o Apical Meristem, **A**TAF1,2, **C**UC2 domain containing transcription factor
NAM	No Apical Meristem domain
Nrf1	Nuclear erythroid 2-related factor 1
NTL	NAC proteins with transmembrane motif-1 like
OD600	Optical density at 600 nanometers
PA200	Proteasome activator 200 Kd
PACE	Proteasome Associated Control Element
PAC1	Proteasome α subunit C
PAGE	Polyacrylamide gel electrophoresis
PBA1	Proteasome β subunit A
PCR	Polymerase chain reaction
pFP	p-fluoro-phenylalanine
PGPH	Post-Glutamyl Peptide Hydrolyzing
pMDC	Marks Gateway TM vector

PP2A	Protein phosphatase 2
PRCE	Proteasome Related *Cis* Element
PSIR	Proteasome stress-induced regulon
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RAD	Radiation sensitive
RING	Really Interesting New Gene
RP	19S regulatory particle
RNA	ribonucleic acid
RNAseq	RNA-sequencing
RPN	Regulatory particle non-triple-A ATPase
RPT	Regulatory particle triple-A ATPase
RPX	Regulator of proteasome expression, aka NAC78
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium dodecyl sulfate
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
SylA	Syringolin A
TAIR	The *Arabidopsis* Information Resource
T-DNA	Transfer DNA
Trp	Tryptophan
TSS	Transcriptional start site
Ub	Ubiquitin
UBC (E2)	biquitin conjugating enzyme
UBP	Ubiquitin protease (DUB)
UBQ	Ubiquitin gene
UIM	Ubiquitin interacting motif
UPS	Ubiquitin proteasome system

WRKY	WRKY protein domain-containing transcription factors
WT	Wild-type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Y1H	Yeast-1-hybrid
Y2H	Yeast-2-hybrid
YFP	Yellow fluorescing protein
YPD	Yeast extract peptone dextrose

CHAPTER 1

THE UBIQUITIN/26S PROTEASOME SYSTEM

INTRODUCTION

There are few biological processes at the molecular level that are highly conserved across several kingdoms of life. Generally these systems are tied to life-essential functions such as cellular growth and division, metabolism, and environmental response. These rare few regulatory pathways provide researchers with a critical look into the commonality over the evolution of life, and how the processes relate back to the structure and regulation of RNA, DNA, and protein present in all organisms. Pivotal to life is the proper maintenance of protein abundance, as it primarily determines proper growth and response of cells to the environment over time. This maintenance can be achieved at the nucleic acid or protein levels, however the most efficient means of regulating proteins is post-translationally, specifically via proteolysis, and in the case of the Ubiquitin/26S Proteasome System (UPS), selective proteolysis.

Protein turnover is a crucial regulatory cornerstone for most cellular activity. Proteolysis is indispensible for (i) the proper removal of many mis-translated or mis-folded polypeptides, (ii) increasing available amino acid pool during periods of growth and starvation, and for (iii) modulating key protein levels required for proper cellular homeostasis, differentiation, growth, and environmental adaptation (King et al. 1996, Hershko et al. 1998, Hochstrasser et al. 1999, Smalle et al. 2004, Finley 2009, Vierstra 2009).

The two major modes of proteolysis in eukaryotic cells are the UPS and autophagy pathways. Autophagy is a bulk degradation pathway for peptides, larger proteins and complexes, and even organelles (Li et al. 2012, Marshall et al. 2015), while the UPS is a target-specific proteolytic pathway, using multiple covalently attached ubiquitin (Ub) moieties as a post-translational modification signal for degradation. The 26S proteasome is the major cellular protease, and is at the nexus of the UPS; it is possibly the most important apparatus for removing proteins in eukaryotes due to the speed and specificity at which it can degrade those targets. For example, the auxin-signaling protein AUX/IAA in plants and UPS transcription

factor Rpn4 in yeast (*Saccharomyces cerevisiae)* can be degraded in mere minutes via the UPS (Zenser et al. 2001, Xie, 2001, Kepinski et al. 2005).

Defects in the UPS or any other inability to properly remove cellular proteins or peptides can have severely deleterious consequences for development and longevity. In humans, the accumulation of mis-folded or abnormal proteins (amyloidogenic) or excessive levels of key regulatory proteins have been linked to a variety of neurological and other pathologies, including Huntington's, Parkinson's, Alzheimer's, and diabetes (Dahlmann 2007, Thomas et al. 2007, Zhang et al. 2009, Cheng et al. 2013). Cancer research and treatment has also focused on the UPS: bortezomib (marketed as Velcade) is an irreversible inhibitor of the 26S proteasome. Bortezomib was designed to specifically interact and inhibit the proteolytic active sites of the 26S proteasome and currently represents a promising anti-cancer therapy to some multiple myelomas and lymphomas (Ling et al. 2002, Richardson et al. 2005, Voorhees et al. 2006, Ruan et al. 2011).

Due in part to the speed and specificity of the UPS, it is not surprising it has been shown to be necessary for many aspects of plant growth and development, including seed development, cell cycle progression, photomorphogenesis, flowering time, most hormone responses, circadian rhythms, self incompatibility, senescence, and disease resistance to name a few (Moon et al. 2004, Smalle et al. 2004, Dharmasiri et al. 2005, Vierstra 2009). Because of this, enhancing our understanding of the structure, assembly, function, and regulation of the UPS is of the upmost importance to make continual advancements in agricultural and medical research.

Ubiquitylation and Ub-binding Proteins

Protein ubiquitylation is dependent upon the sequential shuttling of Ub between the E1, E2, and E3 enzymes (**Figure 1-1)** (Vierstra 2009). However, ubiquitylation actually begins with deubiquitylating enzymes (DUBs). DUBs are responsible for the final step in UB synthesis,

cleaving poly-Ub translational fusions and generating several single Ub moieties from one gene product. DUBs also recycle conjugated Ubs back into the cellular pool for immediate re-use by breaking the bond between the amino acid of an ubiquitylated protein and the C-terminal tail of the Ub (Yan et al. 2000, Yang et al. 2007, Isono et al. 2014). While there are numerous DUB proteins encoded in eukaryotic genomes, only one proteasome subunit, RPN11, has de-ubiquitylating activity that is constantly associated with 26S proteasome function.

In *Arabidopsis*, the ATP-dependent process of protein ubiquitylation begins with one of two E1 Ub-activating enzymes. These enzymes are comprised of an Ub-binding domain as well as an ATP-hydrolysis domain that generate a thioester bond between the Ub and a cysteine residue of the E1 (Hatfield et al. 1997, Vierstra 2009). E1s are responsible for the recruitment of Ubs into the ubiquitylation cycle, but play no role in target specificity.

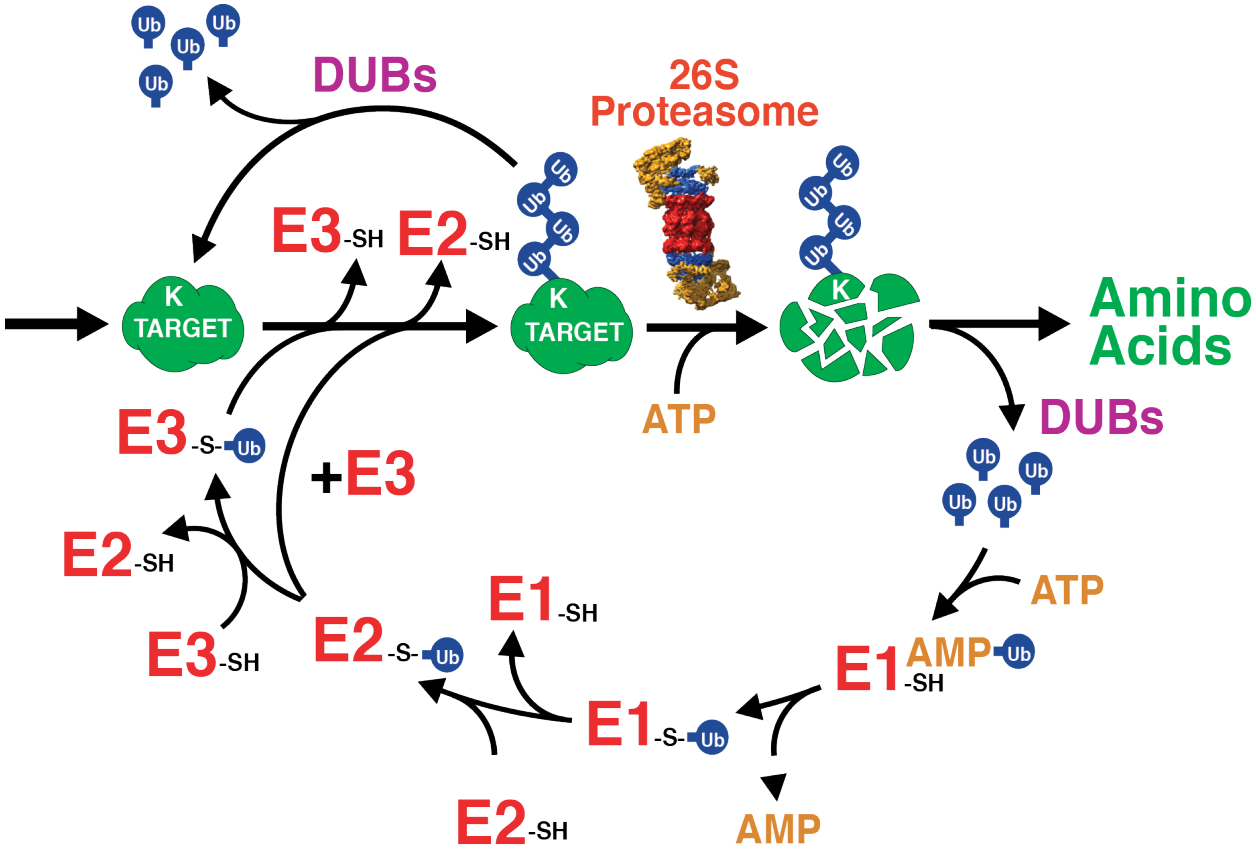
After activation, Ub is shuttled from the E1 to E2 Ub-conjugating enzyme (UBC). The thioester linkage between Ub and the E1 is transferred to an E2 Cys residue. There are more E2 enzymes in *Arabidopsis* thaliana than E1s (~37), however identification of E2s in organisms (as well as E3s) is primarily done via searches for conserved sequence domains from other organisms, so this number could and has changed over time due to improvements in genome annotations (Vierstra 1996, Bachmair et al. 2001, Smalle et al. 2004, Vierstra 2009). UBCs function as an intermediary between Ub activation and target modification and are not responsible for target specificity per se; that lies more with the E3 enzyme.

The E3 Ub ligase is the final step in the conjugation pathway, and it is the enzyme that denotes target specificity to the ubiquitylation cascade. There are four different classes of E3 Ub ligases in plants: HECT, RING (Really Interesting New Gene), U-box, and cullin-RING ligases (CRLs). E3s interact with E2s to covalently transfer the Ub moiety to a target protein Lys (Vierstra 2003, Vierstra 2009). E2-E3 interaction is mediated either through direct binding of the E2 to the E3 protein (HECT, RING, U-box), or via association with other complexed

Figure 1-1. The Ubiquitin 26S Proteasome System

Ub-mediated proteolysis initiates with Ub cleavage, which is performed by DUBs (right side of image). DUBs will release Ub proteins from poly-Ub chains or by cleaving poly-Ub translational fusions. Next, Ubs enter the ATP-dependent portion the system, becoming conjugated to E1s. The Ub-E1 complex then interacts one of several E2 proteins, transferring the Ub moiety via transesterification (SH to S). The E3 ligase mediates the Ub transfer from the E2 to a lysine (K) on a target substrate, forming a poly-Ub chain. Chain length and linkage determines the fate of the Ub-tagged protein, potentially fating it for degradation by the 26S proteasome unless DUBs remove the Ub moieties.

Figure 1-1. The Ubiquitin 26S Proteasome System



proteins that make up the E3 (CRLs). Due to the target-selective nature of E3s, there has been a large expansion of Ub ligases throughout plant evolution (**Table 1-1**). However this genomic expansion of Ub ligases does not seem to correlate to genome size or even genome makeup: there are over ~1500 E3s encoded in *Arabidopsis*, about 5% of the genome, each having one or a handful of target proteins that it can recognize. But other plant species can have wildly different numbers of encoded E3s, even between closely related lineages. One such example are the CRL-type F-box proteins, which have ~900 genes encoded in *Arabidopsis thaliana*, but over 1300 in *Arabidopsis lyrata,* and only ~400 in *Zea mayes* (Hua et al. 2011). Although there are usually hundreds of F-box proteins in any given plant, only a small number of these are conserved over several species, and many of the remaining ligases are psuedogenes or species-specific.

The type of poly-Ub chains also makes a difference in terms of protein destination. Poly-Ub chains are generated via covalent linkages to one of seven exposed Lys on each Ub: K6, K11, K27, K29, K33, K48, and K63. (Dammer et al. 2011), with the best known being the K11, K48, and K63 linkages. K11 linkages play a role in targeting peptides to the Endoplasmic Reticulum-Associated Protein Degradation (ERAD) pathway (Xu et al. 2009) and cell cycle regulation (Jin et al. 2008). K63 linked mono- and poly-Ub conjugation will target proteins for sorting and DNA repair-related functions (Hicke et al. 2003, Bergink et al. 2009). K48-linked poly-Ub chains are the best characterized, and are the hallmark modification of proteins and polypeptides that will be targeted for degradation by the 26S proteasome. Mutation of the Ub K48 lysine will result in cellular lethality, indicating such linkages are essential (Xu et al. 2009).

Table 1-1. UPS Pathway Composition in the *Arabidopsis* Genome

	No. of Genes	
Ubiquitin	15	
E1s	2	
E2s	37	
E3s		
HECT	7	>1500 E3 Genes
F-Box	~897	
BTB	80	
Rbx/Cullin/Ask	33	
APC	20?	
Ring Finger	477	
U-Box	64	
DWD	119	
DUBs	64	
Proteasome	>70	
Estimated total	>1885	
	6.9% of Proteome	

The 26S Proteasome

The main protease in plants is the 26S proteasome, a 2.5-MDa complex responsible for the turnover of Ub-tagged proteins (Vierstra 2009). Proteins fated for degradation by the proteasome require a specific Ub topology in which at least four Ub moieties are linked together in a chain through conserved lysines. A poly-Ub chain is conjugated to targets via a three-step, ATP-dependent E1-E2-E3 enzymatic cascade (**Figure 1-1**) (Thrower et al. 2000, Yang et al. 2004, Duncan et al. 2006, Book et al. 2009, Lin et al. 2011). It is the ability of the 26S proteasome to recognize poly-Ub topologies that makes the UPS a target-specific method for intracellular proteolysis.

The 26S proteasome is composed of two distinct subcomplexes (**Figure 1-2a,b**), the 28-subunit core protease (CP) and the 18-subunit regulatory particle (RP) **Table 1-2**. The eukaryotic 20S CP is made up of four stacked heptameric rings of unique α- and β-subunits in a $\alpha_{1-7}/\beta_{1-7}/\beta_{1-7}/\alpha_{1-7}$ configuration. It assumes a barrel-shaped structure with an internal chamber enclosing 6 proteolytic sites provided by the three β-subunits: β1, β2, and β5 (**Figure 1-2c**) (Baumeister et al. 1998, Fu et al. 1998, Unno et al. 2002, Rabl et al. 2008, Finley 2009). The CP exists in all kingdoms of life, including archaea and eubacteria; although prokaryotic proteasomes are more simplistic than their eukaryotic counterparts, with the 20S subunits often encoded by one to two genes per organism (*e.g.* single α- and β-subunit genes), although there are exceptions (Lowe et al. 1995, Groll et al. 1997).

The three proteolytically active β-subunits are N-terminal nucleophile hydrolases, which contain a single active-site threonine residue, the N-terminal amide of which functions as the catalytic nucleophile, acting as the proton donor for the proteolytic active site (Dick et al. 1998, Book et al. 2010). β1, β2, and β5 are translated as inactive pro-enzymes (Figure 2). The N-terminal sequence that precedes the active site threonine is auto-catalytically

Figure 1-2. 26S Proteasome Structure and Function

A. A crystal structure and cartoon diagram of the 20S CP. The barrel-like particle encompasses the proteolytic active sites. The axial pore into the CP is gated by the N-terminal extensions of the α-ring, which prevents non-specific protein degradation.
B. A cross-section cryo-EM image showing the location of the proteolytic active sites within the CP (Kisselev et al. 2012).
C. A cryo-electron micrograph (EM) image of the yeast 26S proteasome. Coloration indicates the major subcomplexes: red is the CP, blue is the RP base, and gold is the RP lid (Lasker et al. 2012).
D. Cartoon schematic of the 26S proteasome. Denoting the functions of the CP and RP. Ub-target recognition, de-ubiquitylation, unfolding, and translocation are performed by the RP, while peptide cleavage occurs within the CP.

Figure 1-2. 26S Proteasome Structure and Function

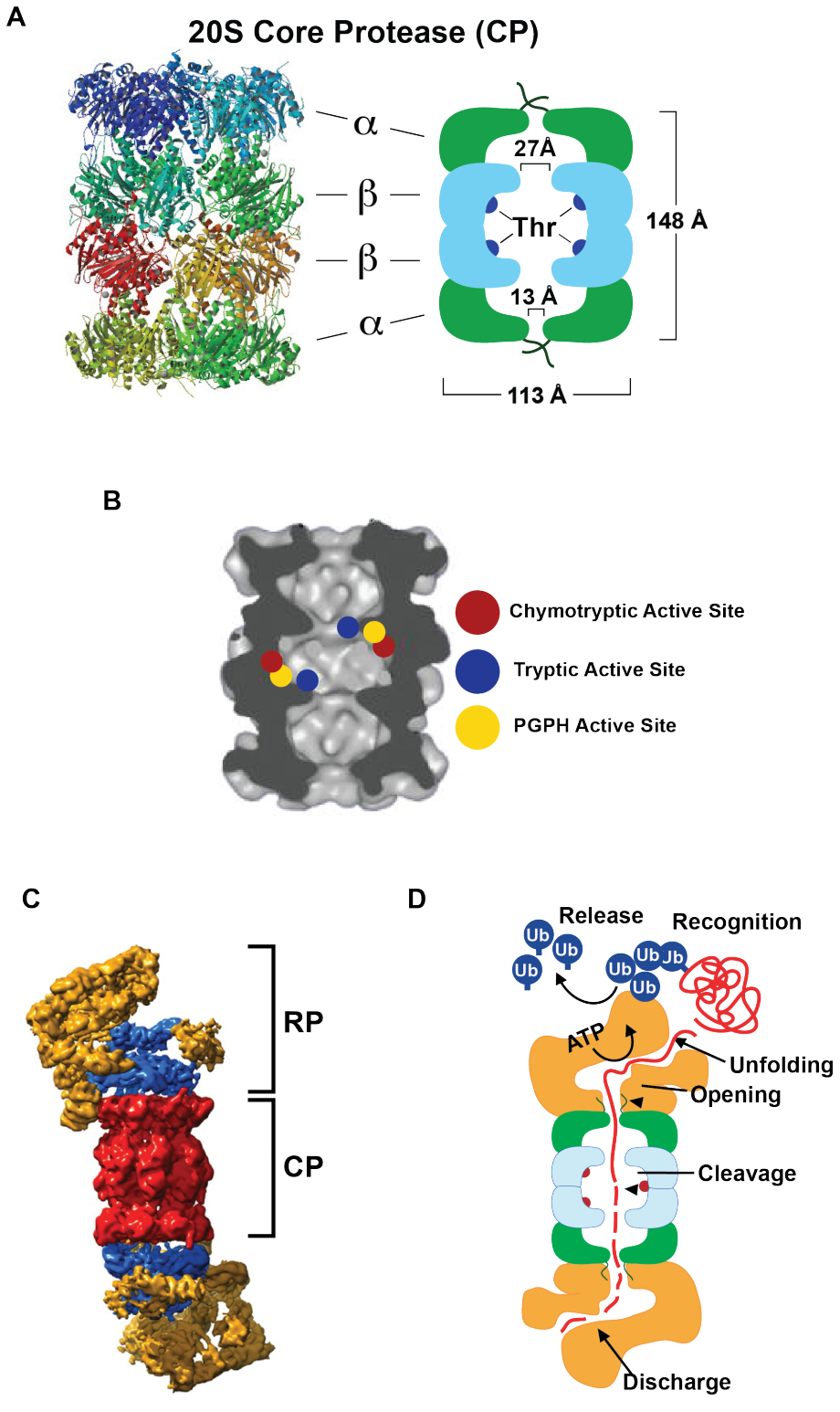


Table 1-2. Subunits of the Eukaryotic 26S Proteasome

20S Core Particle

	Subunit	Yeast	Human	*Arabidopsis*	
Alpha	α1	PRS2	IOTA	PAA1	PAA2
	α2	PRE8	C3	PAB1	PAB2
	α3	PRE9	C9	PAC1	
	α4	PRE6	XAPC7	PAD1	PAD2
	α5	DOA5	ZETA	PAE1	PAE2
	α6	PRE5	C2	PAF1	PAF2
	α7	PRE10	C8	PAG1	
Beta	β1	PRE3	Y	PBA1	
	β2	PUP1	Z	PBB1	PBB2
	β3	PUP3	C10	PBC1	PBC2
	β4	PRE1	C7	PBD1	PBD2
	β5	PRE2	X	PBE1	PBE2
	β6	PRE7	C5	PBF1	
	β7	PRE4	N3	PBG1	

Red = Proteolytic Active Site

19s Regulatory Particle

	Subunit	Yeast	Human	*Arabidopsis*	
Lid	RPN1	RPN1	PSMD2	RPN1a	RPN1b
	RPN2	RPN2	PSMD1	RPN2a	RPN2b
	RPN3	RPN3	PSMD3	RPN3a	RPN3b
	RPN5	RPN5	PSMD12	RPN5a	RPN5b
	RPN6	RPN6	PSMD11	RPN6	
	RPN7	RPN7	PSMD6	RPN7	
	RPN8	RPN8	PSMD7	RPN8a	RPN8b
	RPN9	RPN9	PSMD5	RPN9a	RPN9b
	RPN10	RPN10	PSMD4	RPN10	
	RPN11	RPN11	PSMD14	RPN11	
	RPN12	RPN12	PSMD8	RPN12	
	RPN13	RPN13	ARM1	RPN13	
Base	RPT1	RPT1	PSMC2	RPT1	RPT1b
	RPT2	RPT2	PSMC1	RPT2a	RPT2b
	RPT3	RPT3	PSMC4	RPT3	
	RPT4	RPT4	PSMC6	RPT4a	RPT4b
	RPT5	RPT5	PSMC3	RPT5a	RPT5b
	RPT6	RPT6	PSMC5	RPT6a	RPT6b

processed upon completion of CP assembly, preventing inadvertent proteolysis of cellular proteins before the final particle is built (Frentzel et al. 1994, Yang et al. 1995, Heinemeyer et al. 1997). Each β-subunit confers distinctive peptide cleavage to the CP: β1 has post-glutamyl peptide hydrolyzing (PGPH), or caspase-like, activity, β2 has trypsin-like activity, and β5 has chymotrypsin-like activity (Dick et al. 1998). The chymotryptic-like activity of the CP, which cleaves after hydrophobic residues, is responsible for the majority of protein degradation, and is the predominant target for most proteasome inhibitors (Myung et al. 2001). Once peptides are cleaved into shorter peptides and expelled from the proteasome, they will be further degraded by other cellular proteases such as the tripeptidyl peptidase II (TPPII) into single amino acids (Book et al. 2005).

At either end of the CP barrel, there is a narrow 13-Å axial pore that, along with N-terminal extensions of the α subunits, prevents polypeptides from entering and undergoing unintended degradation (Lowe et al. 1995, Groll et al. 1997, Groll et al. 2000). This small pore remains closed until it is bound by one of several proteasome activators, such as the RP (19S, PA700), PA200 (Blm10 in yeast), CDC48 (p97), PA26, the mammalian-specific PA28α/β, which forms the immunoproteasome, or the archaeal PAN (proteasome-activating nucleotidase) (Stadtmueller et al. 2010). Activator binding results in a conformational shift to a more open orientation of the axial channel, allowing for the entrance of polypeptide chains (**Figure 1-3**). This binding and conformational shift is reliant upon binding pockets formed by the α subunits that are recognized by short C-terminal extensions in the activators (Smith et al. 2007, Rabl et al. 2008, Kusmierczyk et al. 2011).

For most eukaryotes, the CP subunits are encoded only once within their genome, however the majority of *Arabidopsis thaliana* CP proteasome subunits are encoded by two paralogs (5/6 α-subunits and 4/6 β-subunits) that share high sequence identity (>85%). Unsurprisingly, this subunit redundancy occurs in several plants lineages, presumably caused

by the numerous genome duplications that have occurred over plant evolution. This makes it likely that paralog-specific functions have evolved over time. The best example of isoform-specific functionality is with the generation of the metazoan immunoproteasome. Three interferon-inducible β-subunits, β1i, β2i, and β5i, are assembled into the CP instead of their sister paralogs. These new catalytic subunits generate antigenic peptides that can be more easily recognized by the major histocompatibility complex in vertebrates (Kloetzel 2004). Another example of isoform-specific functionality that has recently been discovered is in rice: a single point mutation in an α subunit protein can bestow increased thermotolerance in one species of African rice, *Oryza glaberrima*, compared to other Asian *Oryza* species (Li et al. 2015). This point mutation potentially alters either the active-site pocket or substrate entrance into the CP, resulting in increased proteasomal activity. Few examples of this kind of adaptive change in plant CP subunits have been found, however there are more examples of this kind of evolutionary occurrence in bacterial gene clusters (Kale et al. 2011).

CP Activators

The 19S Regulatory Particle

The 19S RP bestows a variety of functions onto the 26S proteasome, in addition to CP axial channel activation, including Ub-conjugate recognition, recycling of Ubs via one or more DUBs, and substrate unfolding and translocation into the CP (Smalle et al. 2002, Verma et al. 2002, Smalle et al. 2003, Lee et al. 2011). The RP can be subdivided into lid and base substructure (**Figure 1-3c**). The lid directly contacts the CP α-ring and contains a ring of six **R**egulatory **P**article **T**riple-A ATPase (Adenosine Tri-Phosphate) subunits-RPT1-6, and three **R**egulatory **P**article Non-triple-A ATPases subunits-RPN1, RPN2 and RPN10. As the names imply, the RPT subunits consume ATP, which is used to facilitate

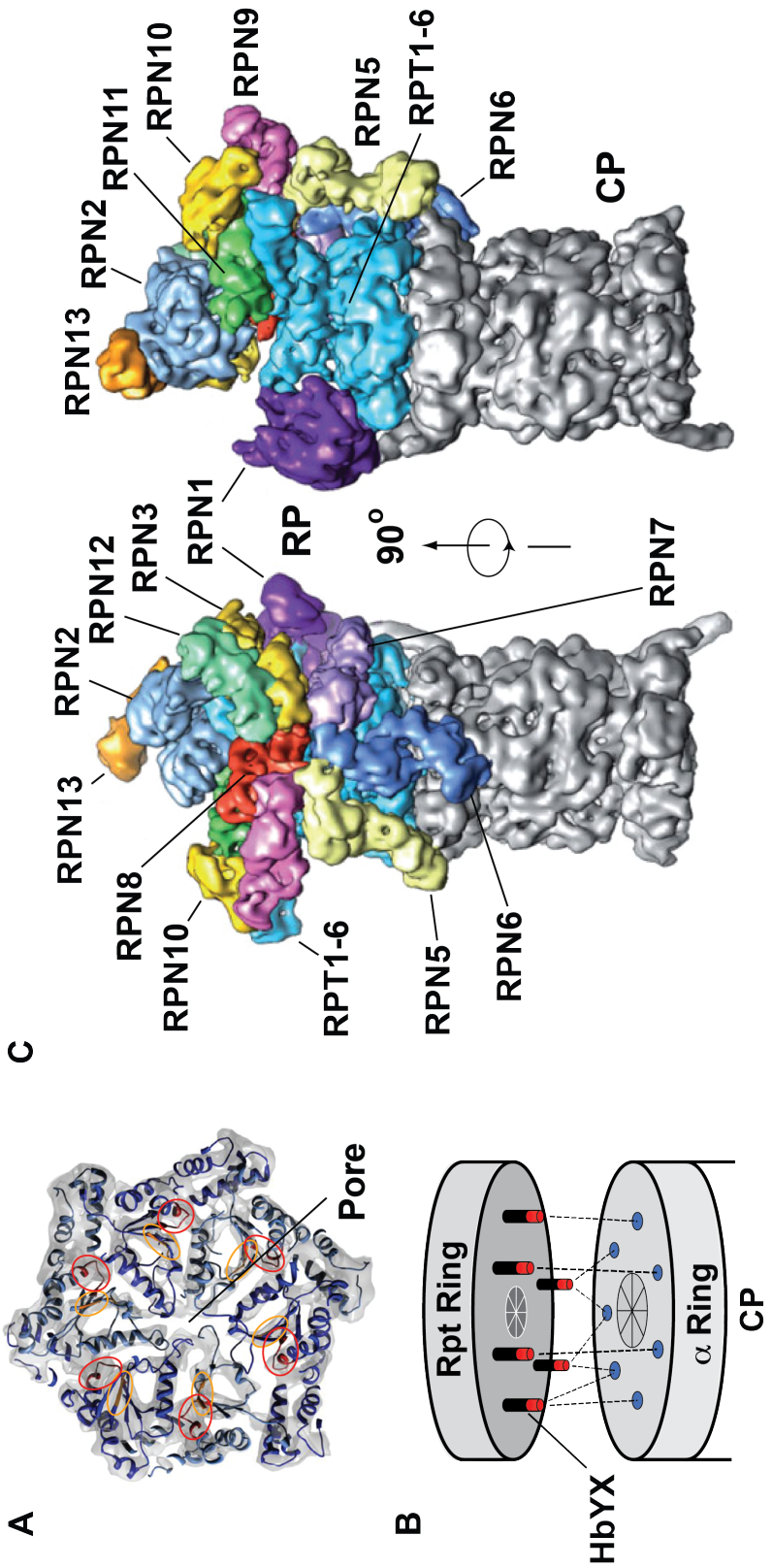
Figure 1-3. 19S Regulatory Particle Organization

A. The structure of the yeast RTP ring (RP base). Red circles highlight the RPT C-terminal extensions that interact with the CP α-ring (Park et al. 2013).

B. Schematic of the RPT ring intercalation into the CP alpha ring. RP-CP formation is mediated by C-terminal extensions (red bars) that include the terminal HbYX motif.

C. A composite three-dimensional EM image of the yeast 19S RP lid and base subunits, incorporated onto the CP. Rpn5 and Rpn6 jut below the RP base, stabilizing RP-CP binding. The area between Rpn10 and Rpn13 form a poly-Ub binding region that recognizes K48-linked Ub chains. Rpn11 is stationed below this poly-Ub binding region, where the subunit removes the Ub moieties from incoming target peptides. These peptides are then moved to the RPT ring, where they are unfolded and translocated into the CP in an ATP-dependent manner (Lander et al. 2012).

Figure 1-3. 19S Regulatory Particle Organization



axial channel opening, target protein unfolding, and translocation of the target into the CP (Smalle et al. 2004, Finley 2009, Vierstra 2009). The N-terminal extensions of the RPT subunits terminate with an evolutionarily conserved HbYX motif, defined as a hydrophobic (Hb), tyrosine (Y), and any amino acid (X) residue. This HbYX sequence helps the RPT ring bind to the CP by intercalating into binding pocket formed by the α subunits. It is this binding that shifts the axial channel into an open conformation (Kusmierczyk et al. 2011). The HbYX motif occurs in proteasome activators of both eukaryotes (RP and PA200) and archaea (CDC48 and PAN), as well as eukaryotic (PBAC2-3 and PIP1-2) and archaeal (Pba1-2) assembly chaperones, indicating a conserved mechanism for α-ring binding throughout proteasome evolution.

The RP lid is made up of at least 12 additional RPN subunits (RPN1-3, 5-13, SEM1), the functions of only some of which are known (van Nocker et al. 1996, Saeki et al. 2002, Elsasser et al. 2004, Husnjak et al. 2008, Schreiner et al. 2008, Gomez et al. 2011, Verma et al. 2011), with only RPN10 showing sub-stoichiometric association with the proteasome (Sakata et al. 2012). In addition to genetic analysis of individual RP subunits, structural information about the RP lid and base structures have helped clarify understandings of RPN function within the proteasome (**Figure 1-3c**) (Lander et al. 2012, Lasker et al. 2012), namely the location and physical interactions of subunits in regards to one another. This elucidates some of the cooperative functions for which certain subunits have been implicated: like DUB activity between RPN11 and RPN8, and where the Ub-shuttling proteins RAD23, DDI1, and DSK2a would be oriented upon their interaction with RPN1 and RPN2 (Farmer et al. 2010). Primarily, most RPN subunits are important for RP stabilization, and a loss of one or more subunits usually results in RP destabilization and 26S proteasome dysfunction (Smalle et al. 2002, Smalle et al. 2003, Book et al. 2009, Book et al. 2010, Pathare et al. 2012, Vilchez et al. 2012).

Many eukaryotic RPN subunits seem to bestow unique functions onto the 26S proteasome. RPN1 and RPN2 act as the main binding/docking sites for the Ub-shuttling

proteins DSK2, DDI1, and RAD23 (Elsasser et al. 2002, Rosenzweig et al. 2012). RPN3 stabilizes the RP and plays a role in RPN11 incorporation and Ub-shuttling proteins association (Bailly et al. 1999, Joshi et al. 2011). RPN5 is responsible for proteasome localization and stability (Yen et al. 2003, Book et al. 2009) as well as involvement in COP9 signalosome function (Yu et al. 2011). RPN6 is crucial for RP lid and base cohesiveness as well as proteasome activity in human embryonic cells (Vilchez et al. 2012), and reduced RPN6 expression can decrease longevity in *C. elegans* (Vilchez et al. 2012). RPN7 and RPN9 have thus far been shown to be involved in RP lid assembly and structural integrity in yeast (Takeuchi et al. 1999, Isono et al. 2004, Hu et al. 2015). RPN8 is part of the de-ubiquitylating activity of the proteasome, dimerizing with RPN11 (Pathare et al. 2014, Worden et al. 2014). RPN11 is Zn^{2+}-metalloprotease and the only core proteasome subunit known to have DUB activity; it removes Ub moieties from substrate peptides prior to unfolding and translocation into the CP (Verma et al. 2002). RPN12a has been shown to be important for RP assembly as well as cytokinin regulation in *Arabidopsis* (Smalle et al. 2002).

RPN10 is responsible for proteasome stability and is the major Ub-receptor protein in the proteasome, containing 3 Ub-interacting motifs (UIM) in *Arabidopsis* and 1 UIM in yeast (Smalle et al. 2003). RPN10 has also been shown to be one of the key mediators, along with the Ub-fold protein ATG8, of autophagy-mediated proteasome degradation in *Arabidopsis* and likely in other eukaryotes (Marshall et al. 2015). RPN13 contains a pleckstrin-like Ub-recognition motif and it interacts with RPN10 to form a poly-Ub chain recognition pocket (Husnjak et al. 2008, Sakata et al. 2012). Sem1 was recently identified as being a stoichiometrically-associated component of the 26S proteasome. This intrinsically disordered protein functions as an assembly chaperone for the RP lid, but remains associated with RPN3 and RPN7 in mature proteasomes (Tomko et al. 2014). Aside from the basic functions listed above, each RP subunit likely has phylogenetically specific roles in organismal growth and development. This can be

inferred from some of the above examples, such as the regulation of the plant-specific hormone cytokinin by RPN12 or the longevity-related functionality of RPN6 in *C. elegans*.

Most RP subunits are essential for proteasome function, and most *Arabidopsis* RP subunits, like those of the CP, are encoded twice within the genome (5/6 RPT, 6/13 RPN), which can and has resulted in unique cellular roles between paralogs. One such difference in paralog activity has also been witnessed in RPN5a/b, where *Arabidopsis* seedlings lacking either the RPN5a or RPN5b display disparate developmental and growth phenotypes (Book et al. 2009). *rpn5a* null plants have numerous pleiotropic developmental and growth defects compared to *rpn5b* mutants, which more closely resembles wild-type (WT) plants. However, both *RPN5a* and *RPN5b* are essential for gametogenesis. Another example of isoform disparity occurs with *RPT2a* and *RPT2b*, which also have distinguishable phenotypes. Plants lacking RPT2a have an increased sensitivity during conditions that inhibit or otherwise limit proteasome capacity compared to *rpt2b* mutants (Sonoda et al. 2009, Lee et al. 2011).

PA200 and Other Activators

PA200, or **P**roteasome **A**ctivator protein **200**, is a 200-kDa solenoid-shaped protein that is conserved among eukaryotes (**Figure 1-4**). A putative nuclear localization signal and several HEAT repeats (**H**untington, elongation factor 3 (**E**F3), protein phosphatase 2A (PP2**A**) and yeast PI3-Kinase **T**OR1) make up the C-terminal half of PA200, whereas no functional domains are evident in the N-terminal portion based on Pfam and SMART protein domain predictions (Letunic et al. 2009, Finn et al. 2010). PA200 is known as Blm10 in yeast due to its initial discovery in a sensitivity screen to the DNA-damaging agent bleomycin (**bl**eo**m**ycin sensitive 10). Investigators found that a plasmid containing *BLM10* suppressed the phenotype of the bleomycin hypersensitive mutant *blm3-1*, and that deletion of *BLM10* led to bleomycin hypersensitivity (McCullock et al. 2006). Additional analysis has confirmed that yeast cells

lacking *BLM10* are more sensitive to other DNA-damaging conditions, such as methyl methanesulfonate, H_2O_2, and 5-fluro-uracil (Doherty et al. 2012).

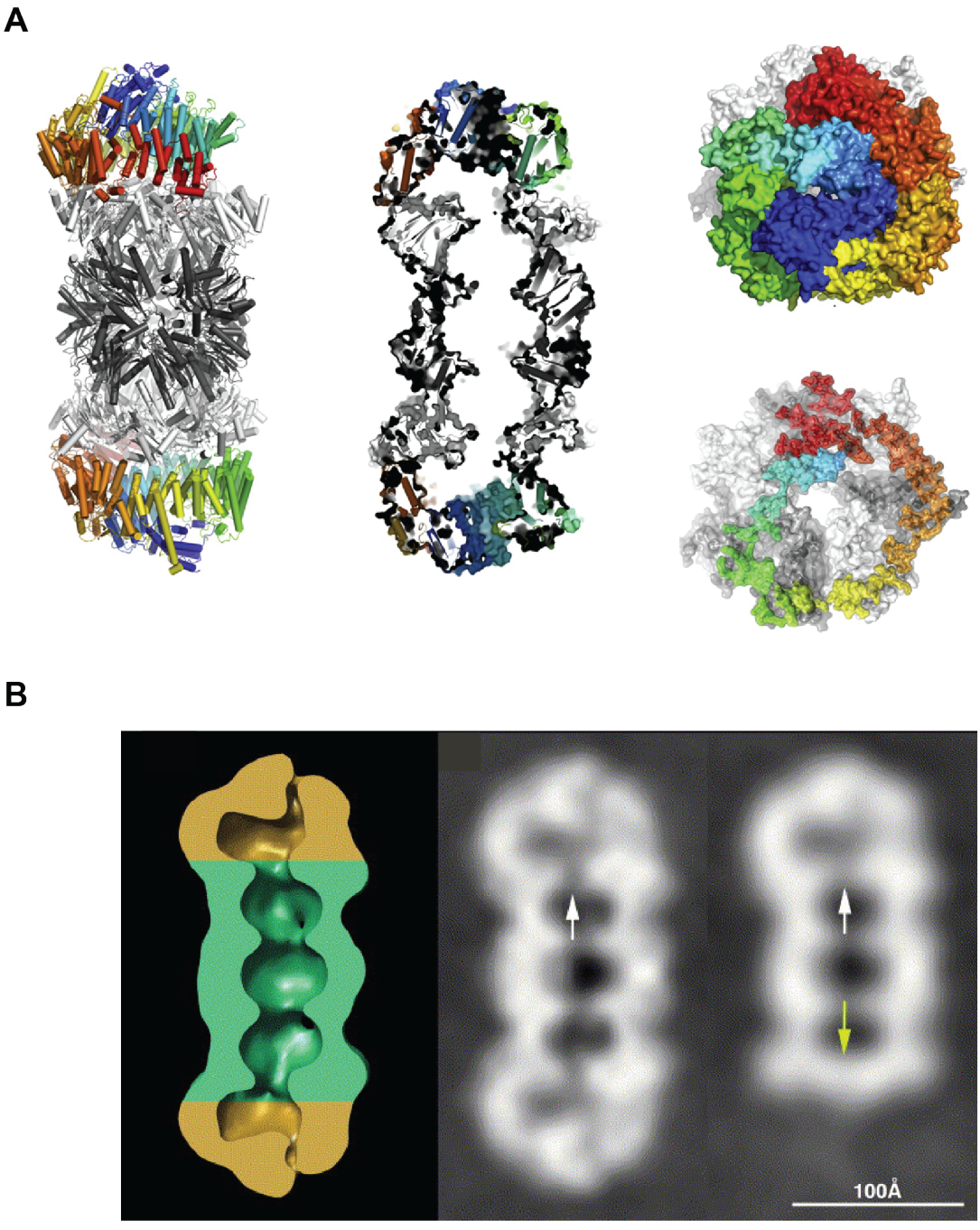
At present, the function(s) of PA200 are not fully understood; it was originally defined biochemically as a RP alternative activator of the proteasome, opening the axial channel of the CP *in vitro* when bound to the α-ring (Sadre-Bazzaz et al. 2010). PA200 is involved in proteasome assembly in yeast, binding to pre-activated CPs half-barrels, although PA200 is not essential or necessary for this, and any other CP-activating particles could possibly function as a similar assembly chaperone (Lehmann et al. 2008, Stadtmueller et al. 2011). However, modeling of the PA200-CP axial channel revealed it is narrower than that created by the RP-CP complex (**Figure 1-4b**), and peptidase assays showed that PA200-CP has much less proteolytic activity than the RP-CP, at least *in vitro* (Iwanczyk et al. 2006, Lehmann et al. 2008).
The lack of peptidase activity would seem to suggest that PA200-CP complexes are not capable of degrading polypeptides, however there is some evidence to imply otherwise. PA200 seemingly lacks many functional domains present in the RP (Ub-tagged target recognition, ATPase activity for unfolding and translocation of polypeptides, *etc*.); however, this lack of functionality could be replaced by a variety of associated factors (Ub-shuttling proteins, DUBs, *etc*.). The presence of Blm10-CP proteasomes increases cellular fitness during respiratory growth under oxidative stress, with *blm10*Δ yeast strains displaying mitochondrial dysfunction and decreased morphological integrity (Tar et al. 2014). This response seems to be related to the Blm10-CP-specific function of degrading the fission protein Dnm1. Additionally, Blm10 has been shown to facilitate the nuclear import of free CP via RAN-GTP and Nup53, a FG-rich nuclear pore protein (Weberruss et al. 2013).

Figure 1-4. The Alternate Proteasome Activator PA200

A. A crystal structure representation of PA200 bound to the CP. The proteasome activator protein PA200 (colorized) bound to the CP (grayscale) from the side (left) with a cross-section of the PA200-CP complex (middle). The far right panel shows the top-view of PA200 sitting upon the 20S CP, with a transparent view of the C-terminal portion of PA200 bound to the CP oriented over the axial channel. PA200-CP binding is mediated by a single HbYX-like motif at the PA200 C-terminus (Iwanczyk et al. 2006).

B. PA200 shifts the CP axial channel into an open conformation. An EM cartoon image (left) of PA200 (yellow) bound to the CP (Kwak et al.), showing the open axial channel (Kwak et al.). The middle panel is a cryo-EM image of the PA200-CP-PA200 complex, with white arrows indicating the axial channel opening that occurs upon binding. The far right panel is the CP bound by only one PA200 protein (top of image). The yellow arrow highlights the “closed” conformation of the CP axial channel when unbound (Iwanczyk et al. 2006).

Figure 1-4. The Alternate Proteasome Activator PA200



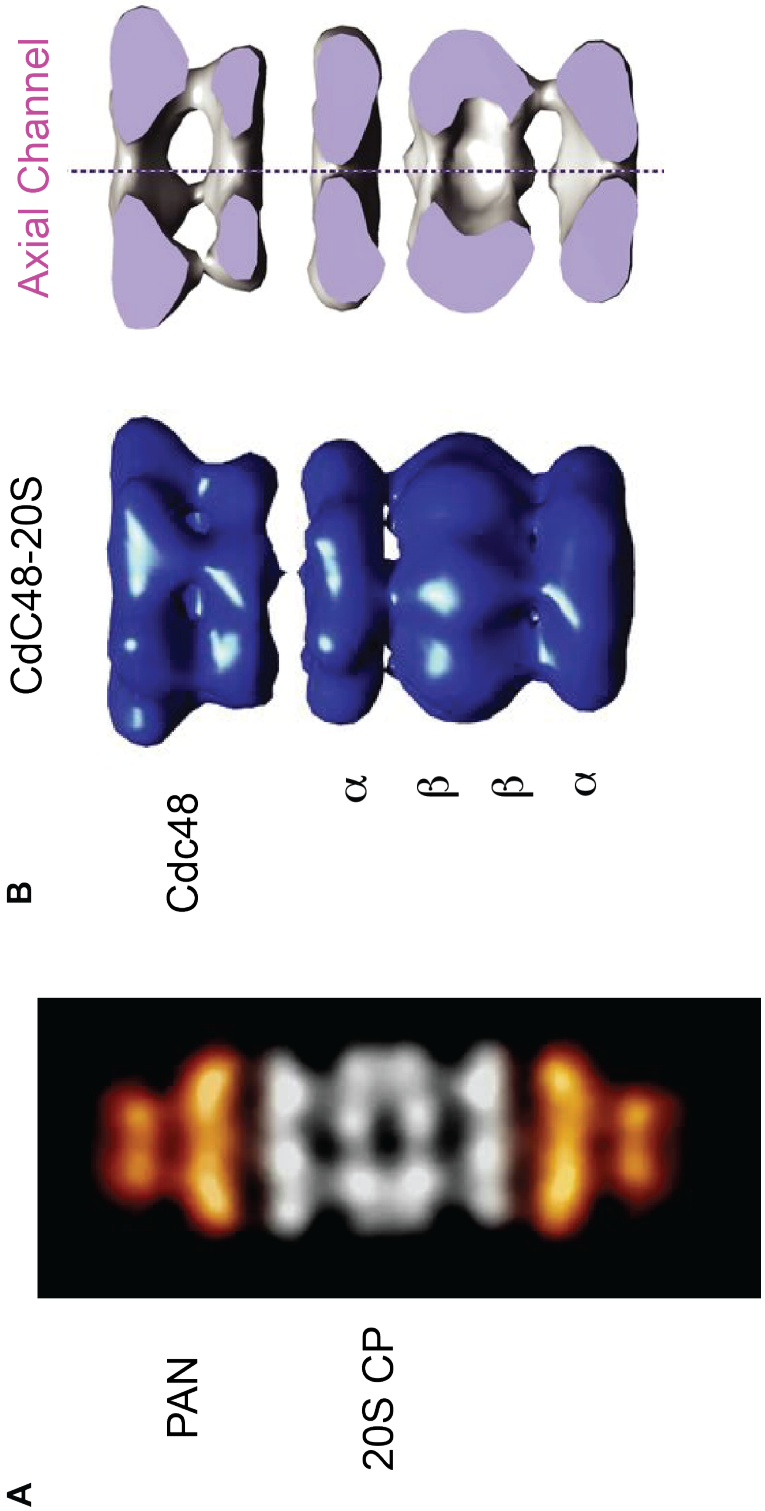
PA200 does not exist in archaea or eubacteria, but other capping particles are still present, including the PAN and CDC48 proteins (**Figure 1-5**) (Stadtmueller et al. 2010, Bar-Nun et al. 2012, Barthelme et al. 2014). PAN and CDC48 also have ATP-dependent "unfoldase" activity, similar to that of eukaryotic RP (Zwickl et al. 1999, Benaroudj et al. 2001, Barthelme et al. 2014). PAN is only present in prokaryotes, but CDC48, also known as p97 or VCP, has evolved a diverse array of functions throughout eukaryotic evolution (Baek et al. 2013). These functions are almost entirely involved with Ub-binding, which occurs through the N-terminal portion of the protein. CDC48 also has the capability to interact with other Ub-fold proteins, such as SUMO in yeast (Hannich et al. 2005). CDC48 appears to be pivotal in the ERAD pathway, assisting in Ub-tagged protein recognition, retro-translocation, ubiquitylation, and degradation of ERAD substrates (Ye et al. 2001, Vembar et al. 2008, Wolf et al. 2012). Finally, CDC48 has been implicated in regulating the membrane-bound UPS transcription factor Nrf1 (Nuclear Factor Erythroid-derived 2-Related Factor 1), which is a major modulator of proteasome subunit expression in mammalian cells (Radhakrishnan et al. 2010, Radhakrishnan et al. 2014).

Figure 1-5. Other Proteasome Activating Particles

A. The PAN-CP complex. An EM image of the archaeal ATPase PAN (orange) bound to the CP (white) (Smith et al. 2005). PAN-CP binding is mediated by the HbYX motif.

B. The mammalian CDC48-CP complex. A cartoon from an EM image showing the placement of the CDC48 ATPase as it would bind the CP barrel, based on structural studies. The right-most panel is a cross-section showing the orientation of the Cdc48-CP axial channel (Barthelme et al. 2014).

Figure 1-5. Other Proteasome Activating Particles



26S Proteasome Assembly

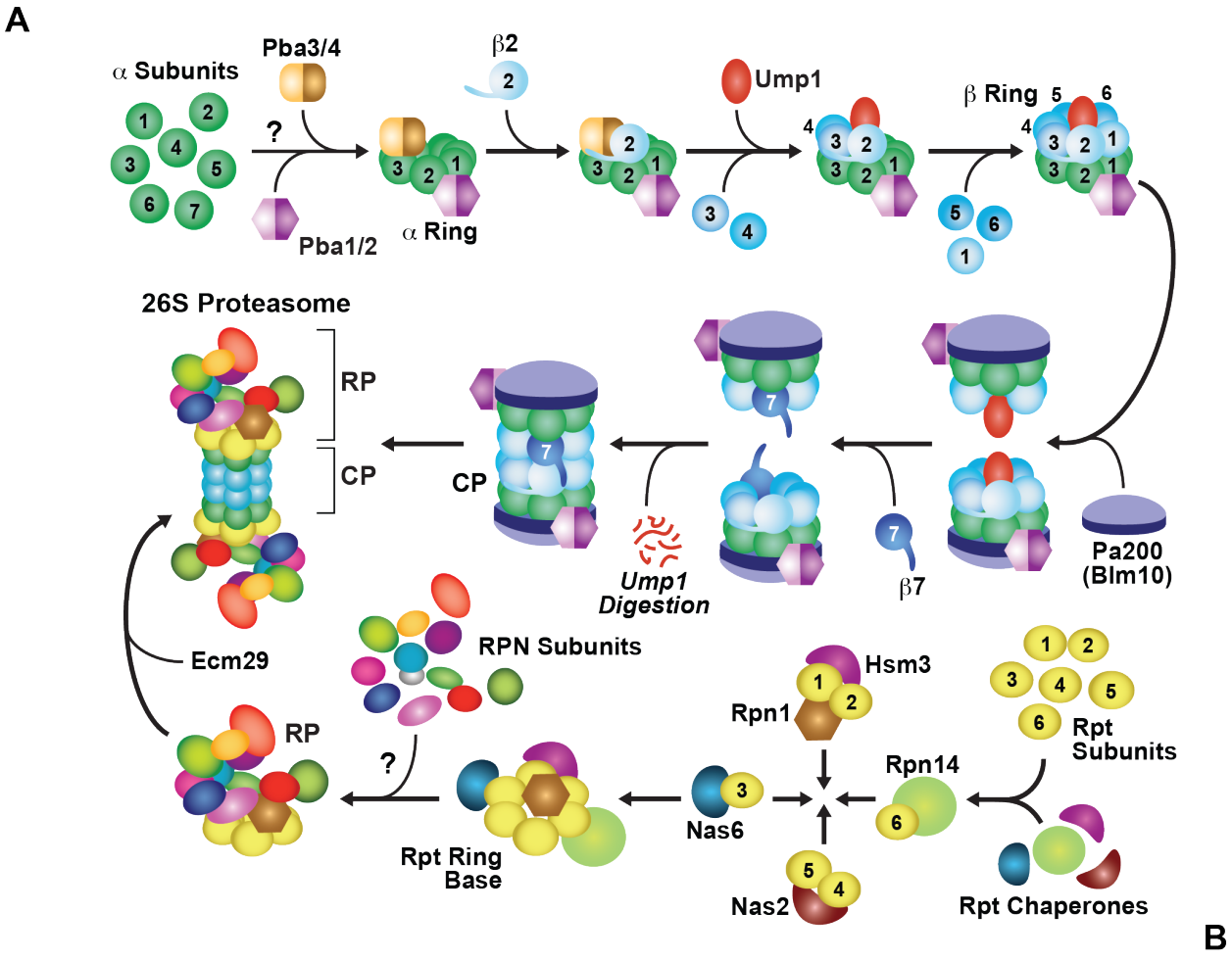
26S proteasome assembly can be broken down into two parts: CP assembly and RP assembly (**Figure 1-6**), which are facilitated by particle-specific chaperones (Tomko et al. 2013). Most studies into proteasome assembly have been conducted in yeast, although very similar mechanisms likely exist in other eukaryotes due to conservation of assembly chaperones. CP assembly initiates with the formation of the α-ring, with α subunits coming together in a seemingly random order (**Figure 1-6a**) (Gerards et al. 1997, Gerards et al. 1998, Yao et al. 1999). The heptameric α-ring serves as a scaffold for β-ring formation, which does have an ordered and sequential incorporation of β subunits (Frentzel et al. 1994, Yang et al. 1995, Nandi et al. 1997, Schmidtke et al. 1997). For α-ring formation in yeast, the HbYX-containing proteasome biogenesis-associated proteins, Pba1 and Pba2 (PIP1 and PBAC2 in *Arabidopsis* and PAC1/2 in mammals), form a hetero-dimer that bind to and incorporate individual α, but not β, subunits into a ring and are released prior to mature CP formation (Hirano et al. 2005, Kusmierczyk et al. 2011). All β-subunits are recruited to the α-ring by the Pbac3/4 hetero-dimer except for the β7 subunit, which is incorporated later (Yashiroda et al. 2008). The formation of the β subunits onto the α-ring is accompanied by the intercalation of Ump1 into the core of the nascent CP half-barrel, preventing premature dimerization of the CP (Ramos et al. 2004, Li et al. 2007, Marques et al. 2007, Hirano et al. 2008). PA200 binds the half-barrel structures and β7 now completes β-ring formation. Finally, the PAA20-1/2CP complexes dimerize and Ump1 is degraded upon proteolytic active site maturation, completing CP assembly (Chen et al. 1996, Ramos et al. 2004).

Figure 1-6. The 26S Proteasome Assembly Pathway

A schematic overview of yeast 26S proteasome assembly.

A. Beginning with CP assembly, the α subunits are formed into a ring in no particular order with the aid of the Pba1/2 and Pba3/4 hetero-dimers. Once the α-ring is formed, it serves as a scaffold for β-subunits to form their ring, again with the aid of the Pba3/4 dimer. β-subunits are incorporated into their ring in a specific order, with β2 starting the process. The Ump1 chaperone is then inserted into the middle of the newly forming half-barrel, along with the β3 and β4 subunits continuing ring formation. Next, the β1, β5, and β6 subunits are recruited to the ring. The CP half-barrel is bound by PA200—or 19S RP—and the β7 subunit completes β-ring formation. The two half-barrels come together to form a mature CP, degrading Ump1 as its first proteolytic act.
B. RP base and lid assembly utilize several chaperones. Rpt1, Rpt2, and Rpn1 form a tetramer with the chaperone Hsm3. Rpt6 binds to Rpn14. Rpt4 and Rpt5 trimerize with Nas2, and Rpt3 binds Nas6. These small complexes come together in a specific order to form the RP base and some of the lid. Lid assembly is not well understood. Ecm29 plays a role in the final incorporation of the 19S RP onto the 20S CP, resulting in the 26S proteasome holoenzyme.

Figure 1-6. The 26S Proteasome Assembly Pathway



The RP requires numerous chaperones for lid and base assembly, with a handful of RP subunits forming the lid and base piecemeal before finally coalescing into the 19S complex (**Figure 1-6b**) (Tomko et al. 2011). In yeast, the base assembly initiates by coupling a set of RPT subunits with one of four separate chaperones: Nas2, Nas6, Hsm3, and Rpn14 (SEM1). Rpt4 and Rpt5, which are bound by Nas2, associates with Rpt3 and Rpt6 that are coupled to Nas6 and Rpn14. Rpn2 and Rpn13 then associate with the 4-member RPT intermediate (Hirano et al. 2005, Funakoshi et al. 2009, Le Tallec et al. 2009, Park et al. 2009, Roelofs et al. 2009, Saeki et al. 2009). Finally, Rpt1, Rpt2, and Rpn1, all bound by Hsm3, completes the base, dislodging the Nas2 chaperone in the process (Tomko et al. 2010).

RP lid assembly is still not fully understood, but the chaperones Hsp90 in yeast and SEM1 in plants have been implicated in the proper incorporation of the lid onto the base (Imai et al. 2003, Tomko et al. 2014). What is known has been elucidated through analysis of lid subparticles; lid assembly can be further broken down into two modules: Rpn5/6/8/9/11 and Rpn3/7/Sem1 (Sharon et al. 2006). The remaining subunits, Rpn10 and Rpn12, are incorporated into the lid/base interface, forming the 19S RP complex. Finally, the 19S RP and 20S CP come together and is stabilized by Ecm29 (extra-cellular matrix 29), tethering the two particles until the CP becomes proteolytically active (Gorbea et al. 2004, Lehmann et al. 2010).

Proteasome Regulation and Stress

Expression of proteasome subunit and other UPS genes is strongly induced by various developmental and environmental situations that challenge cellular proteasome capacity, collectively called proteasome stress (Smalle et al. 2002, Smalle et al. 2003, Lundgren et al. 2005, Kelly et al. 2007, Fuchs et al. 2008, Book et al. 2009, Book et al. 2010, Kurepa et al. 2010, Shang et al. 2011). Proteasome stress is manifested through various circumstances including (i) heat stress, oxidative stress, and ionizing (UV) radiation (Lee et al. 1998, Pajonk et al. 2001, Vierstra 2003, Kim, 2011 #63, Lundgren et al. 2005, Kim et al. 2011, Shang et al.

2011, Livnat-Levanon et al. 2014), (ii) rapid cellular expansion and growth requiring the removal of aberrant proteins arising from synthetic errors (Naujokat et al. 2002), (iii) exposure to amino acid analogs whose translational incorporation makes abnormal polypeptides (Kruegel et al. 2011), and (iv) pathogens that secrete inhibitors of the CP proteolytic active sites (Yang et al. 2004, Lundgren et al. 2005, Schellenberg et al. 2010), such as epoxomicin, lactacystin, and syringolin A (SylA) (Meng et al. 1999, Clerc et al. 2009).

Transcriptomic and translational analyses of organisms undergoing proteasome stress show there is a concerted up-regulation of most, if not all, 26S proteasome subunit genes (Lundgren et al. 2005) along with other proteasome-associated factors, such as PA200, CDC48, and components of the ubiquitylation cascade (**Figure 1-7**). Additionally, the expression of stress-response gene products is altered under proteasome stress, including transcription factors, heat shock response proteins (HSPs), and detoxification-related proteins (*i.e.* glutathione-S-transferases (GSTUs) and o-methyl transferases).

One of the trademarks of proteasome stress is the accumulation of cellular Ub-conjugates that stabilize due to the lack of sufficient proteasome capacity (**Figure 1-7c**). The topology of poly-Ub chains within this accumulated conjugate pool is quite diverse within and between cells types. The overall demographics of Ub-linkages change during proteasome stress, increasing the amount of K11 and K63 poly-Ub chains compared to K48 linkages (Dammer et al. 2011). Additionally, novel proteins and complexes are also ubiquitylated during proteasome stress, specifically during MG132 treatment, with none more interesting than the 26S proteasome itself. Almost every single subunit is modified with Ub during prolonged inhibitor exposure (Kim et al. 2013). There could be multiple reasons for this modification, but purpose is known: proteasome ubiquitylation serves as a signal for degradation via a specific, targeted pathway of autophagy, termed proteaphagy, that is modulated by a single UIM motif (UIM2) in RPN10 along with Ub-fold autophagy protein ATG8 (Marshall et al. 2015).

Transcriptional Feedback Loop and Trans-acting Factors

The transcriptional response to proteasome stress was initially characterized in yeast. A single transcriptional activator, Rpn4, controls the yeast proteasome stress regulon. A C2H2-type zinc-finger transcription factor, Rpn4 binds the DNA recognition sequence PACE (**P**roteasome **A**ssociated **C**ontrol **E**lement) (**Figure 1-8**). PACE is a nonamer present in the upstream promoter regions of 26 of the 33 yeast proteasome subunits (Mannhaupt et al. 1999). Rpn4 was originally identified as being physically associated with the proteasome (Fujimuro et al. 1998), which is why it was given a RPN designation, but it was quickly determined to be a transcriptional activator of proteasome genes during stress, rather than a subunit or associated factor. Rpn4 is regulated post-translationally by the proteasome via a negative feedback loop (**Figure 1-8a**), and thus is in constant proximity to the proteasome. Rpn4 undergoes rapid turnover ($t_{1/2}$~2.5 minutes) through the proteasome, preventing its accumulation in cells when proteasome capacity is in excess (Xie et al. 2001, Dohmen et al. 2007). However, when the protease is challenged with a glut of substrates, Rpn4 turnover is slowed, allowing for it to accumulate rapidly and act upon UPS genes.

A similar proteasome stress response mechanism likely exists in other eukaryotes due to the conservation of proteasome gene expression under stress conditions. Mammalian cells, flies (*Drosophila melanogaster*), and *Arabidopsis* transcriptionally up-regulates a similar cohort of UPS genes as well as increases *de novo* assembly of 26S proteasomes during chemical inhibition (Meiners et al. 2003, Yang et al. 2004, Lundgren et al. 2005, Book et al. 2009, Book et al. 2010, Kurepa et al. 2010). Despite the apparent conservation of this regulon, orthologs of either Rpn4 or PACE remain to be identified outside of yeast and other fungi.

However, other eukaryotic UPS-activating transcription factors have been identified. The mammalian proteins Nrf1 and Airap can up-regulate proteasome genes in mice under oxidative stress and arsenic treatments, respectively (Stanhill et al. 2006, Kensler et al. 2007). Like yeast Rpn4, both Nrf1- and Airap-regulated proteasome gene-activation is induced by

particular stresses, but they do not significantly affect basal expression levels under “normal” conditions. The Nrf1 response in mammals is a p97-dependent mechanism, requiring the ATPase for proper incorporation of Nrf1 into the endoplasmic reticulum (ER) as well as constant degradation via the ERAD pathway. p97 also plays a role in the cleavage of Nrf1 away from the ER membrane, which occurs through a proteasome-independent protease, liberating the membrane-bound transcription factor for its eventual transport into the nucleus where it binds to the upstream antioxidant recognition element (ARE) that is present in many 26S proteasome genes (**Figure 1-8b**).

In *Arabidopsis,* there are members of the NAC (**N**o Apical Meristem, **A**TAF1,2, **C**UC2) domain-containing family of transcription factors that have been shown to bind to and up-regulate proteasome subunit genes *in vitro* and *in vivo*, specifically NAC78 (Yabuta et al. 2011, Nguyen et al. 2013), which binds to the proteasome related *cis* element (PRCE) that exists upstream of most proteasome subunit genes. While some factors that regulate the UPS have been identified, the gene expression stress response still persists to some extent in mutants that lack these *trans-acting* proteins, suggesting that multiple transcriptional regulators, rather than a single “master regulator” like yeast Rpn4, are responsible for activating UPS gene expression under different situations in more complex eukaryotes.

Figure 1-7. The *Arabidopsis* Proteasome Stress Response

A. RNA Northern blots of proteasome subunit transcripts in the *rpn10-1* and *rpn12a-1* mutant backgrounds, which severely limit proteasome capacity and result in increased proteasome subunit transcripts.

B. Subunit immunoblots from the proteasome mutant *rpn12a-1* or WT seedlings treated with the proteasome inhibitor MG132. Only MG132 treatment shows increases in both the PA200 and PBA1 gene products, especially for the immature PBA1 pro-enzyme (white arrow), which has not yet undergone proteolytic processing (black arrow), indicating an accumulation of proteasome assembly intermediates. Figure adapted from (Smalle et al. 2003).

C. An anti-Ub Immunoblot of proteasome mutants or WT Col-0 seedlings (+/- MG132). Only MG132 treatment and *rpn10-1* Ub-recognition subunit mutant background show a stabilization of higher molecular weight Ub conjugates (Ub_c).

Figure 1-7. The *Arabidopsis* Proteasome Stress Response

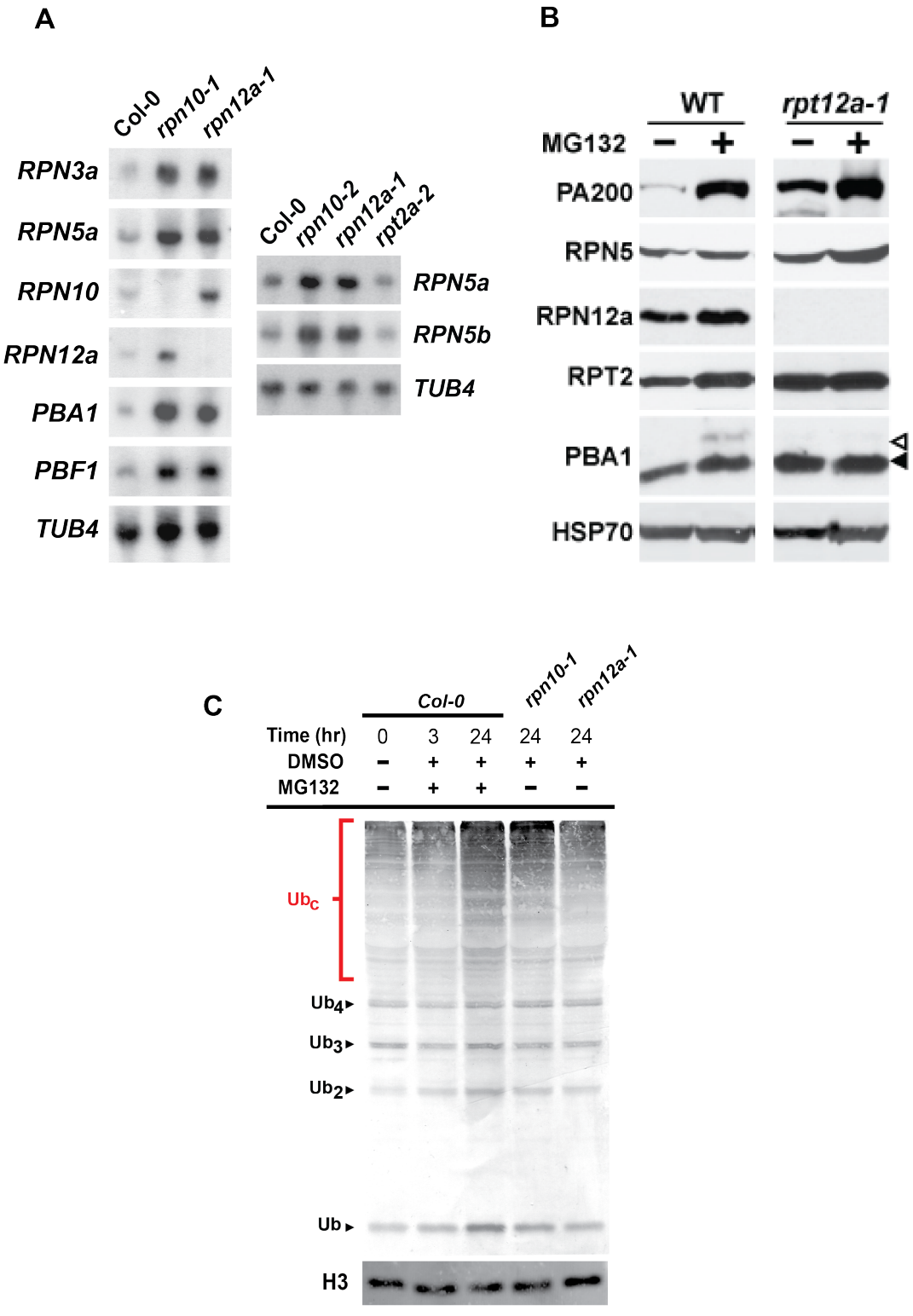
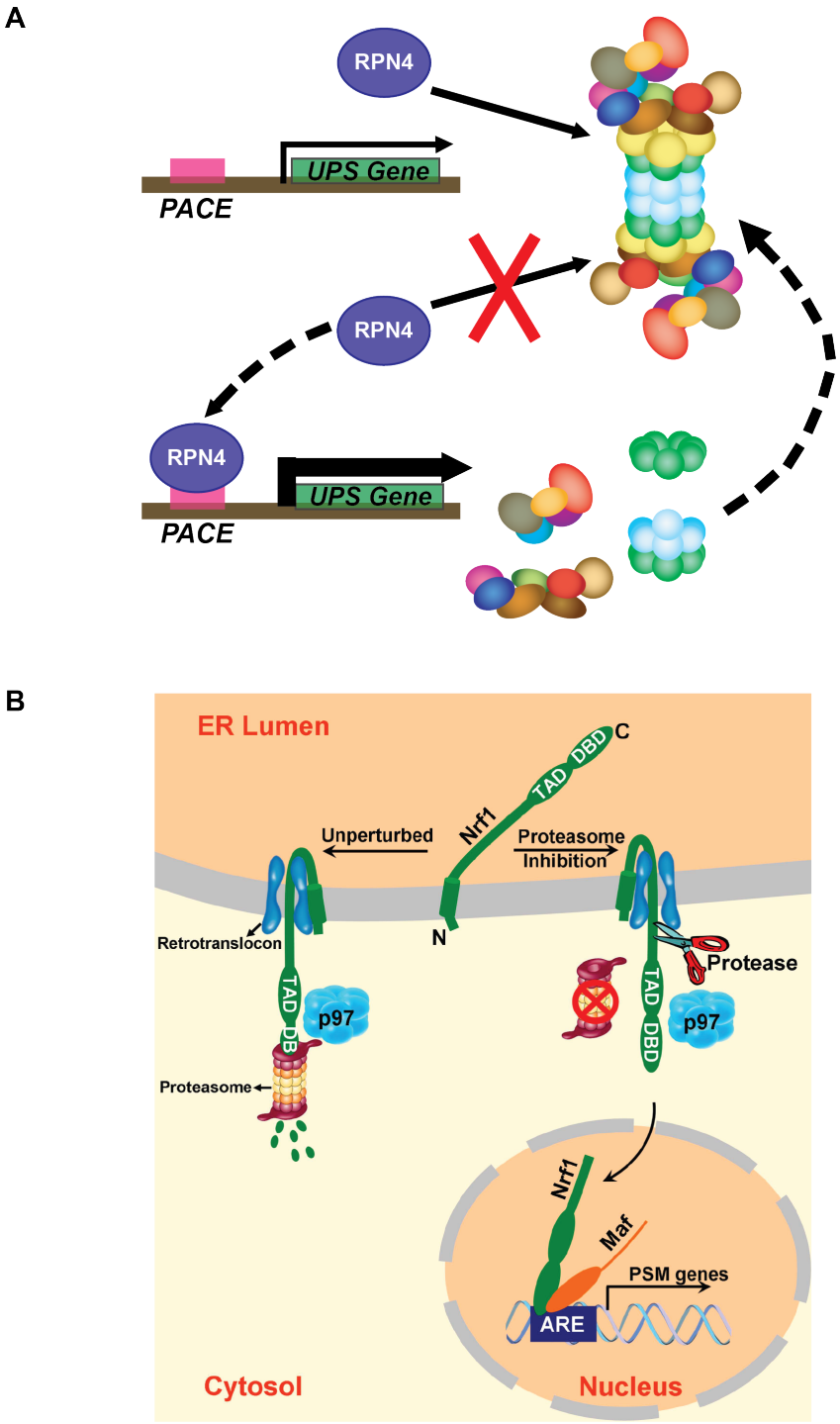


Figure 1-8. Eukaryotic Proteasome-Stress Response Transcriptional Feedback Loops

A. The yeast Rpn4 proteasome stress response feedback loop. The transcription factor Rpn4 is constantly degraded by the 26S proteasome (half-life ~2.5 minutes). When proteasome capacity is limited (red "X") through chemical inhibition, proteasome mutants, and/or a glut of mis-folded/mis-translated proteins, Rpn4 can no longer be degraded efficiently and will accumulate. Rpn4 will then bind to the PACE element and up-regulate proteasome genes.

B. The mammalian Nrf1 proteasome stress response feedback loop. The ER-bound transcription factor Nrf1 is cytosolically oriented and is constantly degraded via the p97-dependent ERAD pathway. When proteasome capacity is reduced, Nrf1 is proteolytically cleaved by a cellular protease (not the 26S proteasome) in a p97-dependent manner, releasing the DNA-binding domains (TAD and DBD)-containing C-terminal portion of the protein into the cytosol. This active form of Nrf1 localizes to the nucleus, where it binds the antioxidant response element (ARE) that exists upstream of several proteasome subunit genes (Radhakrishnan et al. 2014).

Figure 1-8. Eukaryotic Proteasome-Stress Response Transcriptional Feedback Loops



Proteasome Inhibitors

The most common conditions that limit proteasome capacity relate to cellular growth and development, genetic impairment of the proteasome, and environmental response. However, one of the most effective ways to reduce proteasome activity is with small peptide inhibitors (**Figure 1-9**). Intensive research has gone into the field of proteasome inhibitor discovery and development, primarily due to their applications as antagonist to certain cancers like multiple myelomas. Most synthetic and natural inhibitors significantly reduce the chymotrypsin-like activity of the proteasome (Yang et al. 2004), with generally smaller inhibitive effects on trypsin and PGHP activity (Chen et al. 1996, Myung et al. 2001), although there are some inhibitors that significantly decrease tryptic and PGHP proteolysis as well. Proteasome inhibitors can be divided into 2 main groups based on whether they form a covalent or non-covalent bond within the CP active sites with further subdivision based upon chemical structure. All non-covalent inhibitors reversibly bind the active sites, as well as few of the covalent inhibitors. There are at least 5 major classes of eukaryotic proteasome inhibitors that were originally discovered as natural products or contain natural products. These include the aldehydes, β-lactones, α,β-epoxyketones, syrbactins, and lesser-studied cyclic peptides (Kisselev et al. 2012).

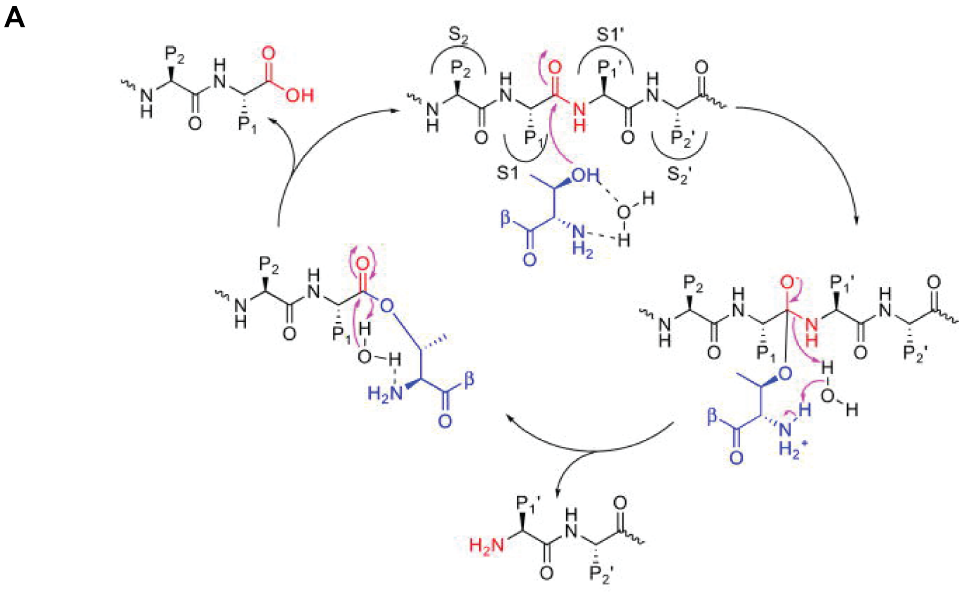
The aldehyde group in particular has the longest and most successful history of research use, primarily due to its low cost and high efficacy (Rock et al. 1994). The most notable inhibitor in this class is the synthetically derived MG132, which is used extensively in laboratory research. MG132 and other aldehydes target serine and cysteine proteases (Tsubuki et al. 1993, Palombella et al. 1994, Adams et al. 1998), and are most sensitive to the chymotryptic activity of the proteasome.

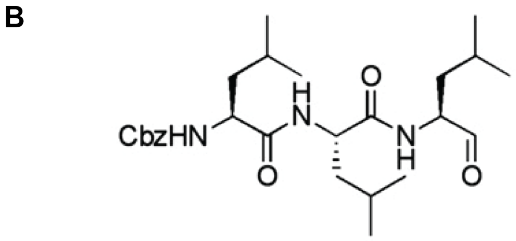
Figure 1-9. Mechanism of Action and Chemical Structures for Common Proteasome Inhibitors

A. Catalytic mechanism of 26S proteasome proteolytic cleavage. Serine protease cleavage within the CP occurs as portrayed by the chemical reaction. The proteasome is blue, substrate is black, and the covalent bond is in red (Kisselev et al. 2012).

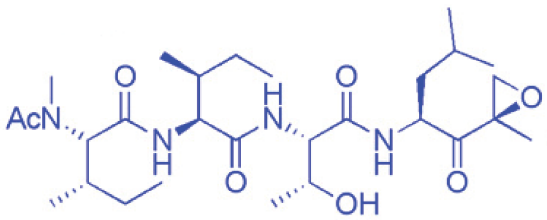
B. Chemical Structures of important proteasome inhibitors that are synthetically derived (black) or natural products (blue) (Kisselev et al. 2012).

Figure 1-9. Mechanism of Action and Chemical Structures for Common Proteasome Inhibitors

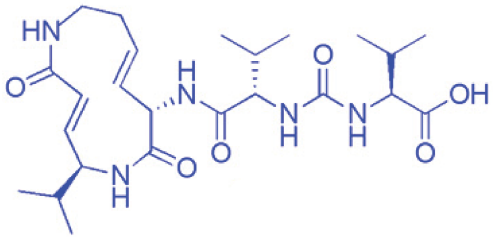




MG132



Epoxomicin



Syringolin A

While MG132 is a potent and specific proteasome inhibitor, the aldehyde class in general has a lower specificity to the 20S CP active-sites than some other inhibitors, and thus likely has off-target effects against other cellular proteases.

The α,β-epoxyketones are the most specific and potent proteasome inhibitors to be discovered, with epoxomicin being the most well known (Meng et al. 1999). Epoxomicin is produced from a strain of *Actinomycetes* and was initially characterized as a proteasome inhibitor through studies on counteracting murine melanoma tumor growth (Hanada et al. 1992). Synthetic derivatives of epoxomicin and other epoxyketones, such as carfilzomib, show the greatest current promise in clinical trials for cancer treatments (Molineaux 2012).

The boronate class of proteasome inhibitors, which include bortezomib, is more potent than the aldehydes, but with this increased potency comes decreased specificity to the proteasome active-sites (Adams et al. 1998). While off-target effects against other serine proteases in boronates are generally orders of magnitude lower than the aldehydes, there are exceptions. In the case of bortezomib, there is a significant reduction in the activity of HtrA2/Omi, an ATP-dependent mitochondrial serine protease (Arastu-Kapur et al. 2011). Modifying other inhibitor classes with boronates can also result in reduced proteasome specificity: MG132-boronate derivatives target the prokaryotic Lon protease and the mammalian mitochondrial ClpXP protease (Fishovitz et al. 2011), indicating a unique set of off-target effects could be specific to certain classes of peptide inhibitors.

In addition to synthetic proteasome inhibitors, naturally produced inhibitors have been studied for decades. These natural products are excreted largely by prokaryotes, however it is likely that fungal species also produce proteasome inhibitors; based on sequence identity to the prokaryotic secondary metabolite gene clusters that encode the small peptide inhibitors. Amongst prokaryotic-produced proteasome inhibitors, the most well known and studied are epoxomycin, lactocystin (clasto-lactocystin-β-lactone)—both secreted by strains of

Actinomycetes—and the more recently discovered natural product SylA, a syrbactin class inhibitor secreted by *Pseudomonas syringae* (Groll et al. 2008, Kisselev et al. 2012). These natural inhibitors are usually produced to aid microorganism infection of plants or other organisms. Prokaryotes get around the issues of self-inhibition because, in many cases, the bacterial proteasome is not essential due to other cellular proteases such as the Lon or ClpXp enzymes (Driscoll et al. 1992, Goldberg 1992, Knipfer et al. 1997). One exception to this is the marine microorganism *Salinispora tropica*, another *Actinomycete*.

Salinispora produces the most potent proteasome inhibitor known to date, salinosporamide A, which shows up to 100% inhibition of the chymotryptic active site for longer durations than other naturally-produced inhibitors (Chauhan et al. 2005, Potts et al. 2011). To counteract this situation of proteasome-dependence while also expressing a proteasome inhibitor, the salinosporamide A metabolite cluster encodes a separate CP subunit that is 50-fold less sensitive to the inhibitor than the sister paralog encoded elsewhere in the genome (Kale et al. 2011). In another turn of medical-based applications from inhibitor research, a salinosporamide A derivative (clinically known as Marizomib) has been shown to be a more potent anti-leukemic drug, with decreased drug resistance exhibited from cancer cells compared to other clinical inhibitors like bortezomib (Niewerth et al. 2014). These types of evolutionary events that result in salinosporamide A-like metabolite clusters are probably not all that rare, and with the increasing number of bacterial and fungal genomes being sequenced and annotated, novel proteasome inhibitor will undoubtedly continue to surface and can be applied to medical and agricultural research.

CONCLUSIONS

The UPS is responsible for the majority of rapid and specific protein proteolysis, and is thus intimately involved in almost all cellular processes. Understanding UPS regulation and functionality within plants could provide new strategies to augment agricultural productivity and sustainability as well as biofuel production. Examples include: (i) enhancing the ability of the protease to degrade foreign pathogens or unwanted endogenous proteins that are inhibitory to growth, (ii) modifying the proteasome when it interferes with plant productivity (seed germination, senescence, fruit ripening and stress tolerance), and (iii) increasing our ability to express proteins transgenically. Additionally, due to the strong conservation of the UPS among eukaryotes, it is likely that further understanding of the *Arabidopsis* UPS could aid in medical treatments for human diseases.

The goal of my thesis is to expand the knowledge of proteasome regulation and function by characterizing proteasome associated factors as well as the regulation of the UPS during conditions that limit proteasome capacity. To accomplish this I used genetic, biochemical, and molecular biological techniques to initially characterize the proteasome activator PA200 in *Arabidopsis* seedlings. Secondly, using various bioinformatics techniques, I performed an in-depth analysis of the *Arabidopsis* transcriptome during conditions that chemically and genetically impair proteasome function. I also described the DNA-binding motif landscape present in the upstream regions of stress-responsive UPS genes, and found multiple *trans*-acting factors that can recognize them and activate UPS gene expression. Finally, I have characterized two transcription factors, NAC53 and NAC78, that act redundantly to regulate UPS gene expression. I demonstrated that plants lacking both NAC53 and NAC78 are hypersensitive to proteasome stress, manifesting severe growth and developmental phenotypes when 26S proteasome activity is inhibited.

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CHAPTER 2

THE *ARABIDOPSIS* PROTEASOME-STRESS REGULON

ABSTRACT

The ubiquitin/26Sproteasome system (UPS) is a highly conserved eukaryotic and archaeal pathway that degrades intracellular constituents. This is accomplished by the post-translational modification of target proteins with a poly-ubiquitin chain, which facilitates their recognition and subsequent degradation by the 26S proteasome. Over 6% of the *Arabidopsis thaliana* genome encodes UPS components, including subunits of the 26S proteasome, components of the Ub-conjugation machinery, de-ubiquitylating enzymes (DUBs), and numerous other UPS-associated factors. Here, I provide a thorough characterization of the *Arabidopsis* proteasome-stress transcriptome via RNA sequencing (RNAseq) analysis. I also describe the DNA-binding motif landscape of UPS and proteasome stress-responsive genes, and identify multiple transcription factor candidates that are capable of activating proteasome gene expression. Together, these data describe a shared gene expression response between proteasome stress and other growth and environmental response pathways.

INTRODUCTION

Eukaryotes, prokaryotes, and archaea rely on the rapid and specific turnover of proteins through the ubiquitin/26Sproteasome system (UPS) to remove aberrant polypeptides and control the abundance of key intracellular regulators (Grau-Bové et al. 2015). The 26S proteasome is a 2.5-MDa proteolytic complex that degrades ubiquitin (Ub)-tagged proteins through an ATP-dependent mechanism. The complex consists of two major subcomplexes; the 20S core protease (CP) that houses the proteolytic active sites, and the 19S regulatory particle (RP) that recognizes, de-ubiquitylates, unfolds, and translocates Ub-tagged proteins and polypeptides into the CP for breakdown (Finley 2009, Bhattacharyya et al. 2014). Approximately 33 distinct subunits comprise the 26S proteasome holoenzyme, and most, but not all, of these subunits and associated factors show a concerted up-regulation in response to situations that compromise proteasome activities, collectively called proteasome stress (Lee et al. 1998, Pajonk et al. 2001, Naujokat et al. 2002, Vierstra 2003, Schellenberg et al. 2010, Kim et al. 2011, Kruegel et al. 2011).

Proteasome stress can be elicited through various circumstances, including (i) heat stress, oxidative stress, and ionizing (UV) radiation (Lee et al. 1998, Pajonk et al. 2001, Vierstra 2003, Kim et al. 2011, Shang et al. 2011, Livnat-Levanon et al. 2014), (ii) rapid cellular expansion and growth that requires the removal of aberrant proteins and polypeptides arising from translational errors (Naujokat et al. 2002), (iii) exposure to amino acid analogs whose incorporation results in abnormal polypeptides, such as p-fluoro phenylalanine (pFP) or canavanine (Kruegel et al. 2011), and (iv) pathogens that secrete inhibitors of the CP proteolytic active sites (Schellenberg et al. 2010), examples of such pathogen-secreted inhibitors are syringolin A and epoxomicin, toxins produced by *Pseudomonas syringae* and *Actinomycetes*, respectively (Meng et al. (1999), (Clerc et al. 2009). Synthetic inhibitors like MG132 and bortezomib are also commonly used to stress proteasome capacity by inhibiting the CP chymotryptic active sites. (Myung et al. 2001, Yang et al. 2004). Similar proteasome stress

response networks exist in other eukaryotes: mammalian cells, flies (*Drosophila melanogaster*), and *Arabidopsis* all transcriptionally and translationally up-regulate UPS genes in concert during proteasome stress (Meiners et al. 2003, Yang et al. 2004, Lundgren et al. 2005, Book et al. 2009, Book et al. 2010, Kurepa et al. 2010).

The proteasome stress regulon in yeast (*Saccharomyces cerevisiae*) is controlled by Rpn4, a C2H2-type zinc-finger transcription factor that binds to the DNA recognition sequence PACE (Proteasome Associated Control Element), a nonamer motif (GGTGGCAAA) present in the upstream promoter regions of 26 of the 33 yeast proteasome subunits (Mannhaupt et al. 1999, Xie et al. 2001, Shirozu et al. 2015). Recently, a conserved *cis*-element termed the Proteasome-Related *Cis* Element (PRCE), was also identified in the upstream regions of over 30 proteasome subunit genes within *Arabidopsis* and other plant species, including *Oryza sativa*, *Ricinus communis*, and *Chlamydomonas reinhardtii* (Nguyen et al. 2013). PACE is recognized by the NAM/ATAF1,2/CUC2 (NAC) family transcription factor NAC78, which activates proteasome gene expression.

To more fully understand UPS regulation during stress, I performed a detailed characterization of the *Arabidopsis thaliana* proteasome-stress regulon via RNA sequencing (RNAseq) analysis of chemically and genetically crippled 26S proteasomes. I also characterize the DNA-binding motif landscape in the upstream sequences of UPS and other proteasome stress responsive genes. Finally, I provide evidence that potentially dozens of plant *trans*-acting factors can bind to and activate proteasome gene expression.

RESULTS

26S Proteasome Subunits Are Up-Regulated During Proteasome-Stress

To initially characterize the proteasome-stress regulon, I evaluated the expression of *Arabidopsis* 26S proteasome subunit genes during two conditions that curtail proteasome activity: (i) via chemical treatment with the proteasome-specific inhibitor MG132 and (ii)

genetically with the two separate exon-trap proteasome subunit mutant lines *rpn10-1* and *rpn12a-1*. Proteasome gene transcripts and products are consistently up-regulated in response to MG132 exposure and in the mutant backgrounds (**Figure 2-1 and Figure 2-2**). Notably, the PBA1 immunoblot shows an accumulation of its full-length, pre-processed form (seen as a higher molecular weight band) upon MG132 treatment, which is a hallmark of proteasome inhibition (**Figure 2-2a**) (Chen et al. 1996, Book et al. 2010). Ub-conjugates significantly accumulate during MG132 treatment and in the *rpn10-1* line, however the *rpn12a-1* line does not show significant obstruction of Ub-conjugate turnover (**Figure 2-2b**). This indicates that while proteasome capacity is challenged in both *rpn10-1* and *rpn12a-1*, it is only RPN10 that participates in Ub-tagged protein recognition (Smalle et al. 2002, Smalle et al. 2003). While most proteasome subunits are up-regulated during proteasome stress, not all paralogs of these subunits are up-regulated similarly (**Figure 2-1b**). This suggests there is a preferential expression, incorporation, or function of certain paralogs into the 26S complex. This is supported by previous in-depth reverse genetic analyses of other *Arabidopsis* subunits, including RPN5a and RPT2 (Book et al. 2009, Lee et al. 2011).

The Proteasome Stress-Induced Regulon (PSIR)

To more fully identify the suite of genes that are regulated in response to impaired proteasome capacity, RNAseq was performed on Col-0 seedlings treated with 100 μM MG132 for 3 hours (hr) or 24 hr, and in the *rpn10-1* and *rpn12a-1* mutant backgrounds. Differentially expressed genes were classified as those whose mRNA levels were significantly different (p-value <0.01, FDR <0.05) from untreated Col-0 tissue (No DMSO treatment), based on negative binomial normalization (edgeR, R-statistical computing language).

Figure 2-1. *Arabidopsis* Proteasome Genes are Up-Regulated in Response to Proteasome Stress

26S proteasome genes are up-regulated in response to proteasome stress.

A. qRT-PCR of 26S proteasome subunits during MG132 treatment or in the *rpn10-1* or *rpn12a-1* mutant backgrounds. Expression was normalized to *ACT2* expression and compared to Col-0 0 hr control. At least three biological replicates are used all samples and error bars represent standard deviation.
B. X-gal staining of Col-0 seedlings expressing a transgenic *GUS* reporter driven by 2 Kb fragments of 26S subunit proteasome 5' upstream regions (+/- MG132).
C. Quantification of GUS reporter staining in (B).

Figure 2-1. *Arabidopsis* Proteasome Genes are Up-Regulated in Response to Proteasome Stress

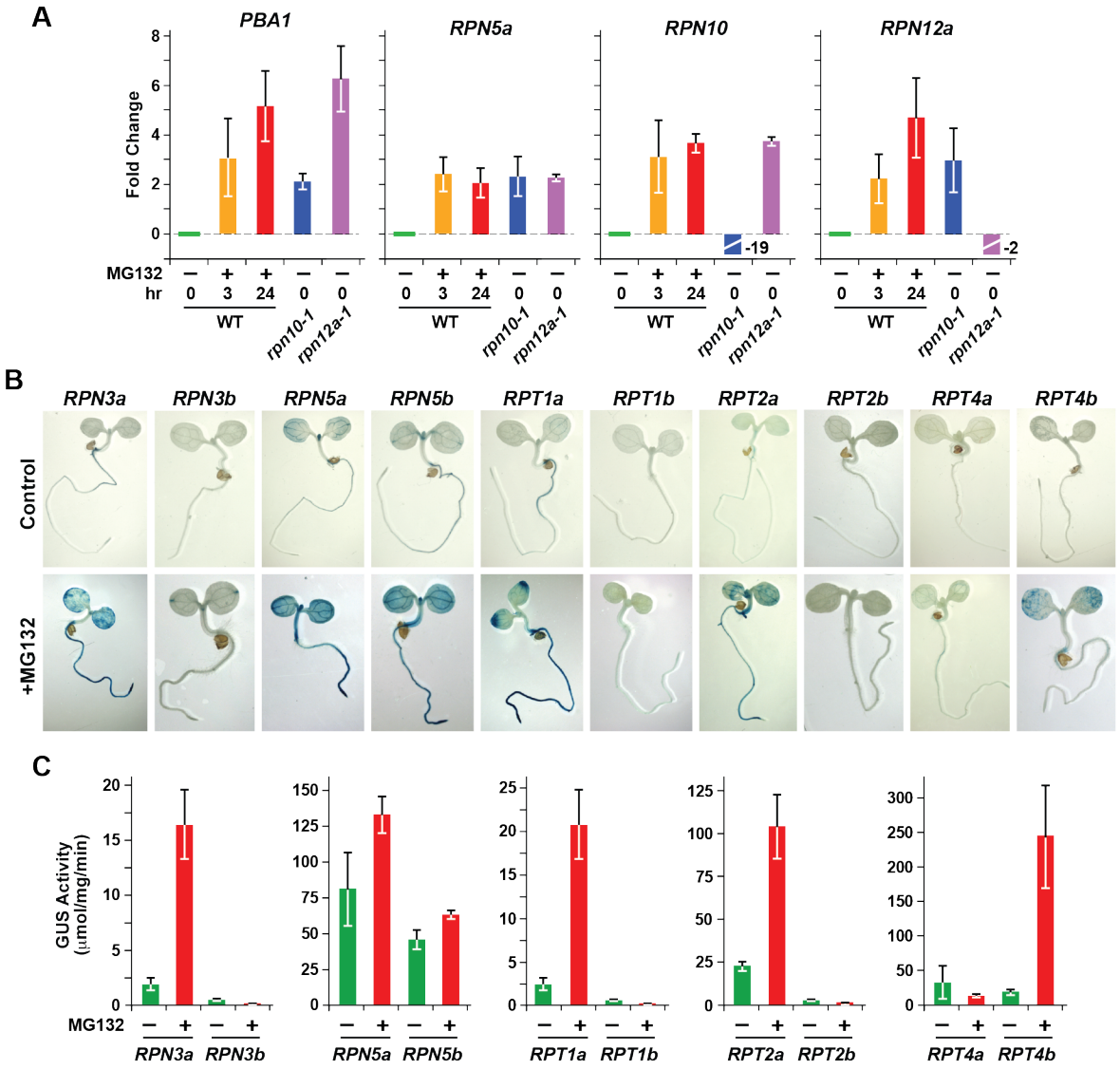
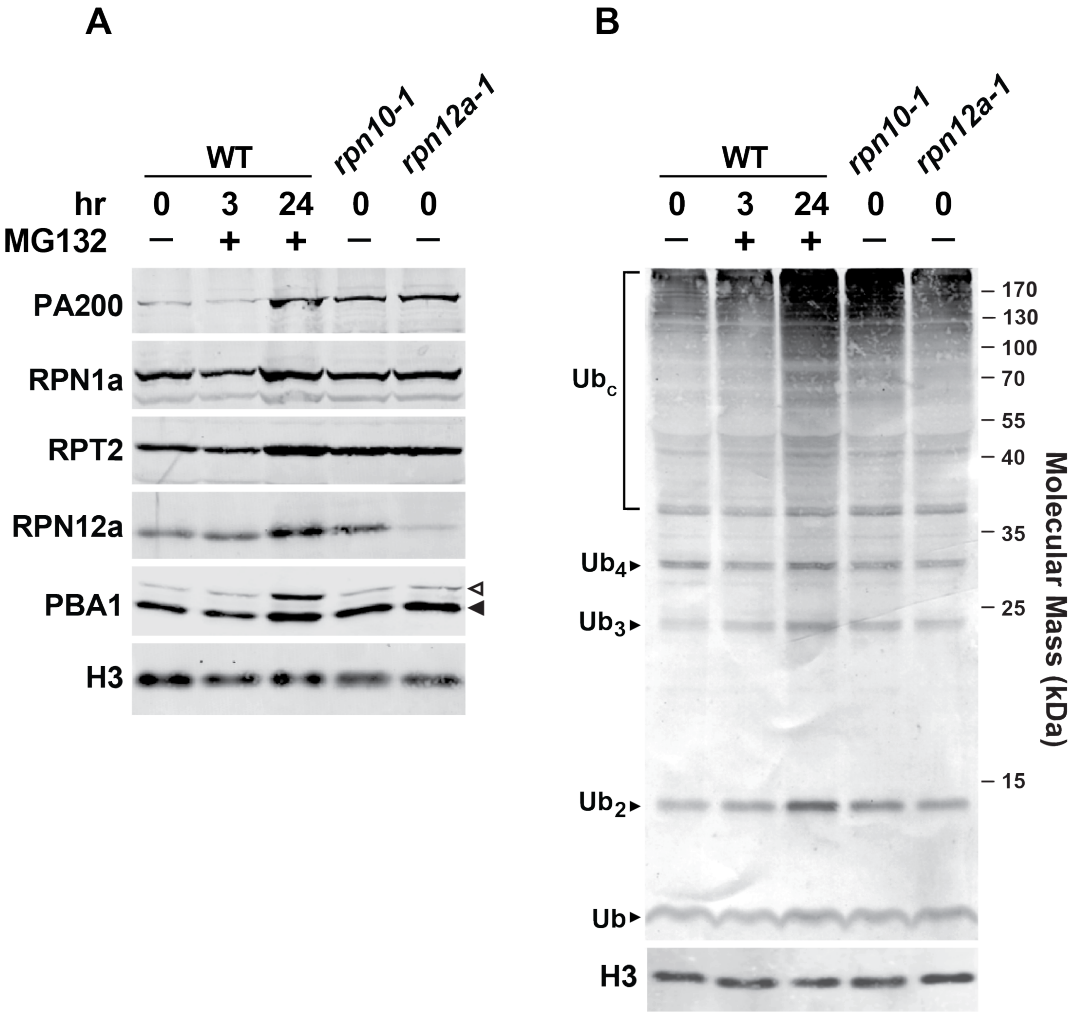


Figure 2-2. *Arabidopsis* Proteasome Proteins Accumulate in Response to Proteasome Stress

A. Protein immunoblots of proteasome subunits during proteasome stress. The black arrow indicates the mature, catalytically active form of PBA1, while the white arrow denotes the un-cleaved proenzyme. Histone 3 (H3) was used as a loading control.

B. Anti-Ub immunoblot showing mono-, di-, tri-, and tetra-Ub moieties, and the higher molecular weight Ub-conjugates (Ub_c) that increase with prolonged exposure to MG132 or in the *rpn10-1* mutant line, but not in the *rpn12a-1* background. H3 was used as a loading control

Figure 2-2. *Arabidopsis* Proteasome Proteins Accumulate in Response to Proteasome Stress



I identified 117 genes to be coordinately up-regulated in both the *rpn10-1* and *rpn12a-1* lines and in Col-0 seedlings treated with MG132 (**Figure 2-3a,b**), with 33 genes being down-regulated. This core set of 150 genes was termed the proteasome-stress induced regulon (PSIR). I did not include in the PSIR any differentially expressed genes that also manifested in the 24 hr MG132 treatment: many UPS and other stress-response genes were up-regulated in this condition; but because prolonged exposure to such a toxic inhibitor could result in off-target gene regulation events that may not be representative of proteasome-specific inhibition, differentially expressed genes from the 24 hr MG132 treatment were not included in the regulon definition. Gene ontology analysis via DAVID (**D**atabase for **A**nnotation, **V**isualization, and **I**ntegrated **D**iscovery) of the 117 PSIR up-regulated genes showed a functional enrichment for proteasome, UPS-related, and general stress response processes (Huang da et al. 2009, Huang da et al. 2009). This was especially pronounced amongst the highest-expressed genes (fold change ≥ 2.0). There was no significant functional enrichment in the 33 PSIR down-regulated genes (**Table 2-1**).

Almost all of the 26S proteasome genes are in the PSIR. A total of 41 proteasome subunit paralogs are significantly up-regulated during all treatments/mutant lines except for *RPN6* and *RPN13* (**Figure 2-3c**), however both of these genes displayed significant up-regulation during the 3hr and 24hr of MG132 treatment, but just not in the *rpn10-1* background (*RPN6*) or in both the *rpn10-1* and *rpn12a-1* backgrounds (*RPN13*). Several proteasome subunits, especially within the RP, display noticeably different fold-change expression between their paralogs (*PAA1/PAA2, PBE1/PBE2, RPN2a/RPN2b, RPN8a/RPN8b,* and *RPT5a/RPT5b*), and some paralogs do not display any significant differential expression compared to the wild-type (WT) Col-0 control *(RPN3b, RPN9a, RPT1b,* and *RPT6a*).

Table 2-1. All genes in the Proteasome Stress-Induced Regulon (PSIR)

All genes that comprise the PSIR with their *Arabidopsis* Genome Identifier (AGI) and their Log_2(fold-change) expression (LogFC) compared to WT (Col-0 No DMSO treatment) for each proteasome stress condition: Col-0 treated with 3 hr MG132, *rpn10-1*, and *rpn12a-1*. UPS genes are highlighted in red. Genes are ranked by their expression in the Col-0 3 hr MG132 treatment condition.

Gene	UPS Gene	Col-0 3 hr MG132 logFC	rpn10-1 0 hr logFC	rpn12a-1 0 hr logFC
AT3G53230	CDC48b	4.64618627	3.089605693	3.49720153
AT1G21520		4.307962676	1.599156832	1.621251229
AT2G21640		4.076297739	2.169852	0.940722965
AT5G20000	RPT6b	3.397449713	3.008672031	3.345726967
AT3G22370		3.058843028	1.040529563	1.305560003
AT3G17611		2.987527526	1.379094636	1.41682212
AT4G03320		2.956279074	1.847239464	2.218354378
AT5G20910		2.869108386	1.901054729	2.390627683
AT3G45730		2.829651758	1.744066723	2.48065995
AT3G13330	PA200	2.627890058	1.779834968	2.138391434
AT1G09100	RPT5b	2.587801007	2.173922446	2.783312955
AT3G50930		2.578991506	1.30166195	1.356699707
AT1G24095		2.553881592	2.099801009	2.029953039
AT2G03430		2.530249431	2.165559695	2.186344632
AT2G05840	PAA2	2.441058879	2.593374306	2.622246822
AT2G30250		2.433418214	1.484137394	1.303491111
AT3G26340	PBE2	2.404052048	1.826774803	2.175223688
AT1G04810	RPN2b	2.156931939	1.395769128	1.81134763
AT5G44390		2.153774969	1.572534065	3.067575422
AT3G09840	CDC48a	2.126239505	1.150971726	1.503212676
AT4G15420		2.112306935	1.495132856	1.634072274
AT3G11270	RPN8b	2.079101886	2.055478933	2.17133526
AT5G56350		2.026607846	0.726154616	1.203473136
AT5G23540	RPN11	2.023745509	1.432782778	1.607878941
AT5G58290	RPT3	1.995346957	1.330338697	1.628475181
AT1G67970		1.981985506	1.754339216	1.302780229

AT3G01600		1.971942879	1.541411907	1.399560726
AT5G40580	PBB2	1.968045919	1.845486344	2.189707256
AT5G66140	PAD2	1.957185344	2.144986381	2.260884509
AT4G29040	RPT2a	1.940369314	1.711880361	2.030571886
AT1G23830		1.898254326	1.772931658	2.350326845
AT5G35690		1.866661744	1.560277498	1.605103699
AT2G46750		1.854932062	1.147594288	1.334556274
AT5G24280		1.777175906	1.678329495	1.588709521
AT4G19006	RPN9b	1.758379392	1.292019869	1.538400416
AT4G14800	PBD2	1.758193279	1.999272682	2.122851886
AT1G53850	PAE1	1.750507997	1.53997244	1.832124373
AT1G67250	UMP1A	1.740814162	1.021661386	1.189172204
AT1G16470	PAB1	1.729496355	1.66808992	1.781488114
AT1G77440	PBC2	1.714104994	1.357073809	1.591165664
AT1G64520	RPN12a	1.672749937	0.942666327	-0.875446134
AT5G42790	PAF1	1.660768241	1.224635771	1.541094553
AT4G12120		1.64284616	1.135015235	1.305684178
AT5G55200		1.632893657	0.898091328	0.783626527
AT5G58210		1.618552721	1.899340801	1.677068907
AT2G20580	RPN1a	1.616648141	1.083855134	1.592124832
AT3G13235	DDI1	1.613037714	0.895117903	1.204960215
AT4G31300	PBA1	1.606868549	1.280189837	1.749392025
AT1G30475		1.598496099	2.074694165	1.651622584
AT4G24820	RPN7	1.572818825	0.996980177	1.308818983
AT4G11740		1.572253596	1.319415861	1.673146387
AT3G25545	PIP2	1.561591176	1.143769665	1.505805961
AT1G53750	RPT1a	1.559244975	1.104038891	1.322428571
AT1G20200	RPN3a	1.557497215	0.883060207	1.442842041
AT1G56450	PBG1	1.540484925	1.231047192	1.499743852
AT4G23040		1.472106061	0.753478805	1.036787216
AT2G48020		1.471244568	1.883610637	1.995387992
AT2G26780		1.45178313	1.007989475	1.203005508
AT5G42010		1.447718999	1.393334632	1.33758038
AT3G15180		1.443003539	2.088299234	2.132721364
AT3G51890		1.434187008	1.321205722	1.46975352
AT1G17960		1.430366181	3.358225948	2.405879234
AT4G38630	RPN10	1.426591034	-2.420071116	1.510739051
AT1G79210	PAB2	1.426571867	1.646224352	1.947903622
AT1G47250	PAF2	1.421245924	1.645862474	1.497011967
AT3G57090		1.420584839	0.633197808	0.837136006
AT5G64760	RPN5b	1.385970755	1.404671049	1.366782531

AT2G27020	PAG1	1.385018279	1.667640409	1.823765803
AT3G07640	PIP1	1.325489591	1.16098162	1.123406185
AT1G13060	PBE1	1.307187603	1.224627302	1.502456104
AT3G53970		1.295818539	1.024546607	1.265031349
AT3G56170		1.294844132	1.058046054	1.125757789
AT4G39740		1.291037261	1.049972631	0.71691208
AT3G22110	PAC1	1.285151924	0.79050672	1.207378699
AT1G21720	PBC1	1.282003278	1.214681793	1.429556807
AT3G18860		1.279098481	1.401640075	1.831392151
AT5G09330		1.274218313	0.872835501	0.937238368
AT3G14290	PAE2	1.272480472	1.403152473	1.488909344
AT5G09900	RPN5a	1.259917237	1.103152469	1.29172798
AT3G18940	PBAC2	1.251382325	0.832477861	1.032963328
AT5G26760		1.249509847	0.927244952	0.948395074
AT3G51260	PAD1	1.242867453	1.369383758	1.587775236
AT5G62230		1.241745113	0.758665434	1.308425676
AT1G74650		1.221756653	0.875855175	2.117445765
AT3G22630	PBD1	1.202883978	0.79366194	1.138208541
AT5G43330		1.189137908	0.618243847	0.815979689
AT5G43010	RPT4a	1.145459355	1.246731896	1.457285786
AT1G45000	RPT4b	1.136717374	1.135207925	1.428817118
AT3G60820	PBF1	1.12891904	1.044027015	1.257736963
AT3G03470		1.063544564	1.147894956	1.789374269
AT3G58270		1.056881003	2.171750945	2.308286057
AT2G35720		1.042064604	0.719031791	0.842959481
AT4G28470	RPN1b	1.021923181	0.713505413	0.847106312
AT5G20880		1.021564953	1.086461605	1.14438812
AT2G17190	DSK2A	0.966838057	0.664066127	0.985857485
AT5G05780	RPN8a	0.932634991	0.80546942	1.078881979
AT3G11780		0.921519027	0.595723243	1.237739027
AT5G23490		0.918065938	1.111333195	0.898409631
AT3G21280	UBP7	0.901176213	0.702863082	0.933453529
AT4G15260		0.890447189	0.87233088	0.842800574
AT1G51710	UBP6	0.83938694	0.637200422	1.030675268
AT2G18600		0.838992205	1.573898402	1.347659831
AT4G17510	UCH3	0.838605174	0.581138205	0.702300763
AT5G16880		0.835094354	0.595994709	0.737588808
AT1G61560		0.832579274	1.127645775	1.006455945
AT2G39650		0.824469261	0.819867923	1.098260754
AT2G34840		0.803191434	0.675165688	0.766677598
AT1G52880		0.745073393	0.826541268	1.030286673

AT2G41040		0.744593708	0.895578838	0.942066408
AT2G20140	RPT2b	0.7222181	0.56738535	0.738980677
AT5G35590	PAA1	0.669318895	1.069385145	1.031992668
AT1G04850		0.662767847	0.750828789	0.93714941
AT1G36180		0.64918902	4.880989737	4.843373295
AT5G27920		0.634681244	0.648474666	0.756156773
AT5G47070		0.561775432	0.962091963	0.821698524
AT1G04990		0.551764674	0.908057375	0.98999141
AT5G39970		0.532495763	0.907901984	0.74706516
AT3G10660		0.512398054	0.950448679	0.983272237
AT2G41640		0.488751558	1.396571585	0.830010303
AT5G40670		-0.4216201	-0.728645145	-0.653012827
AT5G42810		-0.449767905	-0.785048863	-0.571693138
AT1G56020		-0.49473299	-1.405872204	-0.724951594
AT4G37790		-0.513198671	-0.544986903	-0.606399568
AT5G26980		-0.5321612	-2.193400108	-1.670280576
AT5G36120		-0.532514733	-1.627048869	-1.582340943
AT4G04955		-0.555436075	-0.52280963	-0.910830163
AT1G66330		-0.560804571	-1.197417321	-1.149936407
AT5G41260		-0.611156003	-0.856181218	-0.7641322
AT1G78130		-0.613910954	-1.11769659	-0.969338683
AT1G64060		-0.631972739	-0.874693166	-0.651531876
AT1G52260		-0.645674649	-2.001251361	-0.896276507
AT1G60260		-0.650716456	-0.806786316	-2.309684048
AT1G63800	UBC5	-0.675562391	-0.930567425	-1.081793816
AT5G26670		-0.688334897	-1.223364107	-1.116843532
AT4G00880		-0.705066835	-1.457569068	-1.029307387
AT1G72820		-0.713892176	-1.138888976	-1.134993604
AT1G55370		-0.749489789	-1.890154518	-1.916840178
AT4G28250		-0.751101682	-1.268884589	-0.866764626
AT5G38150		-0.762287839	-1.245555357	-1.993724127
AT1G71020		-0.765695172	-0.947859814	-0.871288807
AT5G48850		-0.779672826	-1.839554095	-2.856866306
AT1G60010		-0.784656233	-0.648066348	-0.649577076
AT5G43150		-0.80443004	-2.485352816	-2.362976999
AT1G06830		-0.813844479	-1.086053134	-1.214144927
AT1G49500		-0.821884565	-1.109702442	-0.94959663
AT4G11320		-0.841736239	-2.663810556	-1.394708108
AT1G51400		-0.871842231	-1.197236228	-1.051525461
AT4G12980		-0.891967803	-1.549449474	-1.03333897
AT3G58850		-0.913556246	-1.111941268	-0.856646419

AT2G01830		-0.996616251	-1.040078767	-0.788886584
AT1G68740		-1.084015766	-0.911979399	-0.940438554
AT4G03960		-1.098103862	-1.303090778	-1.467541609
AT1G52290		-1.123690715	-2.110573328	-1.632149234
AT1G70850		-1.129748886	-3.971643018	-1.147440136
AT1G73330		-1.137731911	-5.694848757	-2.426288012
AT1G18870		-1.463839069	-1.388473102	-1.664363092
AT3G48100		-1.59654493	-0.920888338	-0.836010672
AT4G04840		-1.68097873	-1.655860915	-2.30616392
AT3G45070		-1.83170245	-2.0366258	-1.50335132
AT5G41080		-2.074231659	-1.175319518	-1.348777291

It should be noted that the technical aspect of RNAseq transcript level normalization can explain some of the paralog expression discrepancies I observe: transcript levels are normalized to a control. For example, one gene may have a higher WT basal level of expression compared to another gene. A similar change in absolute transcript levels (e.g. reads per million sequenced) for the gene with a lower level of basal expression will result in a greater fold-change difference compared to the same change in absolute transcript levels for the gene with higher basal expression levels. To account for this technical issue, I obtained the expressed sequence tags (ESTs) for each proteasome subunit and compared them to paralog transcript level regulation (**Figure 2-3c**). I observed that some differential expression could potentially be explained by the fact that one subunit paralog has higher basal expression levels compared to the sister paralog (e.g. *PAA1* vs. *PAA2* and *PBB1* vs. *PBB2*), but this still does not explain all paralog expression discrepancies.

An examination of other UPS pathway components revealed that many genes are differentially expressed in one or multiple conditions, but are not consistently up- or down-regulated in all conditions, which would result with their inclusion in the PSIR. Looking at the ubiquitylation pathway, several Ub genes are up-regulated (*AT4G05320*, *AT4G05050*, *AT4G02890*, *AT5G03240*, and *AT5G20620*), primarily upon 3 hr MG132 treatment, but none are consistently over-expressed in all conditions. One E2 Ub-activating enzyme (UBC5) is significantly down-regulated in all conditions. There are several more up-regulated UBCs, but they are not consistently differentially expressed across all conditions. A handful of E3 ligases (*AIP2*, *AT1G71020, AT4G27050,* and *AT5G27920*) are up-regulated and are part of the PSIR. Finally, most proteasome assembly chaperones (*PA200, ECM29 NAS6, HSM3*, *PIP1, PIP2, PBAC2, PBAC3,* and *UMP1a)*, some Ub-binding proteins (*DSK2a, DDI1,* and *UCH3*), and a few DUBs (*UBP6, UBP7,* and *UCH3*) are all up-regulated during proteasome stress and are included in the PSIR (**Table 2-1**).

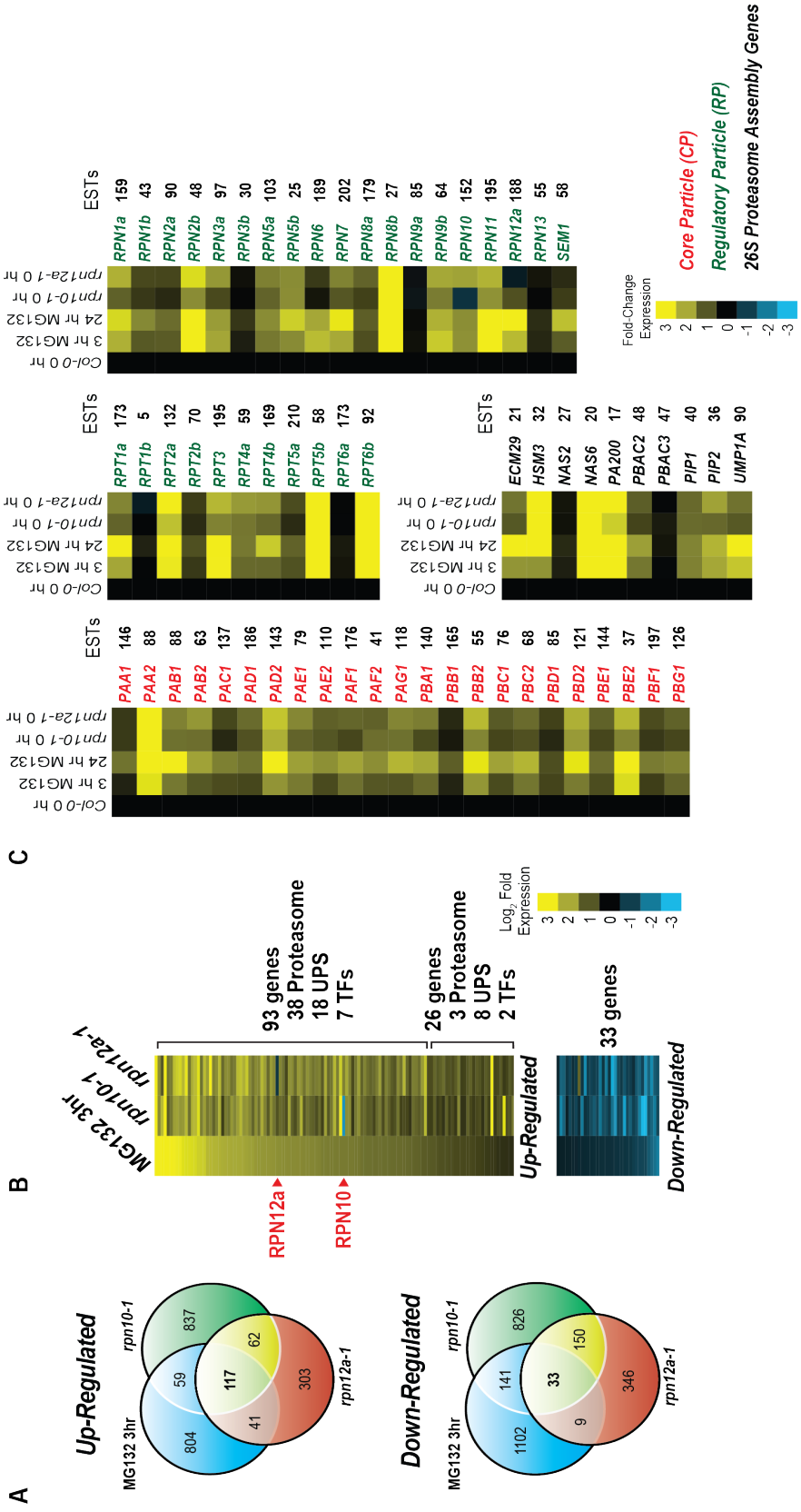
Figure 2-3. The Proteasome Stress-Induced Regulon (PSIR)

A. Venn diagrams of significantly differentially expressed (p-value < 0.01, FDR < 0.05) gene transcripts under 3 different proteasome-stress conditions, based on edgeR analysis: Col-0 after 3 hr MG132 treatment, and the *rpn10-1* and *rpn12a-1* genetic backgrounds. The shared 117 up-regulated and 33 down-regulated genes comprise the PSIR.

B. A heat map of the PSIR ordered by the fold-change expression from the 3 hr MG132 treatment. The brackets in the up-regulated suite delineate the heat map into genes that are expressed more or less than 2-fold (log2(fold-change) = 1) compared to WT, with breakdowns of important genes contained within (TFs = transcription factors).

C. Expression heat maps of 26S proteasome subunit and assembly factor genes from RNAseq analysis normalized to untreated Col-0. Expressed sequence tags (ESTs) were obtained from The *Arabidopsis* Information Resource (TAIR v.10).

Figure 2-3. The Proteasome Stress-Induced Regulon (PSIR)



Turning to the other major cellular proteolytic pathway, only the autophagy gene *ATG8b,* a Ub-fold protein, is significantly up-regulated under all proteasome-limiting conditions (**Figure 2-4**). However *NBR1*, a Ub-binding protein and facilitator of selective autophagy (Kraft et al. 2010), is up-regulated in all conditions except the *rpn10-1* background. Some autophagy genes show significant up- or down-regulation during some stress conditions, but not in others (*e.g. ATG8c, ATG8f, ATG13b* and *ATG18a*), suggesting that only a small subset of autophagy genes could play an important role in proteasome-stress response, or are required for increasing autophagic activity to compensate for decreased proteasome capacity (Li et al. 2012, Liu et al. 2012, Feng et al. 2014).

Looking at stress-responsive DNA-binding proteins, several *WRKY* and *NAC* transcription factor family members displayed transcriptional up-regulation, with *WRKY25, NAC013, NAC018, NAC044,* and *NAC082* being the only members of their respective families to be significantly up-regulated during all experimental conditions compared to WT (**Figure 2-4**). Markedly, the proteasome gene-activating transcription factor *NAC78* is only up-regulated during MG132 treatment, but not in the proteasome mutant backgrounds; this is similar to Nrf1 in mammals, which is also up-regulated during exposure to MG132 in mammalian cells (Sha et al. 2014). A C2H2-like zinc-finger protein (AT4G15420) was up-regulated in all treatments, and although this protein has not been functionally characterized, it has been previously shown to be strongly up-regulated upon heat-shock treatments (Charng et al. 2007).

Other stress responsive genes are also up-regulated. The heat-shock transcription factor HSFA8 and the heat-shock protein (HSP) HSP23.5-M are included in the PSIR. Many other HSPs are up-regulated only during MG132 treatment, including the mis-folded response protein HSP101, as well as several other HSP70 and HSP90 family proteins. Several glutathione-S-transferases (GSTUs) are also up-regulated during proteasome stress. GSTUs have been implicated in re-forming the 26S proteasome after the RP and CP disassociate during oxidative stress or MG132 treatment (Livnat-Levanon et al. 2014).

To observe what, if any, impact that proteasome stress could have on interpreting transcriptional or translational analyses, “housekeeping” genes that are commonly used for qRT-PCR and immunoblot normalization (loading controls) were scrutinized for any significant deviation in expression during MG132 treatment or in proteasome mutant backgrounds. *ACT2, PP2a,* and *TUB2* displayed no significant differential regulation between experimental conditions (**Figure 2-5**), and although *ACT2* does show noticeable down-regulation, it is still not significant ($p > 0.05$). However, when analyzing histone family transcripts, H2B and H4, but not H3.1, showed significantly detectable down-regulation in at least one experimental condition, which agrees with previous research that found decreases in those gene product levels during proteasome stress (Lee et al. 2011).

The MG132-induced Regulatory Gene Network

To better visualize the MG132-stress induced regulon, all available interaction and co-expression data was collected from the STRING database (Jensen et al. 2009) for genes displaying a $2 \geq$ fold-change to WT in the 3 hr MG132 treatment, then mapped out using Cytoscape v3.0.1 (**Figure 2-6**) (Shannon et al. 2003). Of these 336 genes that were input into STRING, only 275 had available interaction/co-expression data. There are three major regions in the co-expressome/interactome where gene nodes are cluster together: (i) a tightly-mapped section containing the majority of 26S proteasome subunits and interacting/assembling factors, (ii) a larger node cluster that is significantly enriched for HSPs and other mis-folded protein response factors, and (iii) a more diffuse grouping that is enriched for detoxification proteins (e.g. GSTU, O-methyltransferases, *etc.*) (**Figure 2-6a**).

Figure 2-4. Expression Heat Maps of PSIR and PSIR-related Genes

Log_2(fold-change) expression heat maps of selected PSIR genes during MG132, *rpn10*-1, and *rpn12a*-1 conditions compared to WT. Several *NAC* and *WRKY* transcription factors are up-regulated in at least one experimental condition, including NAC13 and NAC78. Other UPS and autophagy genes are also differentially expressed.

Figure 2-4. Expression Heat Maps of PSIR and PSIR-related Genes

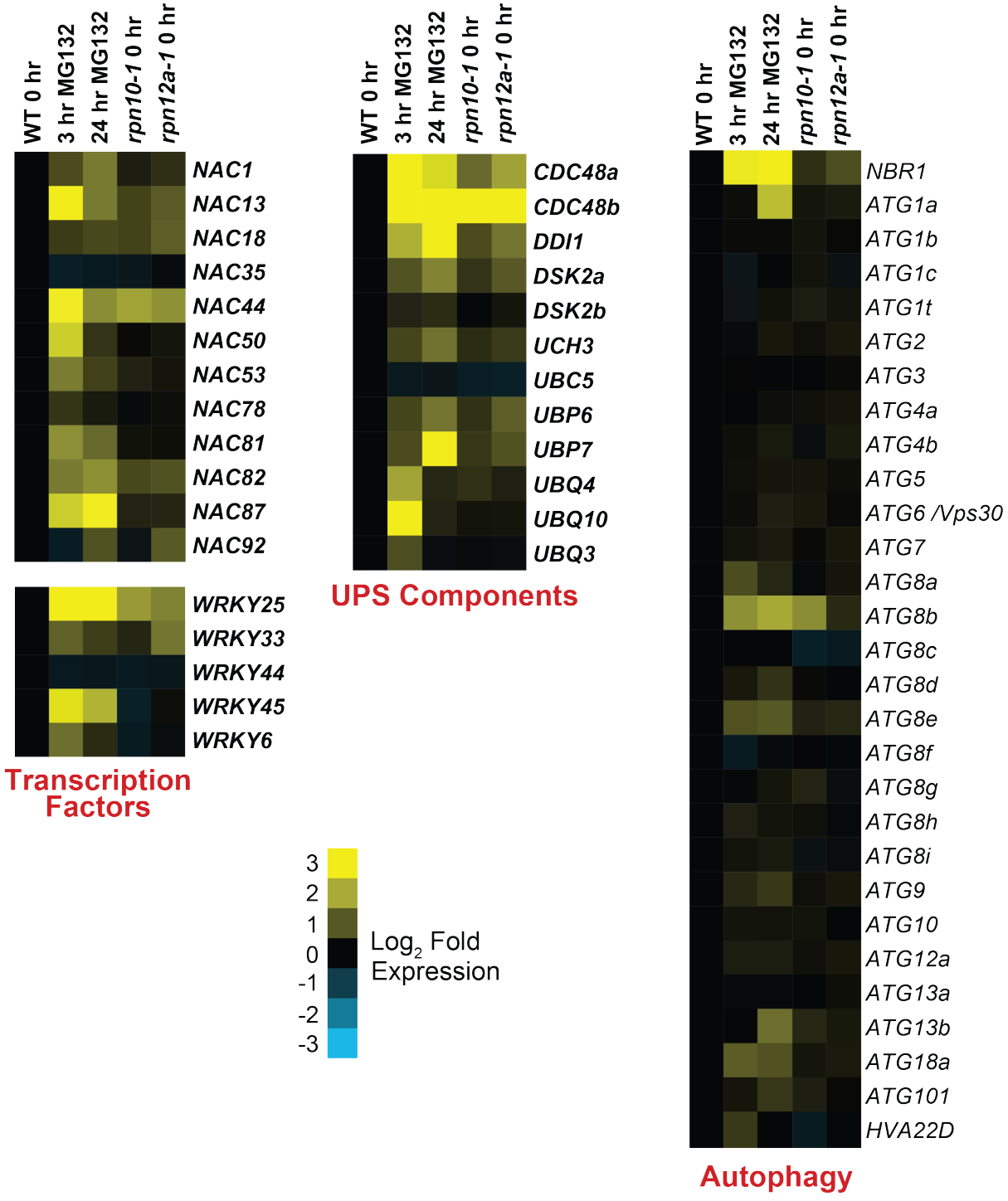


Figure 2-5. **"Housekeeping" Gene Expression During Proteasome Stress**

Fold-change expression of genes commonly used for normalization in qRT-PCR and protein immunoblots (loading controls). Some histone genes show significant down-regulation compared to the Col-0 No DMSO treatment control (asterisks = p-value <0.01). H3 is significantly down-regulated (p-value = 0.008) in the *rpn12a-1* background, but is above the FDR <0.05 cutoff.

Figure 2-5. "Housekeeping" Gene Expression During Proteasome Stress

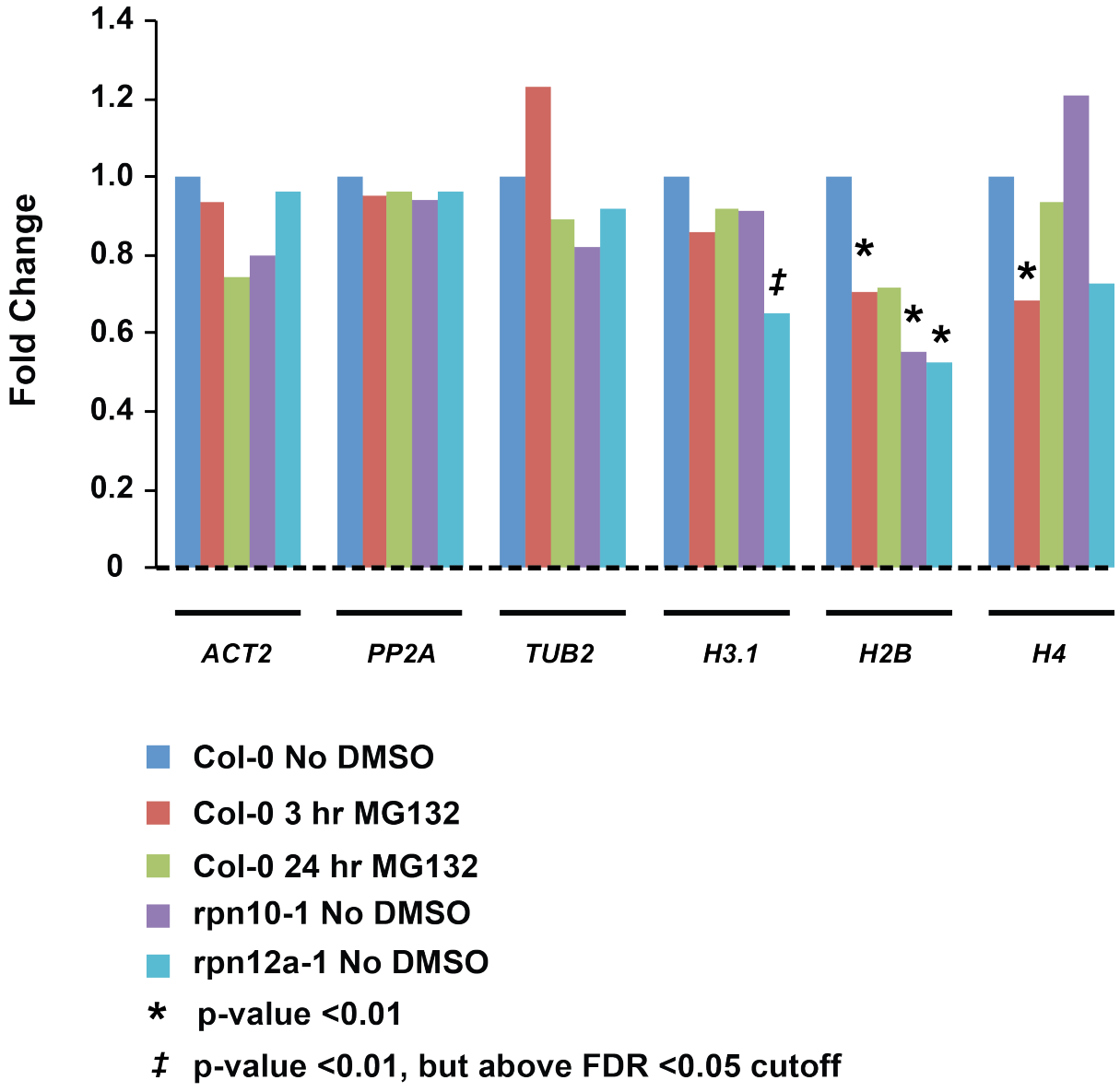
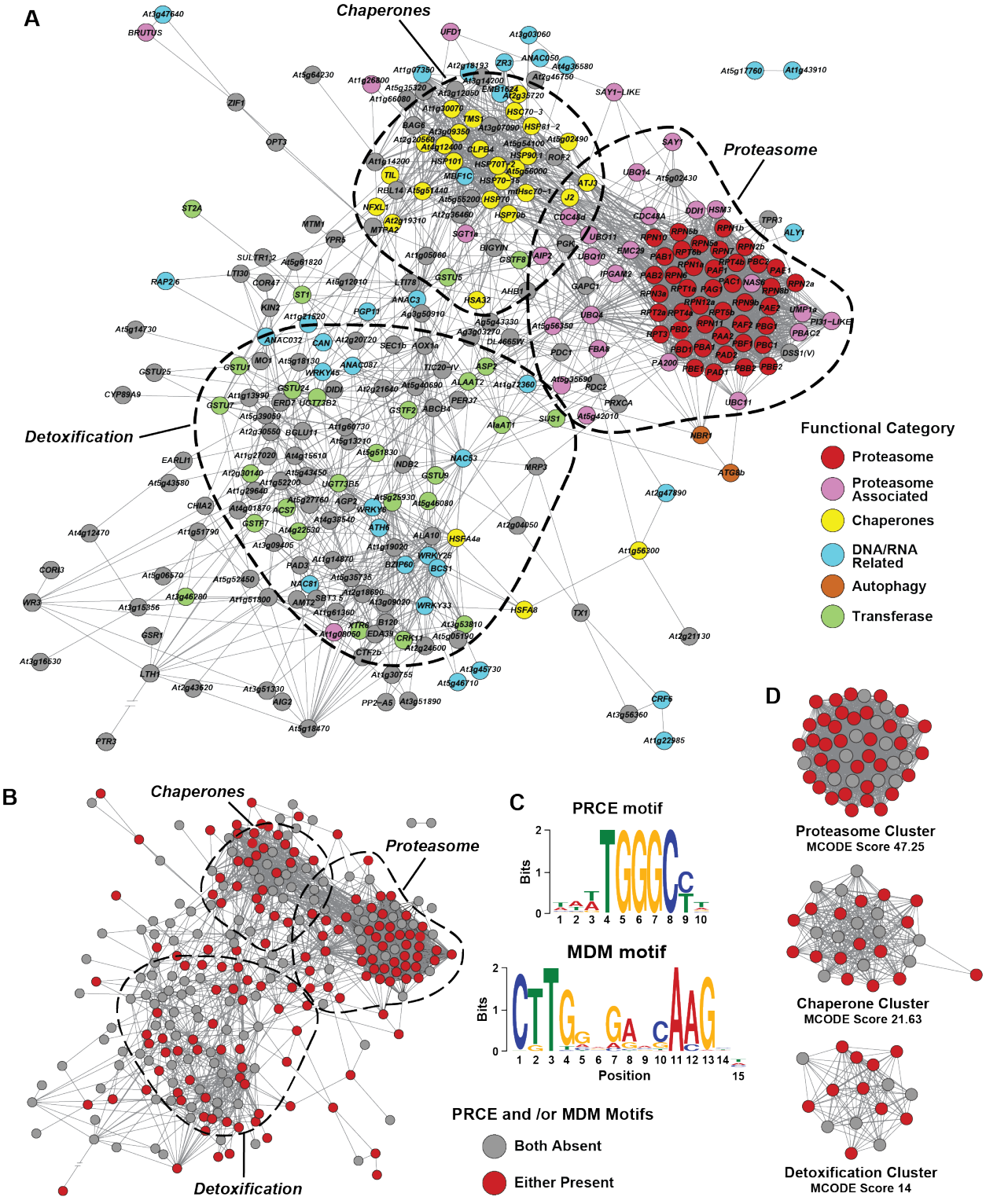


Figure 2-6. The 3 hr MG132 Up-regulated Gene Interaction and Co-expression Network

A. An interaction and co-expression map of proteins encoded by up-regulated genes after 3 hr of MG132 treatment**.** The connections reflect known protein/protein interactions and co-expression data collected from the STRING database for the available 275 of the total 336 significantly up-regulated genes (≥ 2-fold expression) in Col-0 after 3 hr of MG132 treatment. Classifications of major functional groups in the network are highlighted. Portions of the map enclosed by the dashed lines are statistically enriched for genes with the denoted functional categories (DAVID $p < 0.01$).
B. Members of the network that contain the consensus PRCE or MDM *cis* motifs within their promoter regions (motif-containing gene nodes are in red).
C. Sequence descriptions of the PRCE and MDM sequences as determined by MEME.
D. Interactome maps focusing on the statistically significant central hubs present in the Proteasome, Chaperone, and Detoxification clusters as determined by MCODE analysis, and colored for the presence of PRCE or MDM motifs.

Figure 2-6. The 3 hr MG132 Up-regulated Gene Interaction and Co-expression Network



Comparing the 3hr MG132 up-regulated transcripts to other stress-responsive regulatory networks indicates potential overlap of key environmental and developmental systems. A MEME analysis of the 336 genes in the 3 hr MG132 regulon showed an enrichment for both the PRCE, which NAC78 recognizes, and the mitochondrial dysfunction motif (MDM), which NAC13 acts upon during oxidative stress (**Figure 2-6b,c,d**). Both motifs are also heavily enriched in 26S proteasome genes (**Figure 2-7**). The overlap between the oxidative-stress and proteasome-stress response networks has also been observed in mammalian cells, where mitochondrial disruption and MG132 treatment have the same effects on 26S proteasome stability and expression (Kwak et al. 2003, Radhakrishnan et al. 2010, Livnat-Levanon et al. 2014).

DNA-binding Motif Composition of the 26S Proteasome and Proteasome-stress Regulon Genes

While there is a general increase in proteasome subunits transcripts and proteins during stress, the asymmetrical expression of subunit paralogs suggests the possibility for multiple *trans*-acting factors to activate/repress proteasome and other UPS genes during a variety of cellular conditions. However, there appears to be no pattern of motif presence or absence to adequately explain the conservation of asymmetrical expression we observe during proteasome stress: most CP subunits contain either the PRCE or MDM sequences, indicating the potential for regulation by some NAC family proteins like NAC78 and NAC13, respectively (**Figure 2-7**) (De Clercq et al. 2013, Nguyen et al. 2013).

To provide a more complete look at predicted and experimentally verified DNA-recognition sequences that exist in the upstream regions of PSIR and proteasome subunit genes, I performed an enrichment analysis of the AGRIS *cis*-regulatory element database (ATcidDB) and found a significant enrichment (p-value < 0.05) for several known DNA-binding motifs in the upstream regions of both PSIR and 26S proteasome genes, as well for the up-regulated genes from the 3 hr MG132 treatment (**Tables 2-2, 2-3, and 2-4**). Two of the most

interesting identified motifs are the light-induced-response recognition sequences SORLIP2 and T-box. Both occur in roughly half of all proteasome subunit genes (**Figure 2-8**) (Chan et al. 2001, Hudson et al. 2003). Within the PSIR, SORLIP2 is also significantly abundant. The RAV1-B and CArG motifs are also enriched, which are recognized by integrase-type (abscisic acid response) and MADS-domain DNA-binding proteins, respectively (Kagaya et al. 1999, Hepworth et al. 2002, de Folter et al. 2006). The enriched motifs in the 3 hr MG132 up-regulated gene set are unsurprisingly similar to those of the PSIR and 26S proteasome, but there are some unique stress-responsive motifs as well: the WKRY recognition sequence (W-box); ABRE-like (abscisic acid response element); HSEs-binding (heat-shock element); and DRE-like (drought-response element) motifs are also very prominent. Overlaying the SORLIP2, T-BOX, PRCE, and MDM motifs onto proteasome genes reveals that all but 3 subunits contain at least one of these sequences: *PAB2*, *RPN3a*, and *RPN11* (**Figure 2-9**). These 4 motifs, which can be recognized by several different DNA-binding proteins, continue to support the notion that the proteasome, and likely other segments of the UPS, can be flexibly regulated under different cellular conditions by multiple transcription factors.

Trans-Acting Factors of Proteasome Gene Expression

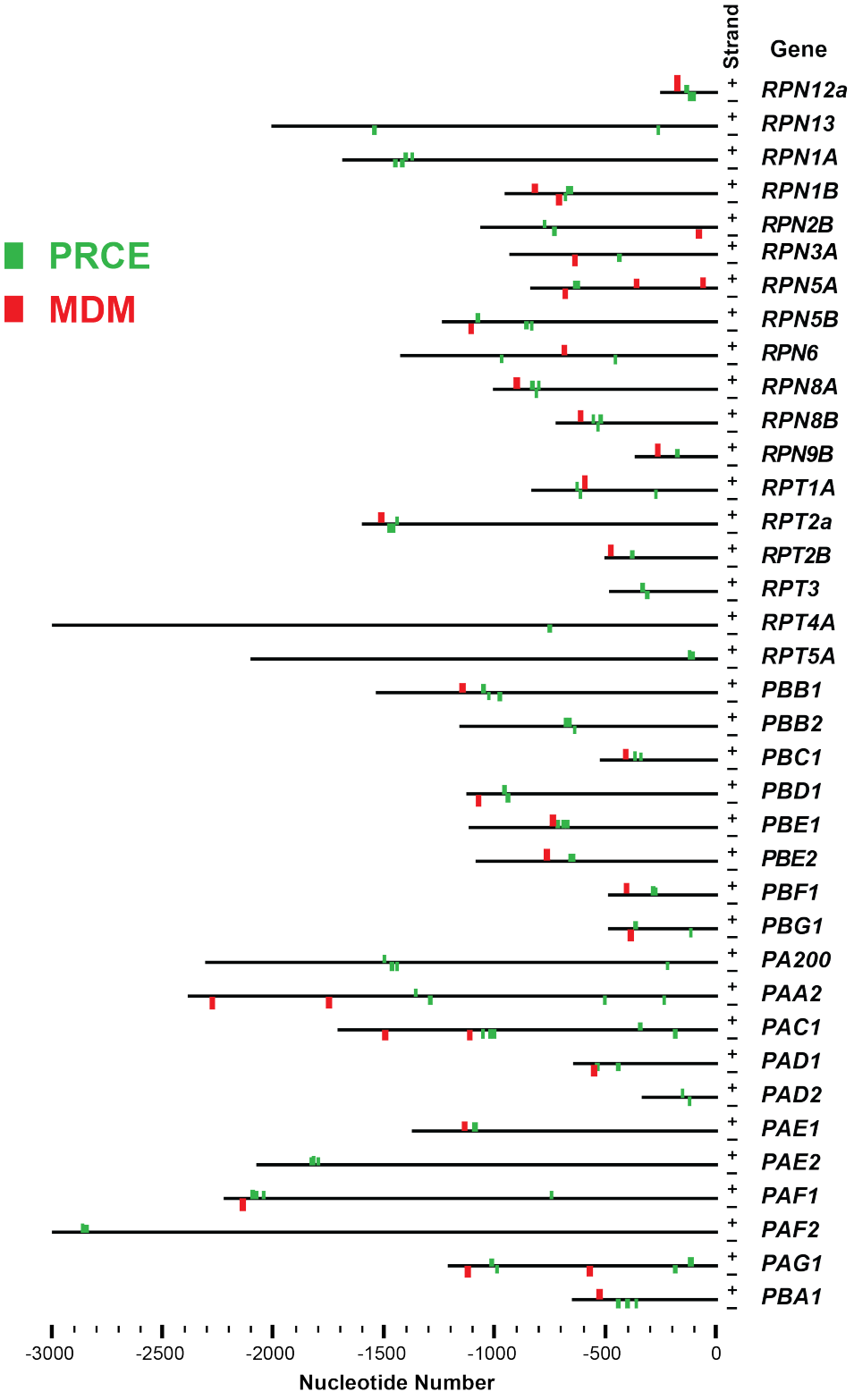
While NAC78 has been shown to activate proteasome subunit expression, I wanted to identify other possible *trans*-acting factors that could recognize and induce proteasome expression. To do this, a directed-yeast-1 hybrid (Y1H) screen was performed testing >1700 *Arabidopsis* transcription factors one-at-a-time (prey) with the upstream DNA sequences of *PA200* and *RPN12a* (bait) to drive luciferase (*LUC*) reporter gene expression (Gaudinier et al. 2011). Several transcription factors were observed binding to one or both promoter fragments and activating *LUC* reporter expression to a significant degree, including the NAC family proteins NAC78, NAC53, and NAC008, several integrase-type DNA binding proteins, and basic helix-loop-helix (bHLH) family proteins (**Table 2-5**). Many of these transcription factors have the

potential to recognize one or more of the motifs identified in the ATcisDB enrichment analysis, indicating that many proteasome gene expression regulators could exist beyond the previously described NAC78.

Figure 2-7. PRCE and MDM Occurrence in the Upstream Regions of 26S Proteasome Genes

Promoter profiles of 26S proteasome subunit genes that show the 5' upstream regions, beginning at the translational start site (nucleotide number = 0) and extending to the translational stop site of the neighboring upstream gene. Green (PRCE) and red (MDM) boxes indicate the promoter position of the stress-response recognition motifs as predicted by MEME analysis. Many PRCE and MDM motifs occur close to each other and often in tandem.

Figure 2-7. PRCE and MDM Occurrence in the Upstream Regions of 26S Proteasome Genes



Tables 2-2, 2-3, and 2-4

Motif enrichment in *Arabidopsis* 26S proteasome genes, the PSIR, and the 3 hr MG132 up-regulated gene set.

A. Binding site motif name as listed in the AGRIS database.
B. Binding motif sequence as defined within the AGRIS database.
C. Motif occurrence in either the 26S proteasome subunit genes, the PSIR, or the 3 hr MG132 up-regulated gene set
D. Motif Occurrence in the whole *Arabidopsis* genome as predicted by AGRIS.
E. p-value of the corresponding motifs indicating significance of enrichment in either the 26S proteasome, PSIR, or 3 hr MG132 up-regulated gene set as compared to the whole genome based on hypergeometric distribution (Scipy).

Table 2-2. Motif Occurrence in 26S Proteasome Subunit Genes

A BINDING MOTIF NAME	B BINDING MOTIF SEQUENCE	C PROTEASOME OCCURRENCE	D AGRIS OCCURRENCE	E p-value
SORLIP2	GGGCC	53	18041	0.0008
T-BOX	ACTTTG	82	31364	0.0016
OBP-1-4-5 in GST6	TACACTTTTGG	1	35	0.0020
AG in SUP	CCATTTTTGG	2	224	0.0091
AP1 in SUP	CCATTTTTGG	2	224	0.0091
E2F-variant	TTTCCCGC	1	84	0.0112
CArG	CC(A/T)(A/T)(A/T)(A/T)(A/T)(A/T)GG	18	5703	0.0141
CCA1 motif 1 in CAB1	AAACAATCTA	2	229	0.0196
RAV1-B	CACCTG	16	5248	0.0245
MYB1	(A/C)TCC(A/T)ACC	9	2584	0.0272
LTRE	ACCGACA	7	1979	0.0365
MYB2	TAACT(G/C)GTT	3	669	0.0392

Table 2-3. Motif Occurrence in Proteasome-Stress Response Regulon

BINDING MOTIF NAME	BINDING MOTIF SEQUENCE	PSIR OCCURRENCE	AGRIS OCCURRENCE	p-value
SORLIP2	GGGCC	122	18041	2.12 E-05
RAV1-B	CACCTG	36	5248	0.00904
HSEs	AGAANNTTCT	26	3570	0.01030
OBP-1-4-5 in GST6	TACACTTTTGG	1	35	0.01146
CArG	CC(A/T)(A/T)(A/T)(A/T)(A/T)(A/T)GG	36	5703	0.02779

Table 2-4. Motif Occurrence in 3 hr MG132 Up-regulated Gene Set (336 genes)

BINDING MOTIF NAME	BINDING MOTIF SEQUENCE	REGULON OCCURRENCE	AGRIS OCCURRENCE	p-value
OBF4-5 BS in GST6	ATCTTATGTCATTGATGACGACCTCC	1	1	0
W-box	TTGAC	755	44377	1.37E-06
DRE-like	(A/G/T)(A/G)CCGACN(A/T)	112	5034	4.84E-06
HSEs binding site	AGAANNTTCT	76	3570	0.0004
T-box	ACTTTG	520	31364	0.0005
CArG	CC(A/T)(A/T)(A/T)(A/T)(A/T)(A/T)GG	112	5703	0.0006
AGL15 in AtGA2ox6	CCAATTTAATGG	1	8	0.0055
ABRE-like	(C/G/T)ACGTG(G/T)(A/C)	182	10757	0.0134
OBP-1-4-5 in GST6	TACACTTTTGG	2	35	0.0138
Z-box	ATACGTGT	23	1067	0.0236
SORLIP2	GGGCC	290	18041	0.0266
LFY in AP3	CTTAAACCCTAGGGGTAAT	683	44441	0.0359
DREB1&2 in rd29a	TACCGACAT	4	129	0.0394
AP1 in SUP	CCATTTTTGG	6	224	0.0447
AG in SUP	CCATTTTTGG	6	224	0.0447

Figure 2-8. Occurrence of Enriched Motifs in Proteasome Genes

Color map diagrams of the 26S proteasome, and PA200, displaying the occurrence of significantly enriched motifs found through MEME or AGRIS enrichment analysis. Colorization indicates the presence of the SORLIP2 (orange), T-box (blue), PRCE (green), or MDM (yellow) sequences in the 5' upstream DNA regions of indicated subunit genes.

Figure 2-8. Occurrence of Enriched Motifs in Proteasome Genes

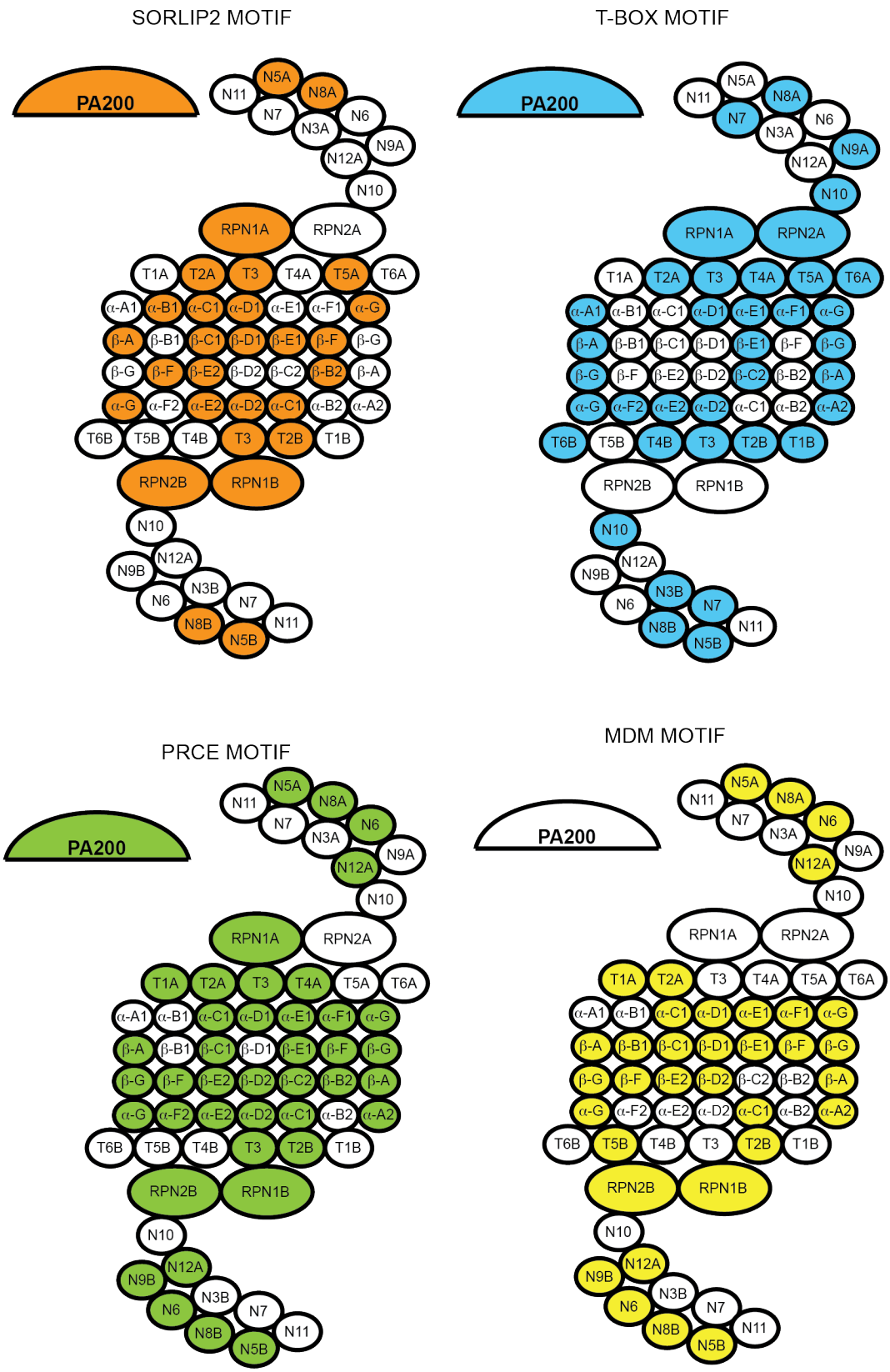


Figure 2-9. Occurrence of the SORLIP2, T-box, PRCE, and MDM Sequences in Proteasome Genes

Color map diagram of the 26S proteasome, and PA200, displaying the occurrence of significantly enriched motifs found through MEME or AGRIS enrichment analysis. Red coloration indicates the presence of one or more of the SORPLIP2, T-box, PRCE, and MDM sequences in the upstream DNA regions of subunit genes. All proteasome genes contain at least one of these DNA-binding motifs, except for *PAB2*, *RPN3a*, and *RPN11*.

Figure 2-9. Occurrence of the SORLIP2, T-box, PRCE, and MDM Sequences in Proteasome Genes

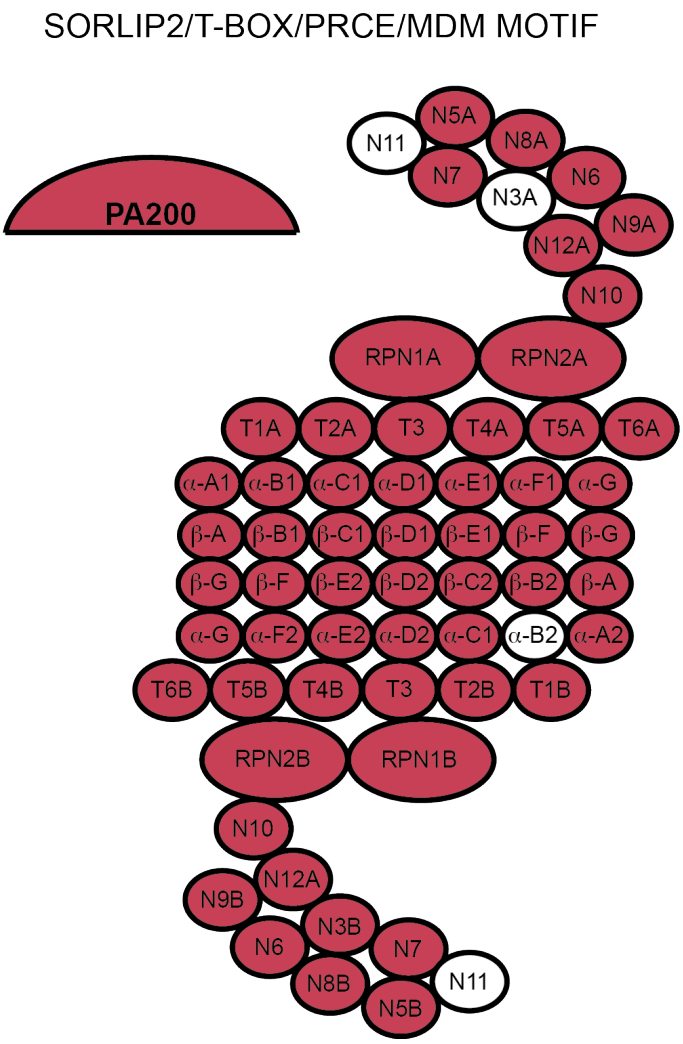


Table 2-5. Proteasome DNA-binding Proteins as Predicted by a Directed-Y1H Screen

A list of *Arabidopsis* DNA-binding proteins (out of a total of 1700 tested) that can recognize proteasome promoters (*PA200* or *RPN12a*) and activate expression of the downstream *LUC* reporter gene in yeast (Y1H). The table contains the AGI of each DNA-binding protein, their transcription factor family, and the relative fold-change in expression of the luciferase reporter gene normalized to the vector-only control. Ranking is by fold-change expression. Transcription factors highlighted in red are shared between both *RPN12a* and *PA200* screens.

Bait: *RPN12a* full-length upstream sequence

AGI	Family	Reporter Expression
AT3G04280	**Orphans a**	**14.38081333**
AT5G18240	G2-like a,b	7.24528891
AT3G16857	**ARR-B a,b**	**6.734748392**
AT5G65130	**AP2-EREBP a,b,c**	**5.217578603**
AT3G07650	C2C2-CO-like a,b	4.744940766
AT3G11100	TRIHELIX a,b	4.720727195
AT1G75490	AP2-EREBP a,b,c	4.699297661
AT1G72570	**AP2-EREBP a,b,c**	**4.204852806**
AT5G61590	**AP2-EREBP a,b,c**	**4.096904262**
AT3G16770	**AP2-EREBP a,b,c**	**4.042367385**
AT1G10120	**bHLH a,b**	**3.947894023**
AT3G23220	AP2-EREBP a,b,c	3.908685485
AT4G33280	**ABI3-VP1 a,b**	**3.836985451**
AT4G00390	GeBP a,b	3.818736788
AT3G10500	**NAC a,b**	**3.541223577**
AT1G28050	C2C2-CO-like a,b	3.532280684
AT1G25580	**NAC a,b**	**3.479881721**
AT5G66350	SRS a,b	3.46744359
AT3G23240	AP2-EREBP a,b,c	3.255658945
AT5G05410	AP2-EREBP a,b,c	3.122737406
AT3G59060	bHLH a,b	3.09905875
AT1G64620	C2C2-DOF a,b,c	3.092035836
AT3G22830	HSF a,b,c	2.994514171
AT1G44830	AP2-EREBP a,b,c	2.860037855
AT4G00270	GeBP a,b	2.823318331
AT1G47870	E2F-DP a,b,c	2.777354433
AT4G14410	bHLH a,b	2.756983358

AT5G06950	bZIP a,b,c	2.750812765
AT1G51140	bHLH a,b	2.73834602
AT1G79430	G2-like a,b	2.662161688
AT2G46680	HB a,b,c	2.622548476
AT4G35040	bZIP a,b,c	2.622123542
AT3G61630	AP2-EREBP a,b,c	2.598997594
AT1G35460	bHLH a,b	2.583271974
AT4G25210	GeBP a,b	2.557612522
AT1G36060	AP2-EREBP a,b,c	2.518899187
AT4G01680	MYB a,b	2.487758436
AT1G28470	NAC a,b	2.484653628
AT5G04410	NAC a,b	2.479226192
AT4G00238	**GeBP a,b**	**2.471372055**
AT3G47500	C2C2-DOF a,b,c	2.453471519
AT5G26650	MADS a,b	2.402309078
AT5G63470	CCAAT a / CCAAT-HAP5 b	2.378408314
AT1G09530	bHLH a,b	2.343812989
AT1G14580	C2H2 a,b,c	2.289511419
AT4G13640	G2-like a,b	2.234049595
AT2G20180	bHLH a,b	2.155617192
AT3G02990	HSF a,b,c	2.116042161
AT2G23660	LOB a / AS2 b	2.100195478
AT4G37750	AP2-EREBP a,b,c	2.09204176
AT2G22200	AP2-EREBP a,b,c	2.085792906
AT3G57800	bHLH a,b	2.084384564
AT5G18450	**AP2-EREBP a,b,c**	**2.060421477**
AT1G04370	AP2-EREBP a,b,c	2.030101869
AT4G29930	**bHLH a,b**	**2.030006404**
AT4G35580	NAC a,b	2.024268006
AT3G04930	GeBP a,b	2.001760784
AT3G24120	G2-like a,b	1.945935647
AT3G57600	AP2-EREBP a,b,c	1.932916645
AT5G01380	TRIHELIX a,b	1.924370369
AT1G77640	AP2-EREBP a,b,c	1.92323714
AT2G38340	AP2-EREBP a,b,c	1.914525152
AT4G38900	bZIP a,b,c	1.893032683
AT3G53200	MYB a,b	1.890772079
AT1G22190	**AP2-EREBP a,b,c**	**1.845293165**
AT5G60440	MADS a,b	1.831827194
AT1G57560	MYB a,b	1.78759265

AT2G20880	AP2-EREBP a,b,c	1.780895109
AT4G38000	C2C2-DOF a,b,c	1.69860317
AT5G61600	AP2-EREBP a,b,c	1.695025979
AT1G28160	AP2-EREBP a,b,c	1.679081695
AT1G13600	bZIP a,b,c	1.623145245
AT1G08000	C2C2-GATA a,b	1.598036911
AT3G28910	MYB a,b	1.590230177
AT1G51600	C2C2-GATA a / ZIM b / CCT c	1.578319273
AT5G21960	AP2-EREBP a,b,c	1.569315006
AT3G28920	zf-HD a,b	1.561255132

Bait: *PA200* 1 Kb upstream sequence (from transcriptional start site)

AGI	**Family**	**Reporter Expression**
AT1G66600	WRKY a,b,c	41.13129512
AT4G23810	WRKY a,b,c	15.27459577
AT4G11070	WRKY a,b,c	14.21044546
AT3G21810	C3H a,b	9.812754851
AT4G18170	WRKY a,b,c	7.616689279
AT1G66550	WRKY a,b,c	6.644891074
AT2G23320	WRKY a,b,c	6.48637965
AT1G13960	WRKY a,b,c	5.350302378
AT2G24430	NAC a,b	5.139007308
AT1G29860	WRKY a,b,c	4.5746772
AT5G13080	WRKY a,b,c	3.911790628
AT3G10500	**NAC a,b**	**3.890071081**
AT3G04280	**Orphans a**	**3.863376922**
AT3G04450	G2-like a,b	3.776811966
AT1G10120	**bHLH a,b**	**3.584313553**
AT3G16770	**AP2-EREBP a,b,c**	**3.55280429**
AT4G17980	NAC a,b	3.234992615
AT2G30590	WRKY a,b	3.214619364
AT5G61590	**AP2-EREBP a,b,c**	**3.12413481**
AT4G01250	WRKY a,b,c	3.064781052
AT3G04670	WRKY a,b,c	3.028131523
AT5G53950	NAC a,b	2.922644122
AT3G16857	**ARR-B a,b**	**2.908031055**
AT5G46350	WRKY a,b,c	2.865694739
AT5G66730	C2H2 a,b	2.677647289
AT1G22190	**AP2-EREBP a,b,c**	**2.604332542**
AT3G01970	WRKY a,b,c	2.587450528

AT1G34180	NAC a,b	2.545390562
AT5G65130	**AP2-EREBP a,b,c**	**2.503975982**
AT5G07100	WRKY a,b,c	2.384496974
AT1G25580	**NAC a,b**	**2.304081285**
AT5G18450	**AP2-EREBP a,b,c**	**2.299159613**
AT2G03340	WRKY a,b,c	2.163935291
AT1G72570	**AP2-EREBP a,b,c**	**2.157967474**
AT1G20980	SBP a,b,c	2.147606893
AT5G57390	AP2-EREBP a,b,c	2.08777088
AT5G52830	WRKY a,b,c	1.998630171
AT1G78080	AP2-EREBP a,b,c	1.990339883
AT5G54230	MYB a,b	1.989078477
AT1G32640	bHLH a,b	1.971750141
AT5G12330	SRS a,b	1.948105361
AT3G13840	GRAS a,b	1.929173697
AT3G50700	C2H2 a,b,c	1.928115592
AT2G44745	WRKY a,b,c	1.863263222
AT2G38470	WRKY a,b,c	1.795300143
AT1G75340	C3H a,b	1.778147594
AT3G61230	LIM a,b	1.723581702
AT4G24240	WRKY a,b,c	1.721282224
AT3G15500	NAC a,b	1.680657366
AT3G27810	MYB a,b	1.659052732
AT1G72830	CCAAT a / CCAAT-HAP2 b / CBFB_NFYA c	1.637715094
AT4G31060	AP2-EREBP a,b,c	1.62839856
AT5G59380	MBD c	1.622405269
AT1G59810	MADS a,b	1.600914266
AT1G12610	AP2-EREBP a,b,c	1.57383467
AT1G75240	zf-HD a,b	1.570618327
AT2G47620	MYB-related a / SWIRM c	1.567030089
AT5G41090	NAC a,b	1.560298995
AT4G00238	**GeBP a,b**	**1.552863074**
AT1G28520	VOZ a,b	1.552834931
AT5G39820	NAC a,b	1.528688116
AT2G22540	MADS a,b	1.522264456
AT1G22985	AP2-EREBP a,b,c	1.516271672
AT3G58710	WRKY a,b,c	1.512646322
AT1G64000	WRKY a,b,c	1.506374214
AT3G60630	GRAS a,b	1.496443751
AT1G76420	NAC a,b	1.495781035

AT4G15420	C2H2 b	1.486637182
AT4G29930	**bHLH a,b**	**1.482230478**
AT3G45880	JUMONJI e	1.476987035
AT1G04500	Orphans a / C2C2-CO-like b	1.465692029
AT3G48600	SWI/SNF-BAF60b a	1.45199202
AT4G33280	**ABI3-VP1 a,b**	**1.448901511**
AT3G05800	bHLH e	1.398733771
AT4G31550	WRKY a,b,c	1.318567925
AT5G19910	SOH1 a	1.307476885

DISCUSSION

Proteasome gene up-regulation is a hallmark of the proteasome-stress response. I confirmed that proteasome subunit expression is asymmetrical between paralogs, and that while some of this disparity can be attributed to technical issues with RNAseq normalization, several subunits display a preferential up-regulation compared to their sister paralog. This partisan expression occurs for both α- and β-subunits, and RP lid and base subunits. This could indicate that the more highly-expressed isoforms (based on fold-change and ESTs), are evolutionarily favorable for 26S proteasome incorporation during proteasome stress. Even among proteasome assembly factor there is unequal regulation, such as with *NAS2* and *NAS6*, which could signify that certain steps in 26S proteasome assembly are more rate limiting than others, or that other redundant assembly chaperones exist. Indeed several uncharacterized "proteasome-interacting" proteins—based on previous co-expression studies (TAIR)—are in the PSIR and could play a role in assembly or other UPS functions.

For this study, "proteasome stress" was broadly defined as any condition that limits proteasome capacity. However, there are discrete transcriptional responses that are linked to particular proteasome stressors. Hundreds of genes are uniquely up- or down-regulated in one or more of the chemical or genetic conditions that reduce proteasome activity. Some of this distinctive regulation could be due to flaws in the RNAseq analysis: insufficient coverage, low quality reads, annotation flaws, *etc.*, but some of it is almost certainly due to a condition-specific response. The PSIR was conservatively defined so as to overcome these complications. However it is quite clear that not all stress is created equal, even stress that specifically impacts proteasome function.

Due to its principal role in cell growth and homeostasis, 26S proteasome transcriptional and translational expression is tightly integrated into most developmental and environmental-response regulatory networks. It has been shown that oxidative stress, heat stress, ionizing radiation, and pathogen infection can trigger expression of 26S proteasome subunits and

associated factors (Lee et al. 1998, Pajonk et al. 2001, Naujokat et al. 2002, Vierstra 2003, Schellenberg et al. 2010, Kim et al. 2011, Kruegel et al. 2011), and I have shown that numerous genes that are characteristically up-regulated during these particular stress conditions are similarly up-regulated in the PSIR (heat shock factors, GSTUs, E3 ligases, stress-responsive transcription factors, *etc.*). We can assume most of the PSIR genes are likely up-regulated under most conditions that would significantly impact proteasome capacity, regardless of the type of stress.

The overlap between multiple regulatory networks and the PSIR makes identifying condition-specific *trans*-acting factors of the regulon more opaque; transcriptional regulators could act cooperatively or redundantly during similar types of stress to ensure adequate cellular proteasome capacity. The diverse and flexibly recognized DNA-binding motif landscape of 26S proteasome subunit and PSIR genes support this. For example, the SORLIP2 motif (the core sequence of which is nearly identical to the PRCE motif) T-box promoter sequences, and other integrase-type motifs can be flexibly recognized by large classes of activators, such as the MADS-box, Myb-type, and NAC/NTL transcription factors (Hudson et al. 2003, Spensley et al. 2009, Dubos, 2010 #57, Smaczniak et al. 2012, De Clercq et al. 2013, Korkuc et al. 2014). Further protein-DNA binding and genetic analyses of DNA-binding proteins during various proteasome-stress conditions will have to be performed to identify UPS-regulating transcription factors.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The T-DNA insertional mutants for *NAC53* (*nac53-1*, SALK_009578C; *nac53-2*, SALK_018311C), *NAC78* (*nac78-1*, SALK_025098; *nac78-2*, SALK_040812C) in the *Arabidopsis thaliana* ecotype Col-0 were generated by T-DNA insertional mutagenesis (Alonso et al. 2003) and obtained from the *Arabidopsis* Biological Resource Center (ABRC) at The Ohio State University (https://abrc.osu.edu/). The *rpn10-1* and *rpn12a-1* exon-trap lines in the C24 background were generated as previously described (Smalle et al. 2002, Smalle et al. 2003). The proteasome promoter:*GUS* transgenic lines were generated by cloning the 5' upstream region for each proteasome subunit, starting at the transcriptional start site. These promoter fragments were cloned into pCAMBIA-3301 or pMDC163 vectors and transformed into *Arabidopsis* via *Agrobacterium*-mediated transformation.

Seeds were either surface sterilized with chlorine gas or treated with 80% ethanol then 50% bleach, stratified in the dark at 4^{o} C for 2 days then germinated on 0.7% agar media containing half-strength Murashige and Skoog medium (½ MS; Caisson Labs), 1% sucrose, 0.5% MES, pH 5.7. The proteasome inhibitor MG132 was procured from SelleckChem and diluted to a stock solution of 50 mM with DMSO. For bulking seed stocks, seeds were plate, grown for 8-12 days, transferred to soil and incubated at 22^{o} C under long day photoperiods (16 h light/ 8 h dark).

GUS Staining

Proteasome promoter:*GUS* fusion transgenic plants were stained for GUS presence after exposure to 24 hr 100 μM MG132. First, a X-gluc histochemical buffer was made (10 mM Na_2EDTA, 100 mM NaH_2PO_4, 0.1% Triton, pH 7.0) and then 5 mg of X-gluc (5-bromo-4-chloro-3-indoyl glucuronide) was dissolved in 100 μL DMSO and added to every 10 mL of X-gluc buffer

that was to be used for staining. Seedlings were grown in liquid culture in 6-well plates. Prior to staining, all ½ MS media was aspirated away from the wells, and then 2-3 mL of X-gluc-containing buffer was added to each well. Samples were incubated overnight and then buffer was removed. 95% Ethanol was added to wells to fix stain and bleach seedlings for visualization. Ethanol washes should be replaced after seedling pigment saturates.

RNA Isolation for RNA Sequencing and qRT-PCR Analysis

Seedlings were grown in constant light for 5 days in ½ MS in 12-well plates. On the fifth day, seedlings were either collected immediately (No DMSO), treated with 100 μL of DMSO for 24 hr or 100 μM MG132 for either 3 hr or 24 hr. Seedlings were pressed between paper towels to remove excess liquid, then frozen in liquid nitrogen and pulverized in 1.5 mL centrifuge tubes with sterilized toothpicks. RNA extraction was performed using the QIAzol Lysis Reagent (Qiagen). cDNA generation was performed following DNAseI treatment (Promega) using Superscript III Reverse Transcriptase (Life Technologies). mRNA enrichment and library generation was performed using the TruSeq RNA library sample preparation kit v2 (Illumina) at the University of Wisconsin-Madison Gene Expression Center (http://www.biotech.wisc.edu/services/gec). Multiplexed sequencing was performed on the Illumina HiSeq 2500 platform using 100 bp single-ended reads. There were at least 2 biological replicates for each condition. Each biological replicate yielded between ~10-25 million raw reads.

Primers for qRT-PCR analysis were generated with Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and are as following: *PBA1* (RP: CCTCCAATGGCAAACGGTTG, LP: TGTGATGTACAACCGTCGCA); *RPN5a* (GAC ATT GGA GGA CTA GAG AAG CAG GTA TG, RP: CTA CTG AAT CCG TCA AGT TGA TTG AGC AAC); *RPN10* (LP: AACCGCAGCTATCCAGATCG, RP: ACATAGCCACTTGCACCCTC);

RPN12a (LP: TCCTTCATGGAGGGTGCCTA, RP: ACGGAATCTCTTTGCACGGT). Amplification was performed on a Roche LightCycler 480 using either Roche LightCycler 480 SYBR Green Master Mix or MidSci Bullseye SYBR Green Master Mix. Primer efficiencies were between 1.9-2.10, based on dilution series analysis.

Analysis of Illumina Reads

FASTQ files were quality-controlled with the Trimmomatic package (Bolger et al. 2014). Between 70-80% of reads were retained in all biological replicates. Trimmed reads were aligned using Tophat-Bowtie 2 to the TAIR 10 genome using Ensemble 2013 *Arabidopsis*.gtf file annotations (Langmead et al. 2012). Read counts were generated through HTSeq (Anders et al. 2015), and quantified via edgeR, normalizing to the Col-0 No DMSO control treatment (Robinson et al. 2010). Genes used for analysis in this investigation were culled via a FDR ≤ 0.05% cutoff. All available gene co-expression and protein interaction data was collected from the STRING database (Jensen et al. 2009) and mapped using the organic layout option from Cytoscape 3.0.1 (Shannon et al. 2003). GO and functional term enrichments were determined using DAVID (Huang da et al. 2009, Huang da et al. 2009).

DNA-binding Motif Enrichment

PSIR and proteasome motif enrichment was performed with the AGRIS *cis*-regulatory element database (ATcisDB) (Yilmaz et al. 2011). Data manipulation was carried out in Python. Statistical enrichment analysis was performed using hypergeometric distribution from the Scipy package in Python (http://www.scipy.org/).

Immunoblot Analysis

Protein immunoblot analysis was performed by first grinding 6 DAG seedling tissue with a sterile toothpick and adding 1:4 weight:volume of 2x sample buffer (30 mM Tris pH 6.8, 3.6%

SDS, 20% glycerol, 10% BME). Samples were separated on 11% SDS-PAGE gels for proteasome subunits and 12% SDS-PAGE gels for anti-UB blots. Separated proteins were transferred onto Millipore P-immobilon or FL-immobilon membrane and immunoblotted according to Smalle et al. (2002). Antibodies against PA200 (Book et al. 2010), Ub (van Nocker et al. 1996), RPN1, RPN5, RPN10, RPN12a, and RPT2a (Smalle et al. 2002, Yang et al. 2004) were as described, and Histone 3 was procured from Abcam (Ab1791).

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CHAPTER 3

NAC53 AND NAC78 ARE ACTIVATORS OF UPS GENE EXPRESSION DURING PROTEASOME STRESS

ABSTRACT

The ubiquitin/26Sproteasome system (UPS) is a highly conserved eukaryotic and archaeal pathway that degrades intracellular constituents. This is accomplished by the post-translational modification of target proteins with a poly-ubiquitin chain, which facilitates their recognition and subsequent degradation. Over 6% of the *Arabidopsis thaliana* genome encodes UPS components, including subunits of the 26S proteasome, components of the conjugation machinery, de-ubiquitylating enzymes, and numerous other UPS-associated proteins. Only a few eukaryotic DNA-binding proteins have been identified as transcriptional activators of some, but not all, proteasome subunit genes during proteasome stress. These include Rpn4, Nrf1, and NAC78 in yeast, mammals, and plants, respectively. Here, I show that the two NAM/ATAF1,2/CUC2 (NAM) transcription factors, NAC53 and NAC78, are responsible for the proper regulation of UPS genes under conditions that challenge cellular proteasome capacity, and that plants lacking both DNA-binding proteins are hypersensitive to proteasome-specific inhibition.

INTRODUCTION

The proteasome stress regulon in yeast (*Saccharomyces cerevisiae*) is controlled by Rpn4, a C2H2-type zinc-finger transcription factor that binds to the DNA recognition sequence PACE (Proteasome Associated Control Element), a nonamer motif present in the upstream promoter regions of 26 of the 33 yeast proteasome subunits (Mannhaupt et al. 1999, Xie et al. 2001, Shirozu et al. 2015). Rpn4 itself is regulated post-translationally through the proteasome via a negative-feedback mechanism, undergoing rapid proteasome-mediated turnover ($t_{1/2}$~2.5 minutes), which prevents its accumulation in cells when proteasome capacity is at equilibrium or in excess of proteolytic need (Xie et al. 2001, Dohmen et al. 2007). However, when the proteasome is challenged with a glut of substrates, Rpn4 turnover is slowed, allowing it to amass rapidly and hence up-regulate UPS gene expression.

Similar proteasome stress feedback networks exist in other eukaryotes: mammalian cells, flies (*Drosophila melanogaster*), and *Arabidopsis* all transcriptionally and translationally up-regulate UPS genes in concert during proteasome stress (Meiners et al. 2003, Yang et al. 2004, Lundgren et al. 2005, Book et al. 2009, Book et al. 2010, Kurepa et al. 2010). However, despite the apparent conservation of this regulatory mechanism, Rpn4 orthologs remain to be identified outside of yeast and other fungi. Yet transcription factors have been identified as regulating UPS expression during stress in other eukaryotes. These include Nrf1 (Nuclear Factor Erythroid-derived 2-Related Factor 1) in mammals and NAC78 (No Apical Meristem, ATAF1,2, CUC2 domain-containing family) in plants (Stanhill et al. 2006, Kensler et al. 2007, Radhakrishnan et al. 2010, Nguyen et al. 2013, Tsuchiya et al. 2013, Radhakrishnan et al. 2014, Sha et al. 2014). Nrf1 is Endoplasmic Reticulum (ER)-bound transcription factor that is constantly degraded through the ER-Associated Protein Degradation (ERAD) pathway via a p97-dependent (CDC48) mechanism, and like Rpn4, when cells lack Nrf1 they become more sensitive to proteotoxic stress (Radhakrishnan et al. 2010, Steffen et al. 2010, Karpov et al. 2013, Radhakrishnan et al. 2014). Likewise, NAC78 is a trans-membrane domain-containing

protein that is likely anchored to the ER and, when overexpressed, induces proteasome subunit gene expression by binding the PRCE motif, which is present in the upstream regions of over 30 proteasome subunit genes within *Arabidopsis*, and occurs in other plant species as well, including *Oryza sativa*, *Ricinus communis*, and *Chlamydomonas reinhardtii* (Nguyen et al. 2013). No one single transcription factor has been shown to up-regulate every single 26S proteasome gene, only a majority of them under certain conditions. There are at least two possible explanations for this: (i) that only a certain subset of UPS/proteasome genes are required to be expressed above basal levels for proper stress response; or (ii) that there remain other potential transcriptional regulators of the system.

Here, I present a detailed characterization of the two membrane-bound *trans*-acting factors, NAC53 (NTL4, AT3G10500) and NAC78 (RPX, NTL11, AT5G04410), and show that both are able to bind and induce transcription of PSIR genes. I also show that at least one of these transcriptional activators is required for the proper expression of PSIR genes, and that plants lacking both NAC53 and NAC78 are deficient in UPS gene expression and have a hypersensitive developmental response to proteasome inhibition.

RESULTS

NAC53 and NAC78 Activate 26S Proteasome Subunit Expression

Previous groups have demonstrated that overexpressing NAC78 (RPX) will up-regulate some, but not all, 26S proteasome subunit genes in *Arabidopsis* (Kim et al. 2006, Kim et al. 2010, Yabuta et al. 2011, Nguyen et al. 2013). To confirm this, I obtained a β-estradiol-inducible XVE promoter-driven *NAC78* transgenic plant line from the TRANSPLANTA collection (*NAC78* #2318, pER8GW vector) (Coego et al. 2014), as well as the *CaMV* 35S promoter-driven *NAC78* lines described in Nguyen, 2013. Seedlings were grown for 5 days in liquid culture, then either 10 μM β-estradiol or 100 μM MG132 was added to the media to observe any difference between a short, acute (β-estradiol-induction) or prolonged, constitutive (*CaMV* 35S)

expression of *NAC78* on proteasome transcript levels. β-estradiol-induced *NAC78* expression does seem to result in proteasome transcript accumulation, although there is variability in the fold-change levels (**Figure 3-1a**). Immunoblots of the inducible and constitutively-expressing *NAC78* lines did not show a very large increase of all proteasome proteins (**Figure 3-1b**), but there was a noticeable accumulation of RPT2 and PA200 in the *CaMV:RPX* lines, both with and without MG132 treatment, as well as a very slight increase of the PBA1 immature and mature gene product. It could be that a prolonged overexpression of *NAC78* over several days is sufficient to generate a visible increase in proteasome gene products, but that a short and acute overexpression of *NAC78* is insufficient to produce similar results. It could also be that the effect of *NAC78* overexpression could be more easily detected during proteasome stress, as the 35S-driven lines appear to show a slightly stronger accumulation of proteasome proteins during MG132 treatment.

To evaluate *NAC78* overexpression on plant fitness, Col-0 and β-estradiol-inducible *NAC78* seedling were germinated and grown in the presence of MG132 and/or β-estradiol to determine if higher levels of *NAC78* would increase seedling tolerance to proteasome inhibition. *NAC78* overexpression did not buoy plant growth or enhance survival under proteasome limiting conditions, but rather, overexpression of *NAC78* during MG132 treatments had a significant negative effect on plant growth (**Figure 3-1c**).

Due to the diverse DNA-binding motif landscape of the proteasome and PSIR, and the numerous transcription factors identified as being activators of proteasome gene expression in the directed Y1H screen (Chapter 2), I predicted that another *trans*-acting factor, one that was probably closely related to NAC78, could also regulate UPS expression. To identify the closest evolutionarily relative to NAC78, I performed a bayesian phylogenetic analysis across a handful of plant lineages with several NAC family proteins that share high amino acid sequence similarity to NAC78. NAC53 was found to be the most closely related to NAC78 based on evolutionary grouping, full-length amino acid sequence identity (~72%), and DNA-binding

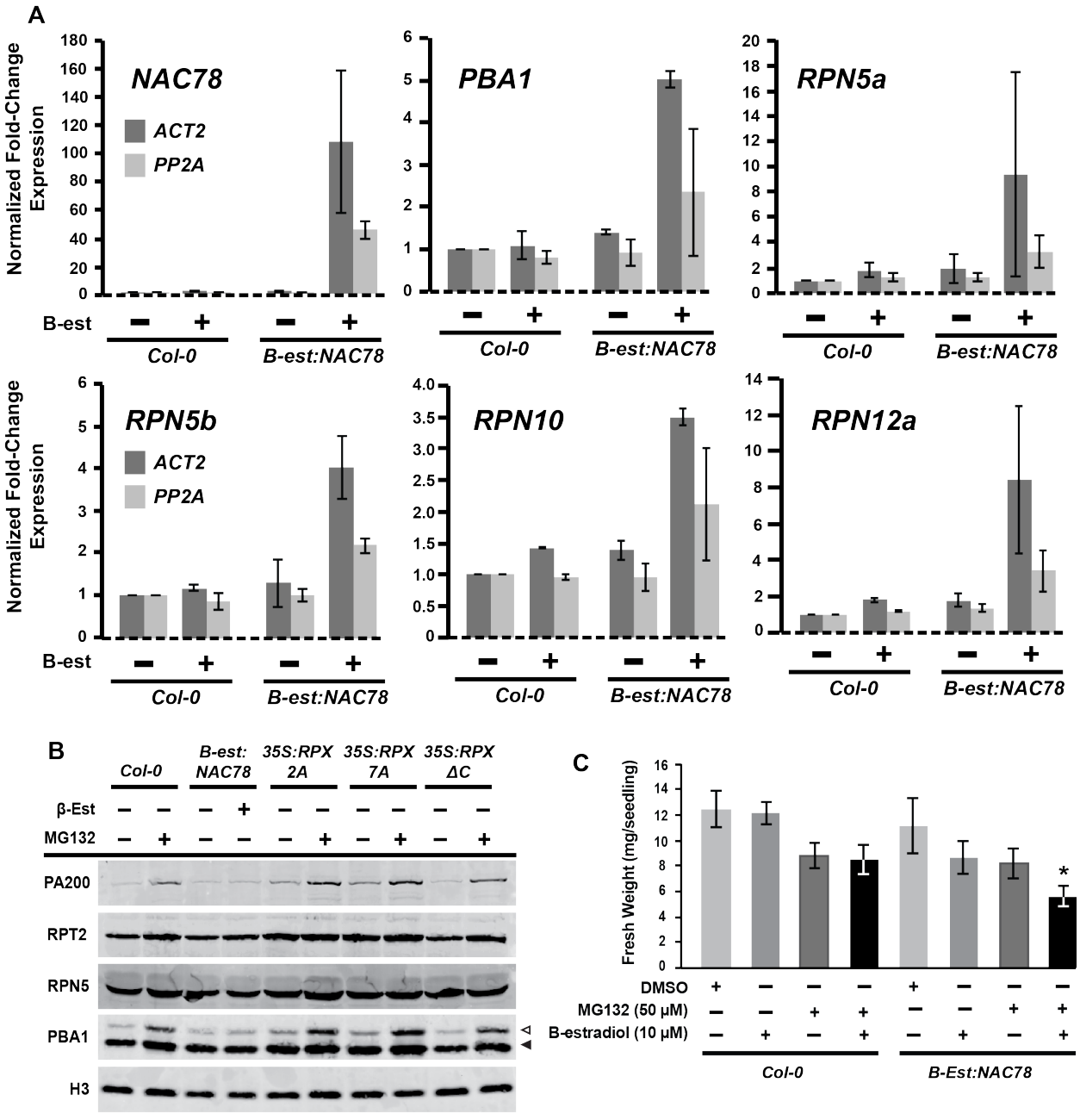
Figure 3-1. *NAC78* Overexpression Induces Proteasome Gene Expression

A. qRT-PCR of *NAC78* and proteasome genes from β-estradiol-inducible *NAC78* transgenic plants normalized to WT Col-0 control (DMSO). mRNA loading was normalized to *ACT2* and *PP2A*.

B. Immunoblots against proteasome proteins in WT (Col-0), β-estradiol-inducible *NAC78* line (β-est:*NAC78*), two 35S-driven full-length CDS *NAC78* lines (35S:RPX 2A and 7A), and a 35S promoter-driven *NAC78* transgenic line that expresses the full CDS except for the 3' end that encodes the trans-membrane domain (35S:RPX ΔC). The black arrow indicates the mature, catalytically active form of PBA1, while the white arrow denotes the un-cleaved proenzyme.

C. Fresh weight of Col-0 and *NAC78* β-estradiol-inducible seedlings germinated and grown for 7 days with and without MG132 and β-estradiol. Asterisk indicates significant difference (p-value <0.01) in fresh weight compared to WT within same treatment.

Figure 3-1. *NAC78* Overexpression Induces Proteasome Gene Expression



domain identity (~93%) (**Figure 3-2**). NAC53 and NAC78 are members of the trans-membrane motif-1-like (NTL) domain-containing subclass of NAC family proteins (Kim et al. 2007), and are thought to be ER-bound, which parallels Nrf1 transcription factor localization in mammalian cells. Like Nrf1, NAC53, and probably NAC78, is proteolytically cleaved away from the membrane to which it is bound during proteasome stress, generating a truncated, "active" form of the protein containing the N-terminal NAM DNA-biding domain. This active NAC protein is then transported into the nucleus where it regulates gene expression (Lee et al. 2012).

Due to such high sequence identity between NAC53 and NAC78, specifically in the NAM binding domain but also across the protein sequences as a whole, it is probable that both proteins could act upon similar regulatory networks, recognizing very similar DNA-binding motifs as either homo- or hetero-dimers (Weirauch et al. 2014). I tested the ability of NAC53 and NAC78 to bind proteasome subunit promoters and observed that both transcription factors can activate proteasome gene expression (**Figure 3-3b**). Additionally, both NACs are able to homo- and hetero-dimerize with each other based on yeast-2 hybrid (Y2H) and bi-molecular fluorescence complementation (BiFC), but not with some other NACs, including NAC13 (NTL1), which has the highest NAM protein domain identity compared to NAC53 and NAC78 within the NAC family (>65%) (**Figures 3-3c,d, 3-4, 3-5, and 3-6**). These NAC53/NAC78 homo- and hetero-dimers are localized to both the nucleus and cytoplasm *in planta* (**Figure 3-6**), however I was not able to determine if they predominantly migrate into the nucleus under MG132 treatment, as has been previously shown for other NTLs during stress (Lee et al. 2014).

Figure 3-2. ClustalW Pairwise Alignment of NAC53, NAC78, and NAC13

BoxShade visualization of the ClustalW pairwise alignment between the protein sequence of NAC53, NAC78, and NAC13. The NAM binding domain (purple) is 93.8% identical between NAC53 and NAC78, but only ~65% identical between NAC13 and either NAC53 and NAC78, which is still greater than any other NAC protein. Overall protein sequence identity is 72% between NAC53 and NAC78, but only ~30% identity is shared between NAC13 and either NAC53 or NAC78. Trans-membrane domains are indicated in green (NAC53) blue (NAC78) and red (NAC13).

Figure 3-2. ClustalW Pairwise Alignment of NAC53, NAC78, and NAC13

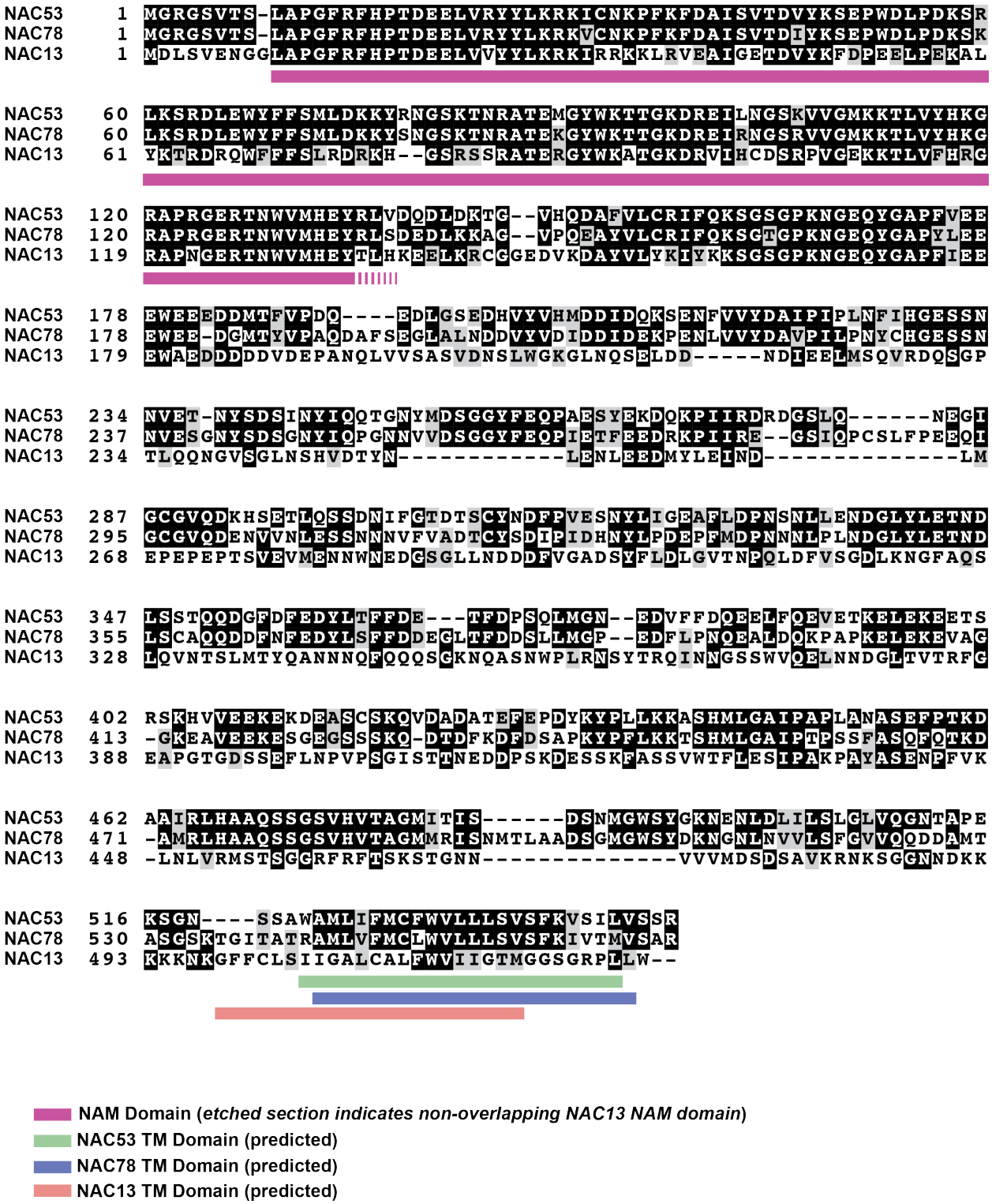


Figure 3-3. *NAC53* and *NAC78* Physically Interact and Activate Proteasome Gene Expression

A. A rooted, bayesian phylogenetic tree of NAC family transcription proteins using a *Physcomatrella patens* NAC protein as an outgroup (Mr.Bayes v3.1). NAC53 and NAC78 group together throughout dicot evolution. All unlabeled branchpoints have support values of 1.0. Os-j = *Oryza sativa japonica*, Os-i = *Oryza sativa indica*, Zm = *Zea mays*, At = *Arabidopsis thaliana*, Al = *Arabidopsis lyrata*, Pt = *Populus trichocarpa*, Rc = *Ricinus cummunis*, Vv = *Vitis vinifera*.
B. NAC53 and NAC78 activate proteasome gene expression. A Y1H quantification (Miller Assay) of full-length NAC53 or NAC78 (prey) binding to the upstream DNA sequence fragments of *PA200* or *RPN12a* (bait). Fold-change values are normalized to promoter-only controls.
C. A Y2H pairwise interaction analysis showing the NAC53 and NAC78 can form homo- and hetero-dimers.
D. Bimolecular fluorescence complementation (BiFC) pairwise interaction analysis using split-YFP reporter proteins that are translationally fused to the N-terminus of NAC53 or NAC78. ½ YFP-NACs (N-YFP or C-YFP) are then transiently expressed in *Nicotiana benthamania* leaves and visualized by confocal fluorescent microscopy. Leaves were treated with DMSO (MG132 treated BiFC images are in Figure 3-4). From left to right, panels indicate (i) the presence of YFP signal if the NAC proteins interact and split-YFP halves fuse and fluoresce, (ii) brightfield image of leaf cells, and (iii) merged images of YFP field and brightfield.

Figure 3-3. *NAC53* and *NAC78* Physically Interact and Activate Proteasome Gene Expression

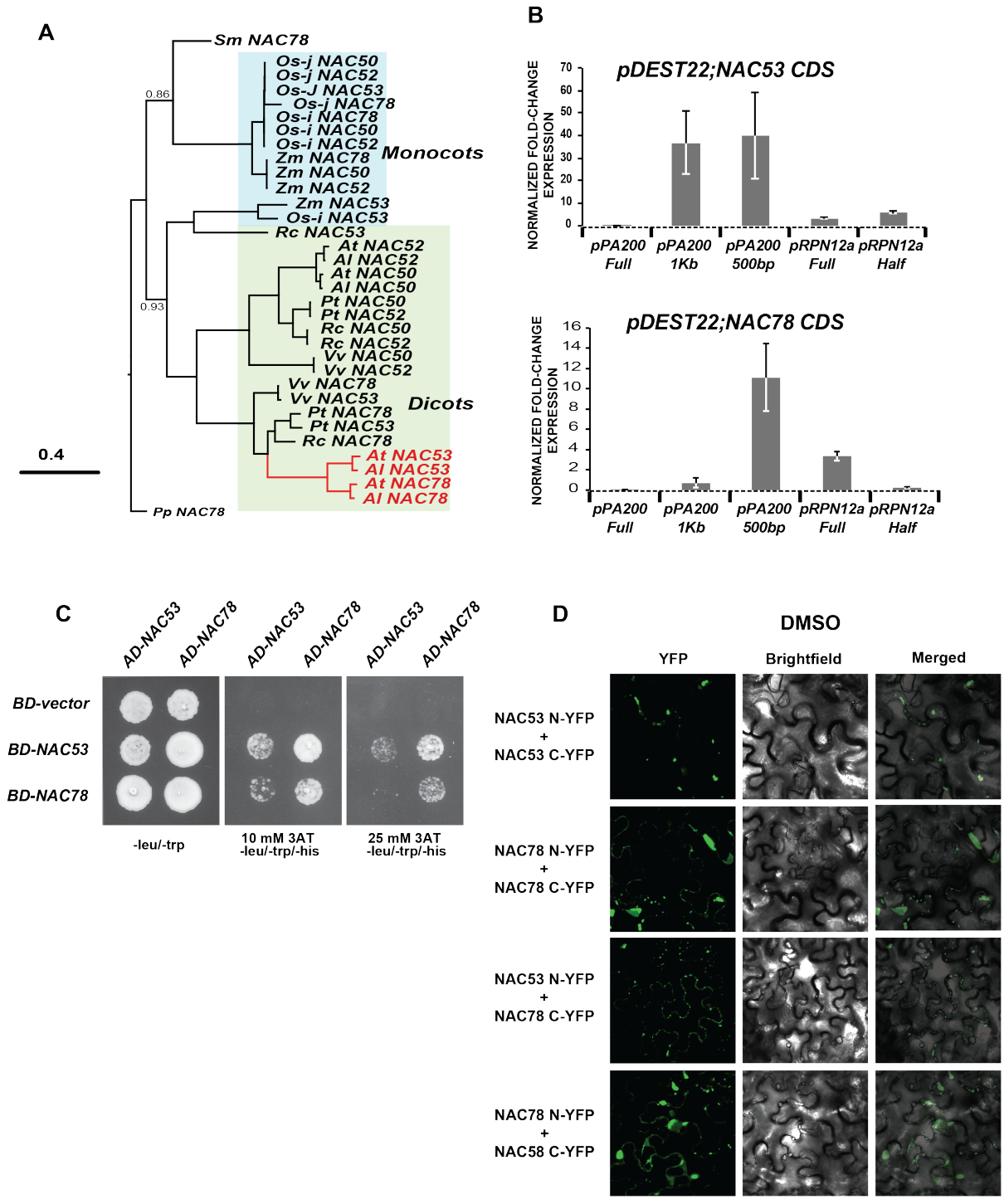


Figure 3-4. NAC53 and NAC78 Physically Interact (BiFC MG132 Treatment and Experimental Controls)

BiFC pairwise interaction assay of NAC53 and NAC78 proteins N-terminally-tagged with split-YFP (N-YFP and C-YFP) and transiently expressed in *Nicotiana benthamania* leaves and visualized by confocal fluorescent microscopy.

A. *Nicotiana* leaves treated with MG132. NAC53 and NAC78 homo- and hetero-dimers persist during inhibitor treatment.
B. Vector-only controls. NAC53 and NAC78 transiently expressed in leaves with empty split-YFP vectors and treated with DMSO. No YFP signal was observed. MG132 treatment also showed no difference in YFP fluorescence (data not shown).

Figure 3-4. NAC53 and NAC78 Physically Interact (BiFC MG132 Treatment and Experimental Controls)

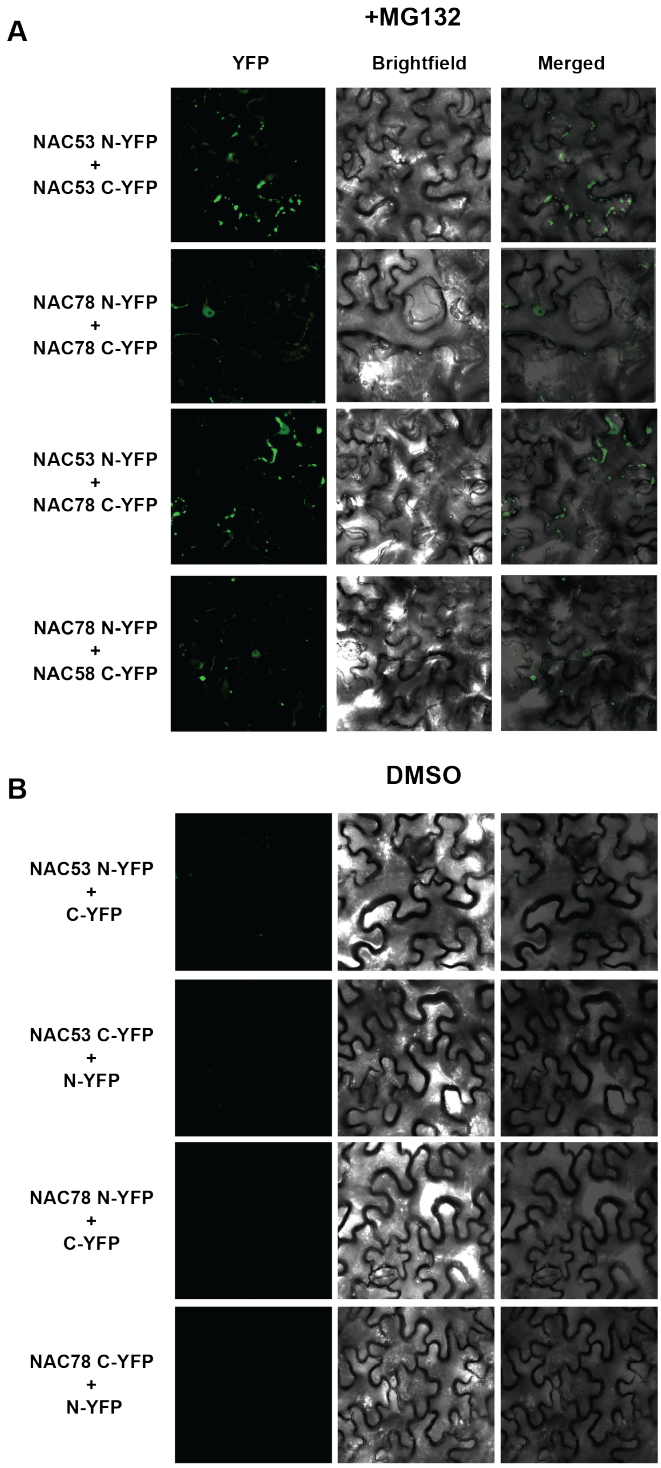


Figure 3-5. NAC53 and NAC78 Do Not Interact with All NAC Proteins *in planta*

BiFC pairwise interaction assay of NAC53, NAC78, NAC13, and NAC101 proteins N-terminally-tagged with split-YFP and transiently expressed in *Nicotiana benthamania* leaves and visualized by confocal fluorescent microscopy. No interaction was detected between NAC53 or NAC78 with either NAC13 and NAC101. Only one split-YFP orientation for each pairwise interaction is shown, but no YFP signal was observed any other pairwise combination (data not shown).

Figure 3-5. NAC53 and NAC78 Do Not Interact with All NAC Proteins *in planta*

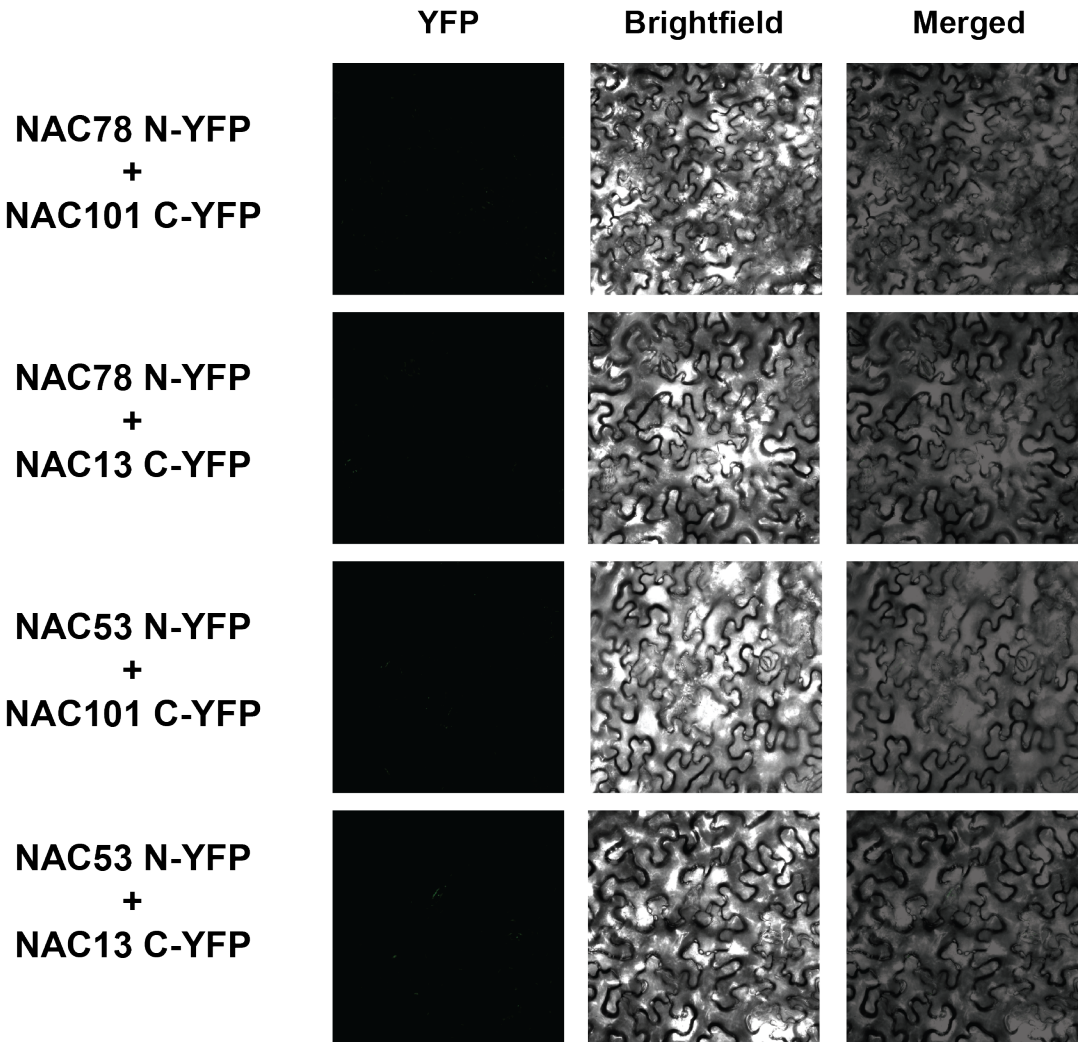
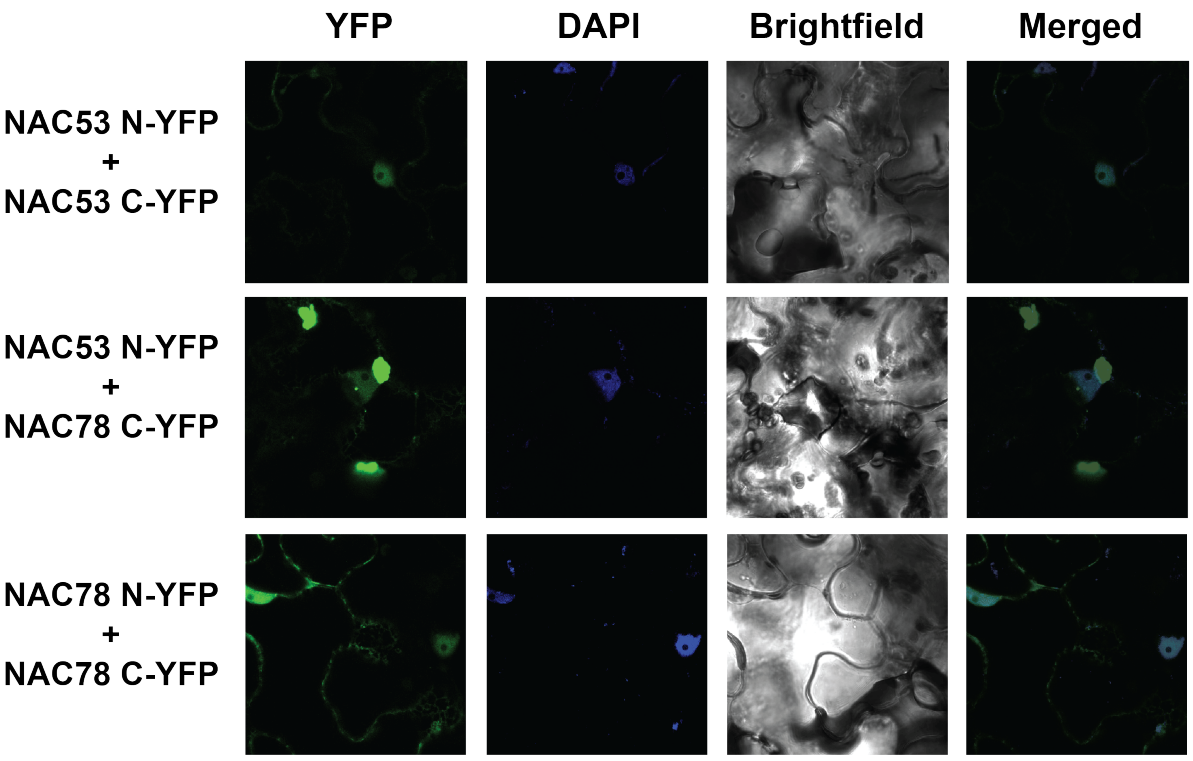


Figure 3-6. NAC53 and NAC78 Dimers Localize to the Nucleus

BiFC pairwise interaction assay of NAC53 and NAC78 proteins N-terminally-tagged with split-YFP and transiently expressed in *Nicotiana benthamania* leaves and visualized by confocal fluorescent microscopy. Cells were infiltrated with DAPI (DNA fluorescent stain) and show that NAC53 and NAC78 homo- and hetero-dimers localize to the nucleus.

Figure 3-6. NAC53 and NAC78 Dimers Localize to the Nucleus



Only NAC53 or NAC78 is Required for Sufficient Proteasome Expression During Stress

To determine if either/both NAC53 and NAC78 are required for adequate proteasome subunit expression during proteasome-stress, I performed a genetic analysis with two separate exonic TDNA insertional mutant lines for both *NAC53* and *NAC78* (**Figure 3-7a**). One TDNA insertion is localized in the extreme 3' end of each gene (87 and 21 bp upstream of the translational stop site for *NAC53* and *NAC78*, respectively), just before the putative trans-membrane domain. The other TDNA insertion is more 5', in second exon of either gene, disrupting the NAM domain sequence (735 and 372 bp downstream of the translational start site for *NAC53* and *NAC78*, respectively). Partial transcripts of each *NAC* gene are produced in all mutant backgrounds based on RT-PCR (**Figure 3-7b**), however it is likely that the *nac53-1* and *nac78-1* lines are the functional null alleles, since they likely do not produce mRNAs that contain a full-length NAM domain, whereas the *nac53-2* and *nac78-2* backgrounds produce transcripts containing the NAM domain, resulting in a functional DNA-binding protein.

To determine if NAC53 and NAC78 are proteasome-stress responsive *trans*-acting factors of the PSIR, *nac* mutant seedlings were treated with MG132 and analyzed via qRT-PCR for difference in proteasome subunit gene expression (**Figure 3-7c**). Only the *nac53-1xnac78-1* background showed significant proteasome subunit gene mis-regulation: mRNA in this double mutant line showed lower basal levels of some proteasome gene transcripts (~30-40%) during control treatments and a significant reduction or complete ablation of subunit expression during MG132 treatment compared to WT. While some *nac* single TDNA mutants, and the *nac53-2xnac78-2* double mutant, showed reduced expression for some UPS genes, there was still a comparable fold-change increase compared to WT, indicating that the overall stress-induced gene activation was largely maintained in those mutant lines. Additionally, proteasome subunit immunoblots showed that only the *nac* double mutant *nac53-1xnac78-1* displayed a slightly reduced accumulation of the unprocessed form of PBA1 and full-length PA200 (**Figure 3-7d**). The other double mutant line (*nac53-2xnac78-2*) did show a slight decrease in PA200 levels

during MG132 treatment, but no decrease in PBA1 proenzyme levels. Interestingly, *CDC48a*, but not *CDC48b* (not shown) expression was reduced in *nac53-1xnac78-1* seedlings. Both paralogs are strongly up-regulated in the PSIR, but only *CDC48a* appears to be regulated by NAC53 and/or NAC78.

To determine if any *nac* single or double mutant backgrounds have deficiencies in the ubiquitylation pathway or Ub-conjugate processing, an anti-Ub immunoblot of all *nac* mutants (+/-MG132) was performed. A slight increase of ubiquitylated proteins was observed in both *nac* double mutant lines during MG132 treatment, but not in any *nac* single TDNA insertion background (**Figure 3-8**), although this result is not consistently observed between biological replicates. This suggests there could be a mild deficiency in Ub-tagged proteins processing in *nac53-1xnac78-1* and *nac53-2xnac78-2* backgrounds, likely from decreased 26S proteasome levels.

To evaluate if the lack of UPS up-regulation in *nac53-1xnac78-1* plants resulted in growth sensitivity or other conditional phenotypes when proteasome capacity is challenged, seedlings were germinated and grown for 6 days in the presence of MG132 or pFP—a phenylalanine analog that increases mis-folded proteins within the cell. The *nac53-1xnac78-1* and *nac53-2xnac78-2* 6-days-after-germination (DAG) seedlings are generally smaller, by fresh weight, compared to WT or other mutant backgrounds when grown under control conditions (DMSO) (**Figure 3-9**). Taking this into account, there was no significant normalized growth sensitivity between the single-TDNA mutants, the double mutants, or WT during pFP treatment (**Figure 3-9b**). However for the MG132 treatment, only the *nac53-1xnac78-1* mutant showed significant growth retardation compared to WT (**Figure 3-9c,d**). Surprisingly, the *nac53-1xnac78-1* mutant largely failed to proceed past germination, only breaking the seed coat, resulting in very low fresh weight measurements at higher concentrations of MG132 (**Figure 3-9e**). There was a noticeable, but not significant, difference in *nac78-1* and *nac53-2xnac78*-2 seedling fresh weight compared to Col-0 after prolonged growth on MG132 (10 DAG) (**Figure 3-**

9f), but the *nac53-1xnac78-1* background still showed the greatest growth sensitivity to long-term proteasome inhibition.

To determine if the *nac53-1xnac78-1* growth sensitivity was due to germinating already weaker *nac* double mutants on MG132, which can block cycle progression, WT and *nac* mutant seedlings were grown under normal conditions for 6 days (1/2 MS salts, 1% sucrose, 0.5 mg/L MES-hydrate, pH 5.7), then transferred to plates containing either MG132 or DMSO and grown for another 6 days. This showed a similar result: *nac* double mutant seedling growth was significantly slower, essentially halted, compared to WT after transferring to plates containing MG132 (**Figure 3-9g,h**).

To determine if *nac53-1xnac78-1* growth hypersensitivity was MG132-specific, the same experiment was repeated using a different chemical class of proteasome-specific inhibitor, bortezomib. Uniform results were observed: the *nac53-1xnac78-1* mutants also displayed a hypersensitive growth phenotype on media containing bortezomib compared to WT seedlings (**Figures 3-10**). These data taken together seem to suggest that *Arabidopsis* requires either NAC53 or NAC78 to properly regulate UPS genes, and that lacking functional, full-length copies of both transcription factors abolishes proteasome gene up-regulation and severely obstructs seedling germination and growth during proteasome inhibition.

Figure 3-7. *nac53-1 x nac78-1* Double Mutants Are Deficient in Proteasome Gene Expression During Stress

A. Gene diagrams of NAC53 and NAC78 with TDNA insertional sites (red triangles) and genotyping primers (red arrows) for RT-PCR (B). Green = exons, blue = UTRs, yellow-orange gradient = NAM DNA binding domain.

B. RT-PCR showing a lack of full-length transcript production in most *nac* mutant lines, but also partial transcript production in most lines as well (PCR cycles =35). *ACT2* was used as a loading control. Genotyping primers are designated in (A).

C. (Continued in panel (Cα)) qRT-PCR of PSIR gene expression (CP and RP lid and base subunits, *CDC48a*, and *PA200*) in *nac* mutant backgrounds (+/- MG132) compared to WT. Fold-change expression was normalized to *ACT2* and *PP2A* gene products (dark and light grey bars). Only the *nac53-1xnac78-1* double mutant failed to significantly express UPS genes during proteasome stress. *CDC48b* expression data is not shown, but expression levels were unchanged when compared across all treatments and genetic backgrounds.

D. Immunoblots of PBA1, RPN12a, PA200, with H3 as a loading control, from *nac* TDNA seedlings treated with or without MG132. PA200 and RPN12a levels are slightly decreased in *nac* double mutant lines. Mature PBA1 levels (black arrow) are relatively unchanged in *nac* mutant seedlings, but the proenzyme PBA1 levels (white arrow) are noticeably reduced in the *nac53-1xnac78-1* line.

Figure 3-7. *nac53-1 x nac78-1* Double Mutants Are Deficient in Proteasome Gene Expression During Stress.

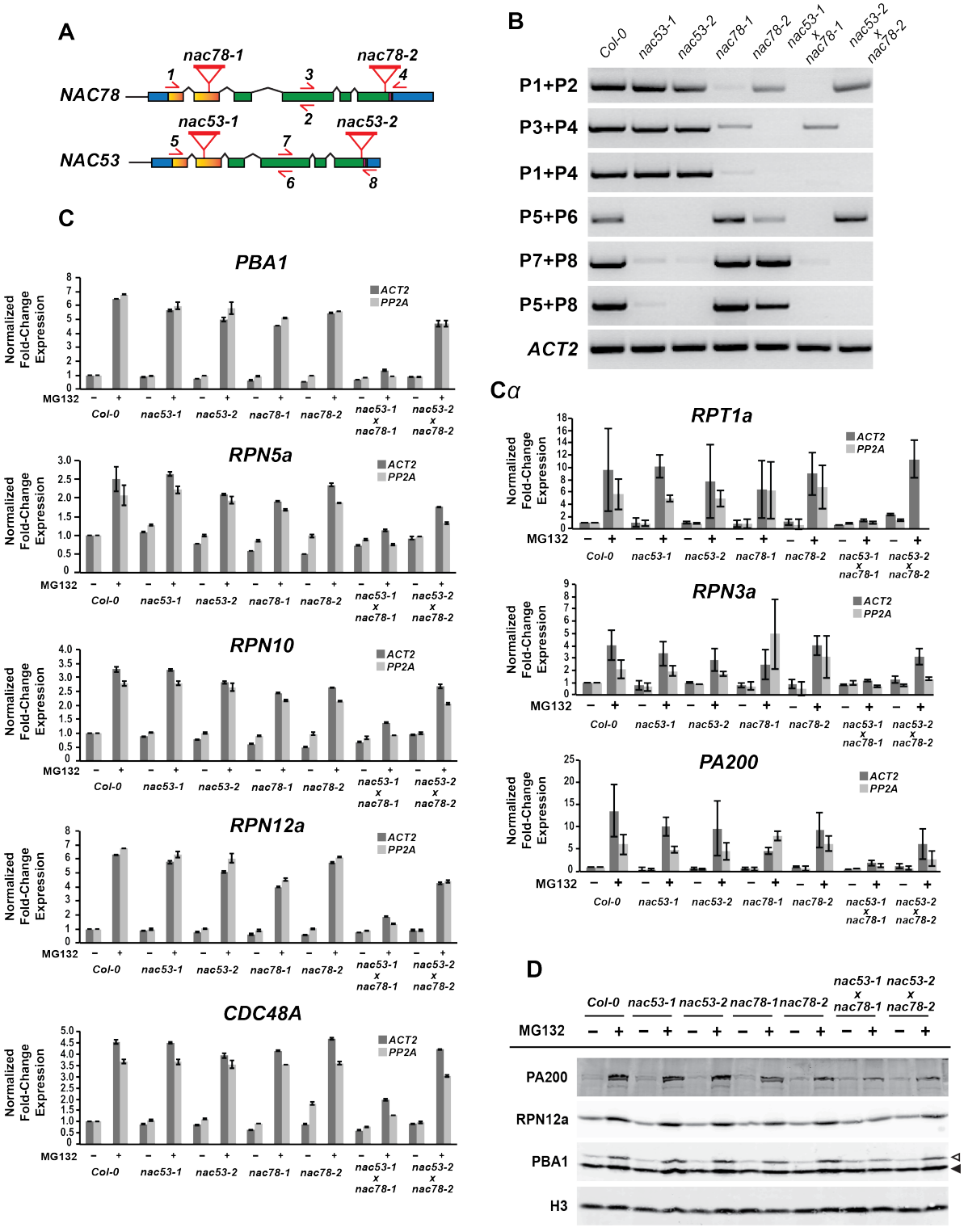


Figure 3-8. Elevated Ub-conjugate Levels in *nac* Double Mutant Backgrounds

Anti-Ub immunoblot of *nac* mutant seedlings treated with and without MG132. Histone 3 (H3) is used as a loading control. Both *nac* double mutant lines show a slight increase in Ub-conjugate levels (Ub_c), suggesting there could be a mild deficiency in Ub-tagged protein processing via the proteasome.

Figure 3-8. Elevated Ub-conjugate Levels in *nac* Double Mutant Backgrounds

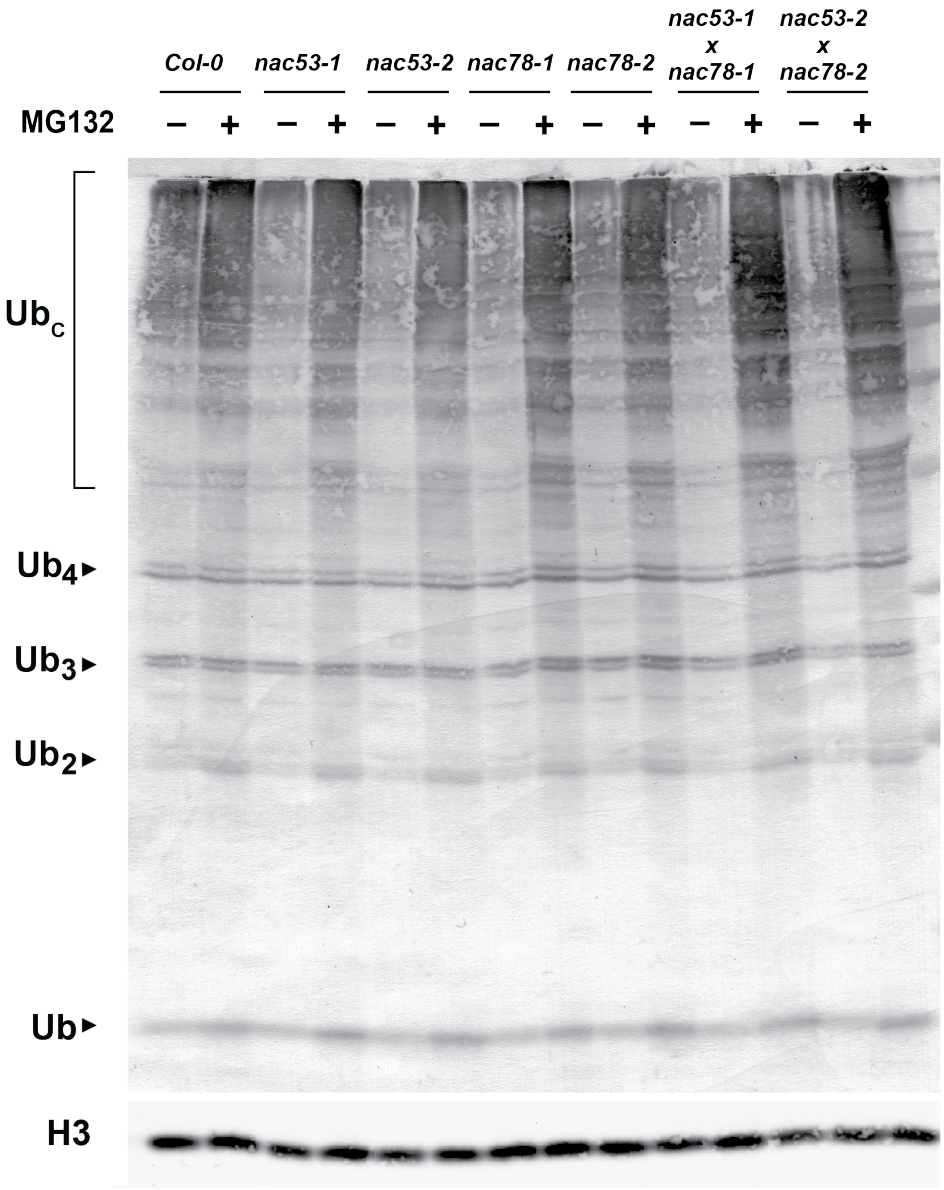


Figure 3-9. The *nac53-1xnac78-1* Double Mutant is Hypersensitive to Proteasome-specific Inhibition

The 6 DAG *nac* double mutants are generally smaller, by fresh weight, compared to WT or single-TDNA *nac* mutant lines when grown under control conditions (DMSO).

A. Fresh weight of 6 DAG Col-0 and *nac* mutant seedlings germinated and grown in the presence of pFP.
B. Averaged seedling growth sensitivity to pFP normalized to DMSO-treated WT control from (A).
C. Fresh weight of 6 DAG WT and *nac* mutant seedlings germinated and grown in the presence of MG132. There is a significant difference (red asterisk) in fresh weight values between WT and *nac53-1xnac78-1* seedlings grown on MG132-containing media (p-value <0.01).
D. Averaged seedling growth sensitivity to pFP normalized to DMSO-treated WT control from (C). There is a significant difference in growth sensitivity between WT and *nac53-1xnac78-1* seedlings (red asterisk) grown on MG132-containing media, but not for any other *nac* mutant line (p-value <0.01).
E. WT and *nac* 10 DAG seedlings grown on ½ MS media containing either DMSO, 30 μM MG132, or 50 μM MG132. *nac53-1xnac78-1* plants barely progress past germination when grown in the presence of a proteasome inhibitor.
F. Quantification of seedling fresh weights from (E). There is a significant difference in both *nac* double mutant lines compared to WT (p-value <0.05).
G. Single seedling images of 12 DAG plants grown for 6 days on ½ MS media, then transferred to ½ MS plates containing either DMSO or 50 μM MG132 and grown for another 6 days.
H. Quantification of seedling fresh weight from (**G**). Asterisk indicates a significant difference in fresh weight between WT and *nac53-1xnac78-1* seedlings (p-value < 0.01).

Figure 3-9. The *nac53-1xnac78-1* Double Mutant is Hypersensitive to Proteasome-specific Inhibition

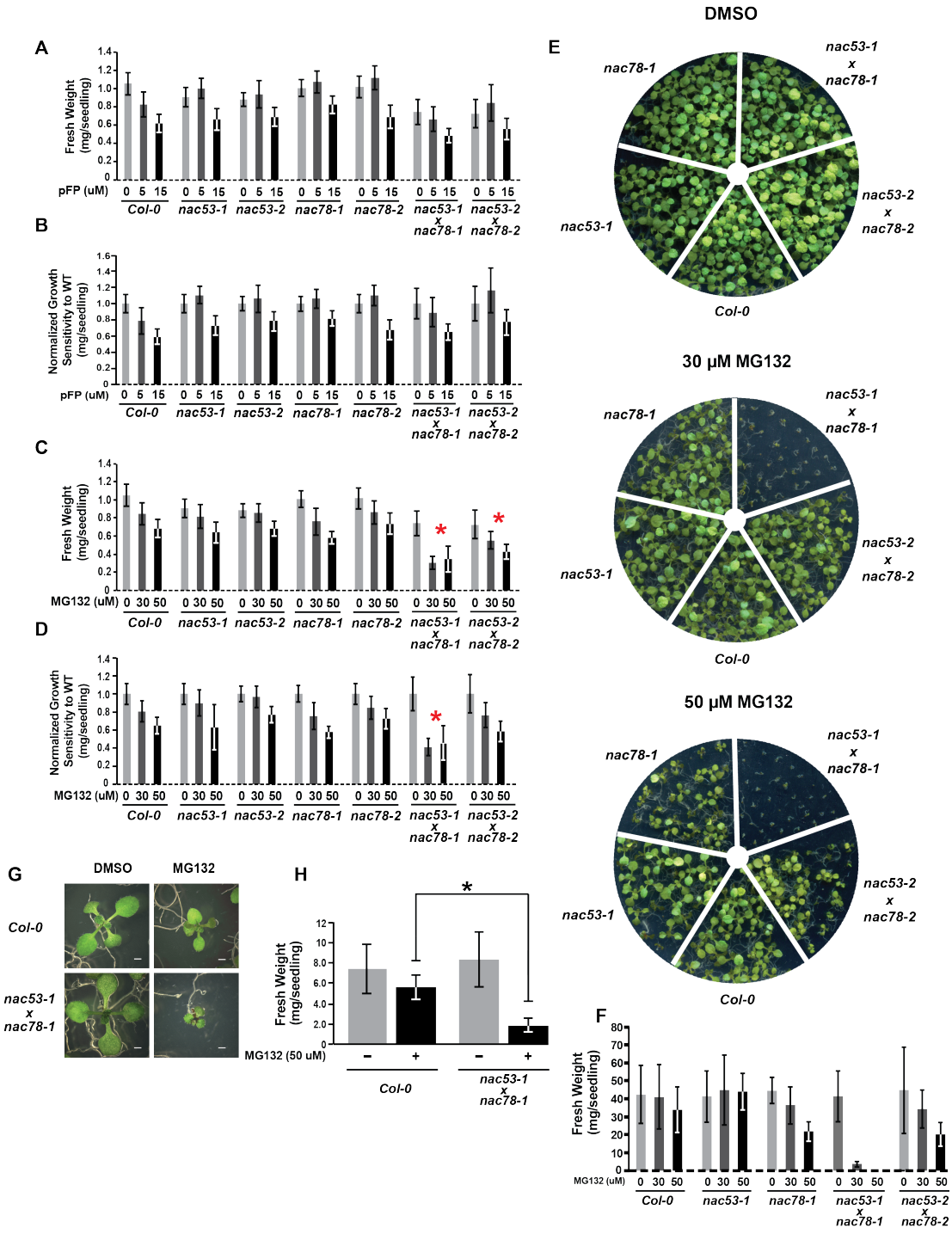


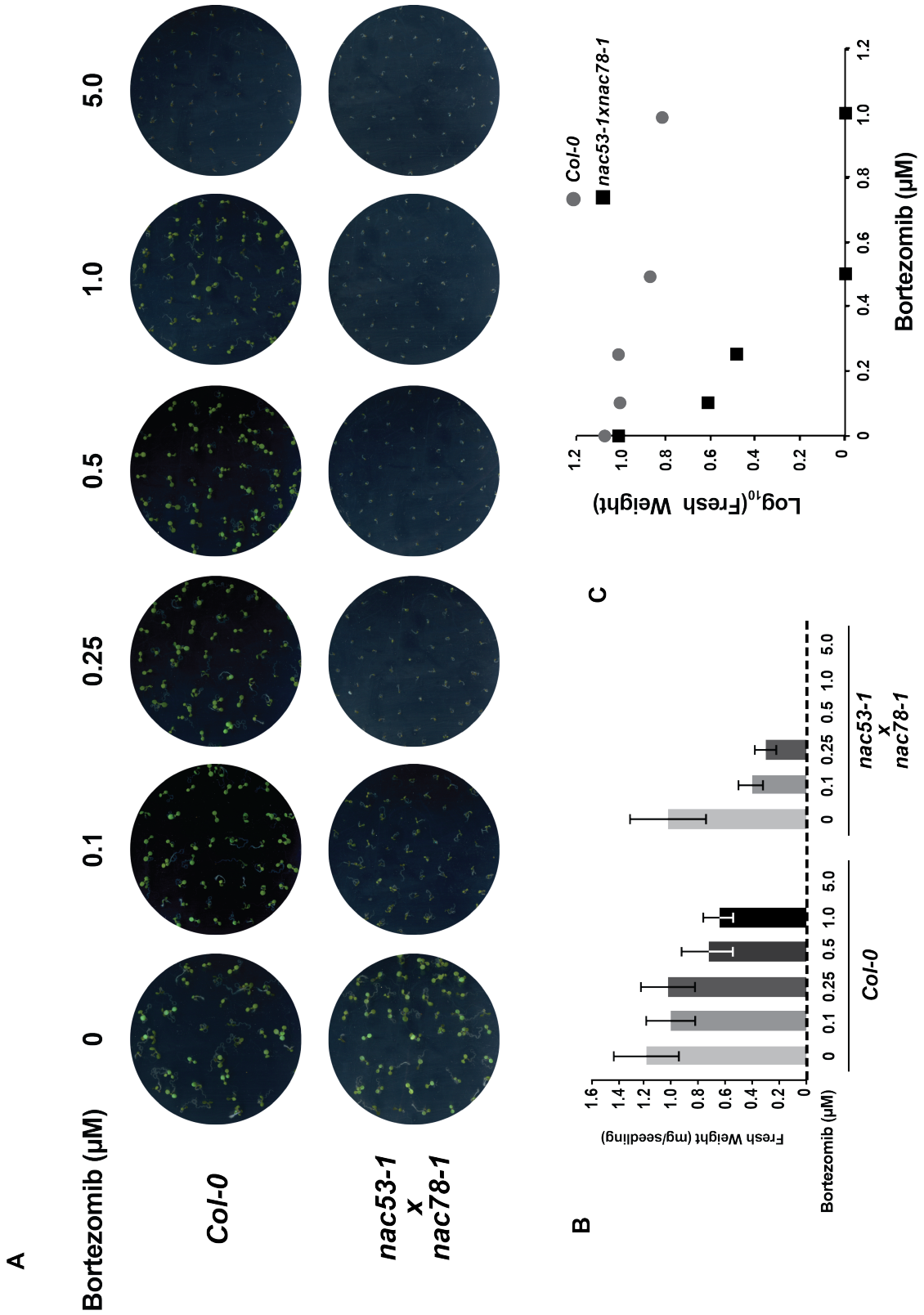
Figure 3-10. **The *nac53-1xnac78-1* Double Mutant is Hypersensitive to Bortezomib**

A. WT and *nac53-1xnac78-1* 6 DAG seedlings germinated and grown on ½ MS media containing either DMSO or varying concentrations of the proteasome-specific inhibitor bortezomib. *nac* double mutant growth is significantly reduced compared to WT at lower concentrations of bortezomib. Both Col-0 and *nac53-1xnac78-1* fail to progress past germination initiation on 5.0 μM bortezomib.

B. Quantification of seedling fresh weight from (A) displaying the significant difference between WT and *nac* seedlings at the lowest concentrations of bortezomib treatment. The *nac* double mutant seeds fail to progress much beyond germination at lower concentrations of bortezomib compared to WT. The difference between fresh weights of *nac53-1xnac78-1* compared WT seedlings during exposure to any concentration of bortezomib is statistically significant (p-value <0.01).

C. Scatter plot of Log_{10}-tranformed fresh weight values from (B) against bortezomib concentrations (grey circles = Col-0, black squares = *nac53-1xnac78-1*). The efficacy of bortezomib sensitivity is significantly higher in the *nac* double mutant background compared to WT Col-0.

Figure 3-10. The *nac53-1xnac78-1* Double Mutant is Hypersensitive to Bortezomib



DISCUSSION

I was able to confirm that *NAC78* overexpression induces the transcriptional up-regulation of UPS genes, but the lack of proteasome subunit protein accumulation during *NAC78* overexpression in β-estradiol-inducible transgenic plants could indicate that only prolonged expression of proteasome transcripts via the *CaMV 35S:NAC78* transgenic lines will result in noticeable proteasome subunit production. However, prolonged overexpression of *NAC78* does not reduce the sensitivity of seedlings grown on MG132-containing media, rather it seems to result in a hypersensitive response to MG132 compared to Col-0. This suggests that *NAC78* expression alone cannot adequately increase proteasome capacity during stress, or that *NAC78* overexpression could actually have a negative impact on growth during proteasome inhibition; possibly by impeding cell-cycle progression through a combination of off-target effects from prolonged MG132 exposure and constant gene activation from increased NAC78 levels. Based on the coordinate/redundant roles of NAC53 and NAC78 in regulating UPS genes, it is also possible that overexpression of both together, rather than just one or the other, would increase proteasome protein levels and generate more robust seedling growth during MG132, bortezomib, or other proteasome-stress treatments.

NAC53 and NAC78 proteins can homo- and hetero-dimerize, localize to the nucleus, and bind to and activate proteasome transcript expression. While the nuclear localization of the NAC53/NAC78 hetero-dimer is strong evidence of them acting together in gene regulation, I was not able to determine if proteasome stress (MG132) results in the translocation of the homo- or hetero-dimer pairs from the cytosol to the nucleus. The cytosolic to nuclear localization of NAC53 and other NTLs, but not NAC78, has been observed in prior studies from *Arabidopsis* protoplasts upon exposure to a variety of stress conditions (Lee et al. 2014).

The presence of either NAC53 or NAC78 is sufficient for UPS gene expression during chemical inhibition of the proteasome (MG132 and bortezomib). Plants lacking both NAC53 and NAC78 are unable to transcriptionally and translationally up-regulate UPS genes during

proteasome inhibition, resulting in significant retardation of seedling development and growth. It should be noted that while basal UPS gene expression is also lower in *nac53-1xnac78-1* seedlings, it is not completely gone. This fact, along with the observation that the PSIR gene *CDC48b*, but not its sister paralog *CDC48a*, expresses normally in the *nac53-1xnac78-1* background suggests there are other transcription factors regulating the PSIR during growth and/or other stress conditions.

Since NAC53 and NAC78 are membrane-bound transcription factors, it is reasonable to assume that the plant proteasome stress-response feedback loop could be similar to that of the mammalian Nrf1. Like Nrf1, NAC53/NAC78 could be localized to the ER and constantly degraded through the ERAD pathway in a CDC48-dependent mechanism. If this is the case, it is interesting that only one *CDC48* paralog (*CDC48a*) is transcriptionally regulated by NAC53/NAC78, while the sister paralog (*CDC48b*) is unaffected by the presence or absence of both *trans*-acting factors. This indicates an evolutionarily distinct relationship between the CDC48 paralogs regarding the UPS response during stress. CDC48a and CDC48b only share 41% identity, so it is likely there are paralog-specific functions in the ERAD pathway, and for 26S proteasome or NAC53/NAC78 post-translational regulation during stress.

Quizzically, I observed that *nac53-1xnac78-1* plants, while sensitive to MG132 and bortezomib, display no growth phenotypes when exposed to pFP. One possible explanation for this is that the autophagy pathway can compensate for the cellular increase in mis-folded proteins, but it cannot compensate for conditions that specifically inhibit proteasome activity (*e.g.* MG132 or bortezomib). Autophagy can sufficiently degrade mis-folded polypeptides, while still allowing existing 26S proteasome complexes to degrade signaling or checkpoint proteins that are essential for growth. Oddly, all *nac* TDNA lines, except *nac53-1xnac78-1*, show a slight hyposensitivity compared to Col-0 at low pFP concentrations (5 μM). This disparity is not significant, but it is a conserved trend that cannot be fully explained.

The *nac* single TDNA mutants and the *nac53-2xnac78-2* double homozygous mutant do not display a hypersensitivity to either MG132 or pFP that is as severe as *nac53-1xnac78-1*. Growth sensitivity does manifest after long-term exposure (10 DAG) to MG132, suggesting that the single mutants and *nac53-2xnac78-2* are not as fit as WT, but still more robust than the *nac53-1xnac78-1* background. This is supported by the fact that the 3' TDNA insertions (*nac53-2* and *nac78-2*) generate at least partial *NAC* mRNA transcripts, likely encoding a functional NAM domain and lacking a trans-membrane domain, which could make for a small population of active transcription factors that can sufficiently activate UPS gene expression.

It is clear that seedlings lacking both NAC53 and NAC78 cannot regulate proteasome genes during chemical inhibition of the CP active sites. Despite this, based on (i) the persistence of basal UPS gene expression in seedlings lacking NAC53 and NAC78, (ii) the continued expression of other UPS/PSIR genes during proteasome inhibition, (iii) the existence of other closely related NAC proteins throughout plant evolution, (iv) the expansive DNA regulatory motif landscape, and (v) the list of potential DNA-binding factors identified through the directed Y1H screen (Chapter 2), it would seem that NAC53 and NAC78, while largely crucial, are probably not the sole regulators of UPS or PSIR genes. Based on the conservation of trans-membrane-bound transcription factors (NAC53/78 and Nrf1), it is possible that other NTLs or NACs could also activate UPS gene expression in *Arabidopsis* or other plant lineages.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The T-DNA insertional mutants for *NAC53* (*NAC53-1*, SALK_009578C; *NAC53-2*, SALK_018311C), *NAC78* (*NAC78-1*, SALK_025098; *NAC78-2*, SALK_040812C) in the *Arabidopsis thaliana* ecotype Col-0 were generated by T-DNA insertional mutagenesis (Alonso et al. 2003) and obtained from the *Arabidopsis* Biological Resource Center (ABRC) at The Ohio State University (https://abrc.osu.edu/). The *rpn10-1* and *rpn12a-1* exon-trap lines in the C24 background were generated as previously described (Smalle et al. 2002, Smalle et al. 2003). The *NAC78* β-estradiol-inducible XVE promoter-driven overexpression transgenic lines (*NAC78* #2138 and #2319) were provided by the TRANSPLANTA resource (Coego et al. 2014), and the RPX (NAC78) *CaMV* 35S-driven overexpression lines were from Nguyen *et al.*, 2013.

Seeds were either surface sterilized with chlorine gas or treated with 80% ethanol then 50% bleach, stratified in the dark at 4^{o} C for 2 days then germinated on 0.7% agar media containing half-strength Murashige and Skoog medium (½ MS; Caisson Labs), 1% sucrose, 0.5% MES, pH 5.7. For growth experiments with MG132, bortezomib, or pFP on plates, media was cooled to 55^{o} C and MG132 (50 mM stock solution in DMSO; SelleckChem), bortezomib (50 mM stock solution in DMSO; SelleckChem) or pFP (10 mM stock solution; Sigma-Aldrich) was added to 25 mL of media and immediately poured into petri dishes. Seeds were grown in liquid culture under continuous white light for RNA and protein isolation, or in long day photoperiods (22^{o}C, 16 hr light/ 8 hr dark) for growth assays and seed bulking. For growth assays, 6-10 DAG seedlings were weighed individually or in batches and then averaged to determine per-seedling fresh weight. At least 15 seedlings were used for all growth quantifications. One-way ANOVA was used to determine statistical significance between WT and mutant seedling fresh weight.

RNA Isolation for RT-PCR and qRT-PCR Analysis

Seedlings were grown in constant light for 5 days in ½ MS in 12-well plates. On the fifth day, seedlings were either collected immediately (No DMSO), treated with 100 μM DMSO for 24 hr or 100 μM MG132 for either 3 hr or 24 hr. Seedlings were pressed between paper towels to remove excess liquid then immediately frozen in liquid nitrogen and pulverized in 1.5 mL centrifuge tubes with sterilized toothpicks. RNA extraction was performed using QIAzol Lysis Reagent (Qiagen). cDNA generation was performed using DNAse1 treatment (Promega) and Superscript III Reverse Transcriptase (Life Technologies).

Primers for qRT-PCR analysis were generated with Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and are as following: *PA200* (LP: GTTTGTCTCGTCACCCTGCT, RP: GCCACCTGCTCATCCTTAGA); *PBA1* (RP: CCTCCAATGGCAAACGGTTG, LP: TGTGATGTACAACCGTCGCA); *RPT1a* (LP: CGATTTGGAAATCCGGCGAC, RP: TGATCTTCTTGGCGAGATCCT); *RPN3a* (LP: TCATCGTCACTTTCTCCCGG, RP: CACAGCACGAGCAATACGAC); *RPN10* (LP: AACCGCAGCTATCCAGATCG, RP: ACATAGCCACTTGCACCCTC); *RPN12a* (LP: TCCTTCATGGAGGGTGCCTA, RP: ACGGAATCTCTTTGCACGGT); *CDC48a (*LP: TGAAGCTGGCTGAAGATGTG, RP: AGATCAGCACCGACGTAACC); *CDC48b* (LP: ACAGAGATTTGCCAGCGTTC, RP: TCCTCTTCCATTGCTTCTGG);*NAC53* (LP: TGT TGG GTG CTA TTC CTG CT, RP: CTG ATG ATT GTG CTG CGT GT); *NAC78* (LP: CAT GAC TCT AGC AGC GGA CA, RP: ATC GCA TCA TCC TGT TGG AC). qRT-PCR amplification was run on a Roche LightCycler 480 using either Roche LightCycler 480 SYBR Green Master Mix or MidSci Bullseye SYBR Green Master Mix.

Phylogenetic Comparison of NAC Family Proteins

Phylogenetic analysis of full-length amino acid sequences of NAC proteins was performed using MrBayes 3.1. bayesian inference program (Ronquist et al. 2012) using the mixed amino acid model (aamodelpr = mixed) until convergence (average standard deviation of split frequencies) reached below 0.05 or plateaued. The rooted tree was generated using Java TreeView.

Immunoblot Analysis

Protein immunoblot analysis was performed by first grinding 6 DAG seedling tissue with a sterile toothpick and then adding 1:4 weight:volume of 2x sample buffer (30 mM Tris pH 6.8, 3.6% SDS, 20% glycerol, 10% BME). Proteasome subunit blots were separated on 11% SDS-PAGE gel and Ub blots on 12% SDS-PAGE. Separated proteins were transferred onto Millipore P-immobilon (Ub-blots) or FL-immobilon (26S proteasome subunit blots) membrane and immunoblotted according to Smalle et al. (2002) with polyclonal antibodies. Antibodies against PA200 (Book et al. 2010), Ub (van Nocker et al. 1996), RPN1, RPN5, RPN10, RPN12a, and RPT2a were as described (Smalle et al. 2002, Yang et al. 2004), and the monoclonal Histone 3 was procured from Abcam (Ab1791).

Bimolecular Fluorescence (BiFC) and Yeast Hybrid Analysis

GV3101 *Agrobacterium tumafaciens* transformed with *NAC53* or *NAC78* full-length CDS cloned into either the CD3-1648 (N-YFP) or CD3-1649 (C-YFP) vectors. Overnight *Agrobacterium* cultures were then diluted to OD600 = 0.5 in re-suspension media (10 mM $MgCL_2$, 10 mM MES-S pH5.6, 100 μM acetosyringolin) and syringe-infiltrated into mature *Nicotiana benthamania* leaves for at least 36 hr prior to visualization. For MG132 treatment or DAPI staining, a re-suspension solution without acetosyringolin was made up with 100 μM

MG132 or 1 μM DAPI and infiltrated into the same leaves 24 hr or 1.5 hr, respectively, prior to visualization on either a Zeiss LSM 510 or 780 confocal laser scanning microscope.

For Yeast 1- and 2-hybrid analysis, MaV203 yeast cells were transformed with the full-length CDS of either *NAC53* or *NAC78* cloned into the Gateway-compatible *pDEST22* or *pDEST23* (Life Technologies). For Y1H, proteasome promoter fragments (bait) were cloned into p*LacZ* vector. A β-Galactosidase assay (Miller assay) was used to quantify transcription factor activation of the reporter *lacZ* gene as described in (Zhang et al. 1996) using at least 3 biological replicates for each measurements. The Y2H analysis was performed by diluting overnight cultures, or picked colonies from plates, to OD_{600} =0.1 in water and then plating yeast cells onto either YPD *leu/trp* to quantify unselective growth or *leu/trp/his* with 3-Amino-1,2,4-triazole to select for pairwise interaction. Vector only controls were also plated to test for spurious activation.

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CHAPTER 4

MOLECULAR CHARACTERIZATION OF THE *ARABIDOPSIS* CP ACTIVATOR PA200

ABSTRACT

The 20S CP has many eukaryotic activating particles. Amongst them, PA200 (Blm10 in yeast) is one of the most well known, having been identified as a CP-binding protein in yeast and characterized as an assembly chaperone. Here, I provide the initial preliminary molecular characterization of *Arabidopsis* PA200 and show it to be a non-essential component of the UPS. Plants lacking PA200 display no discernable growth or developmental phenotype during a variety of cellular stress conditions when compared to WT, however PA200 is highly up-regulated during proteasome stress, indicating that it does play a role in the UPS and that there exists a possible redundancy for its function(s) in plants.

INTRODUCTION

PA200, or **P**roteasome **A**ctivator protein **200**, is a 200-kDa solenoid-shaped protein that contains a putative nuclear localization signal and several HEAT repeats (**H**untington, elongation factor 3 (**E**F3), protein phosphatase 2A (PP2**A**) and yeast PI3-Kinase **T**OR1) make up the C-terminal half of PA200, whereas no functional domains are evident in the N-terminal portion based on Pfam and SMART protein domain predictions (Letunic et al. 2009, Finn et al. 2010). PA200 is known as Blm10 in yeast due to its initial discovery in a sensitivity screen to the DNA-damaging agent bleomycin. Additional analysis has confirmed that yeast cells lacking Blm10 are more sensitive to treatment with DNA-damaging chemicals and conditions, such as methyl methanesulfonate, H_2O_2, and 5-fluro-uracil (Doherty et al. 2012).

At present, the function(s) of Blm10/PA200 are not fully understood; it was originally defined biochemically as a RP alternative activator and a CP assembly chaperone in yeast: opening the axial channel of the CP *in vitro* when bound to the α-ring and binding to CP half-barrels during proteasome assembly (Sadre-Bazzaz et al. 2010). PA200 is likely involved in plant proteasome assembly, based on the conservation of other assembly chaperones; although it is not essential or necessary for this, and any other CP-activating particles could possibly function as assembly chaperones (Lehmann et al. 2008, Stadtmueller et al. 2011).

PA200 seemingly lacks many functional domains present in the RP (Ub-tagged target recognition, ATPase activity for unfolding and translocation of polypeptides, *etc.*); however, this lack of functionality could be replaced by a variety of associated factors (Ub-shuttling proteins, DUBs, etc.). Indeed, some functions of PA200 have been elucidated in yeast. Blm10 has been shown to facilitate the nuclear import of free CP via RAN-GTP and Nup53, a FG-rich nuclear pore protein (Weberruss et al. 2013). Additionally, Blm10-CP proteasomes increase cellular fitness during respiratory growth under oxidative stress, with *blm10*Δ yeast strains displaying mitochondrial dysfunction and decreased morphological integrity (Tar et al. 2014). To understand the importance of PA200 in UPS function, I performed the initial molecular

characterization of PA200 in *Arabidopsis*, shedding some light on its role regarding proteasome regulation and response during proteasome stress.

RESULTS

Arabidopsis PA200 shares only 22% amino acid sequence identity with both its yeast and human counterparts but can be easily recognized by the organization of HEAT repeats which help form its signature solenoid structure (**Figure 4-1a**). To better understand the functions of PA200 in plants, our lab developed a collection of T-DNA insertion mutants that potentially block expression of the single *Arabidopsis* PA200 gene (Book et al. 2010). Both RT-PCR and immunoblot analysis show that two mutant lines, *pa200-2* and *pa200-3*, fail to accumulate full-length PA200 mRNA and protein and thus likely represent null alleles (**Figure 4-1b,c**). *pa200* mutants are phenotypically indistinguishable to wild type under a variety of growth conditions (**Figure 4-1d,e**), including darkness, short and long day photoperiods, continuous red or far-red light, exposure to MG132, and growth in the presence of amino acid (pFP and canavanine) (Smalle et al. 2003). This indicates that PA200 is not essential for UPS function and/or it is redundant to another CP activator.

The levels of Ub, free poly-Ub chains, and Ub-protein conjugates were also unaffected in *pa200* backgrounds as compared to wild type (+/- MG132), suggesting the PA200-CP complex works independently of ubiquitylation (**Figure 4-2a**). Whereas yeast *blm10* mutants are modestly hypersensitive to the DNA damaging agent bleomycin, *pa200-2* and *pa200-3* seedlings were not more sensitivity to this drug when compared to WT. The only response I observed for PA200 was a substantial increase of the protein upon MG132 exposure to *Arabidopsis* seedlings or in the *rpn12a-1* mutant background (**Figure 4-2b**). This accumulation implies that PA200 is either a proteasome substrate, or more likely that PA200, like subunits of the CP and RP (Yang et al. 2004), is part of a regulatory system that coordinately responds to proteolytic demand. The increase in PA200 resulted in more PA200-CP complexes based on

affinity-purified 26S proteasomes, as described in (Book et al. 2010), compared to unstressed plants (**Figure 4-2c**).

DISCUSSION

Seedlings lacking *PA200* do not result in lower fitness during normal or proteasome stress conditions. This indicates PA200 is non-essential for UPS function during conditions that limit proteasome capacity. Our attempts to identify a conditional phenotypic response for *pa200* mutants proved fruitless; I could not identify a nutrient or chemical stress condition that resulted in differential responses between seedlings lacking *pa200* and WT. However, the conditions I tested were modeled from previous investigations into PA200 from other organisms, like yeast and mammalian cells. So it could be possible that the role of PA200 has changed over UPS evolution, and specifically because of the genome duplications in plants, there may be other activators or pathway redundancies that can compensate for the lack of PA200. One such example would be the 19S RP: it is able to bind the CP α-ring and could plausibly act as an assembly chaperone, interacting with CP half-barrels. In either case, more thorough investigations into PA200 are required, and a focus should be given to its potential role in plant proteasome assembly, based on the role of Blm10 in yeast as a CP-maturation factor.

Figure 4-1. Genetic and Phenotypic Characterization of *Arabidopsis* PA200

A. Diagram of the *Arabidopsis PA200* gene. Yellow and white boxes denote coding and 3' UTR exons, respectively. Lines show introns. Coding regions for the HEAT repeats and NLS are in blue and green, respectively. T-DNA insertion sites for the pa200-1 to *-6* mutants are shown in red. Arrows locate the positions of the primers used for RT-PCR analysis in (B).

B. RT-PCR analysis of 2-week-old *pa200-2* and *pa200-3* seedlings. Total RNA isolated from WT and mutant seedlings was subjected to RT-PCR using the *PA200* primers located by the arrows in (A). A primer pair specific to *PAE2* was used as an internal control.

C. Immunoblot analyses of total protein from 1-week-old WT and *pa200-2* and *pa200-3* mutant seedlings with anti-PA200 and anti-UBC1 (loading control) antibodies.

D. Ten-day-old etiolated *pa200-2* and *pa200-3* seedling as compared to WT and *rpn12a-1* seedlings.

E. Twelve-day-old green WT, *pa200-2*, *pa200-3*, and *rpn12a-1* seedlings grown under a long-day photoperiod (16-hr light/8-hr dark).

Figure 4-1. Genetic and Phenotypic Characterization of *Arabidopsis* PA200.

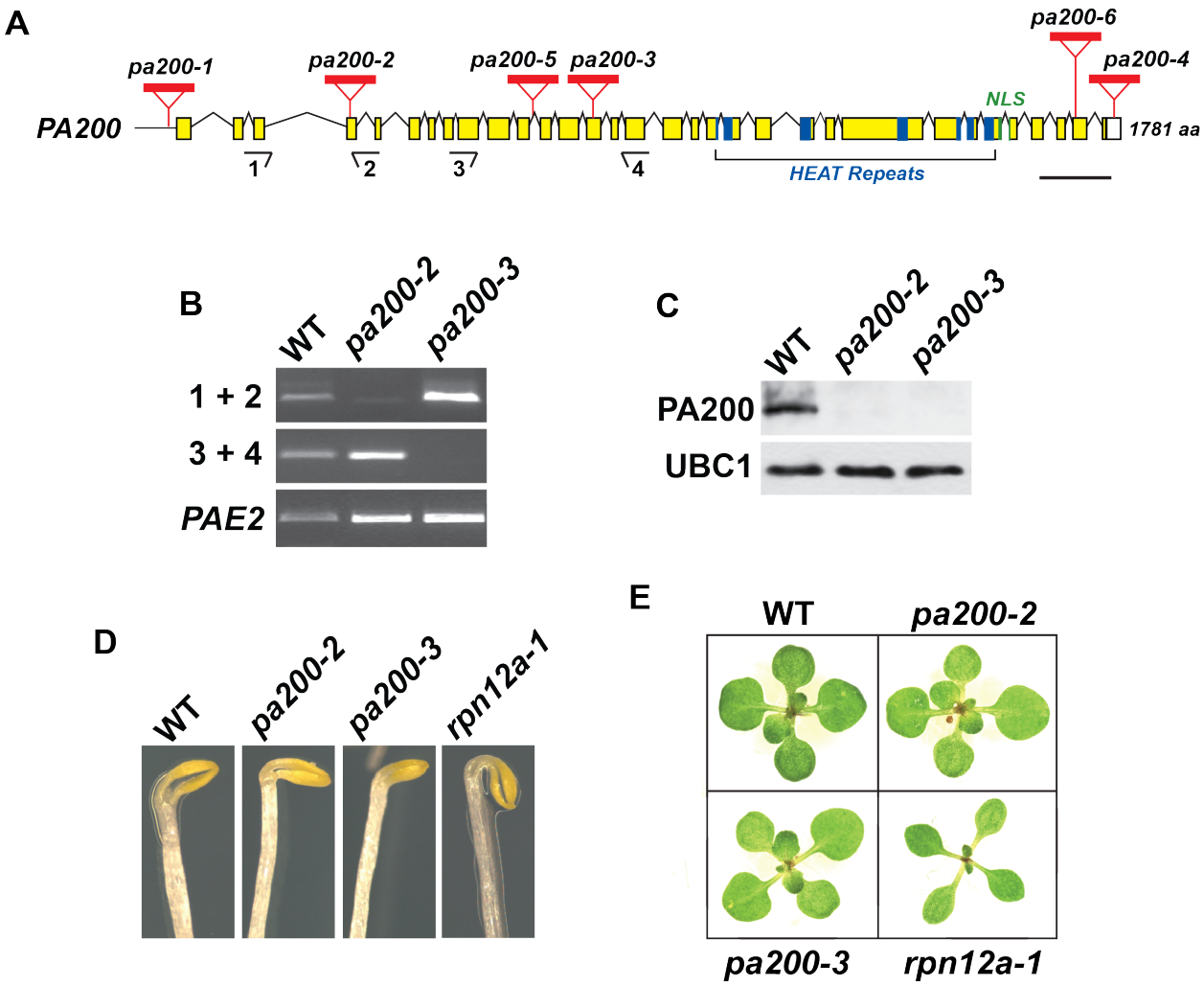


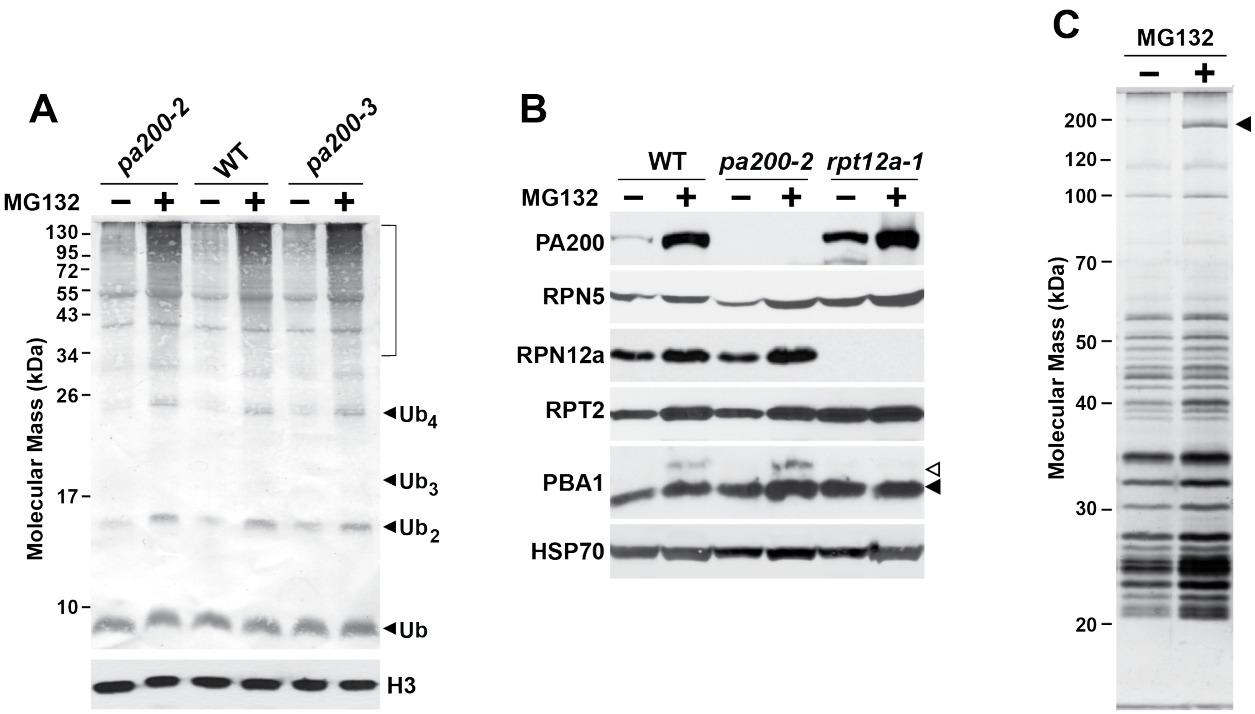
Figure 4-2. PA200 Accumulates During Proteasome Stress

A. Immunoblot analysis of Ub-conjugate levels. Seedlings were treated either with DMSO or 100 μM MG132. Equivalent amounts of total protein were subjected to SDS-PAGE and immunoblotted with anti-Ub antibodies and confirmed by probing with anti-histone 3 (H3) antibodies. Arrowheads indicated free Ub, Ub dimers, trimers, and tetramers. The bracket indicates Ub conjugates.

B. Increased PA200 protein levels in response to proteasome inhibition. Four-day-old WT, *pa200-2*, and *rpn12a-1* seedlings were treated for 30 hr with DMSO or 100 μM MG132. Equivalent amounts of total protein were subjected to SDS-PAGE and immunoblotted with antibodies against PA200, RPN5a, RPN12a, RPT2a, and PBA1, and HSP70 (loading control). The open and closed arrowheads identify the unprocessed and mature forms of PBA1, respectively.

C. PAG1-Flag affinity purification of proteasomes from 10-day-old seedlings treated with or without 100 μM MG132 for 30 hr (as described in Book, Gladman et al., 2010) shows an increase in PA200-CP association during proteasome stress. The arrowhead locates PA200, the identity of which was confirmed by MS/MS analysis of the excised gel slice.

Figure 4-2. PA200 Accumulates During Proteasome Stress



MATERICALS AND METHODS

Plant Materials and Growth Conditions

The TDNA insertional mutants *pa200-2* (SALK_095870) and *pa200-3* (SALK_070184) were acquired from the Arabidopsis Biological Resource Center (ABRC) and tracked by genomic PCR and RT-CPR using the TDNA-specific left border primer (Lba1) in combination with the following gene-specific primer pairs: *pa200-2* (CGTAAGAAGCTGTCTTTGAA and TCTACTGAAAAGCATGGGCAA) and *pa200-3* (CAGTTGCTGCAGCAGCATCCATGTTG and CTGGAATGCGCCACCTGCTCATCCT). The *rpn12a-1* mutant was described by (Smalle et al. 2002). The mutants were backcrossed 3 times to the Col-0 parent and then made homozygous by selfing. Plants were germinated and grown under sterile conditions on solid Gamborg's B-5 growth medium (GM) containing 2% sucrose and 0.7% agar (Gibco BRL, Gaithersburg, MD). For etiolation assays, seedlings were exposed to white light for 24 hr and then transferred to the dark for 10 days before visualization. For MG132 treatment, seedlings were germinated and grown in liquid GM media under constant light for 4 days, then fresh media was exchanged and 100 μM MG132 was added. Seedlings were collected after 30 hr of treatment, pressed flat between paper towels to remove excess liquid and then immediately frozen in liquid nitrogen for further processing.

Immunoblot Analysis and PA200 Antibodies

Protein immunoblot analysis was performed by first grinding 6 DAG seedling tissue with a sterile toothpick and then adding 1:4 weight:volume of 2x sample buffer (30 mM Tris pH 6.8, 3.6% SDS, 20% glycerol, 10% BME). Proteasome subunit blots were separated on 11% SDS-PAGE gel and Ub blots on 12% SDS-PAGE. Separated proteins were transferred onto Millipore P-immobilon (Ub-blots) or FL-immobilon (26S proteasome subunit blots) membrane and immunoblotted according to Smalle et al. (2002) with polyclonal antibodies.

α-PA200 antibodies were generated against partial PA200 fragments and are able to

detect the full-length 200-kDa protein from crude plant extracts. A coding sequence of approximately 800 aa residues of the C-terminus of PA200 was cloned into a pET23b vector and expressed in bacteria and purified over a nickel column. The purified peptide was injected into a rabbit for polyclonal antibody production (Harlan Laboratories, Madison, WI). Antibodies against RPN12a, RPT2a, and PBA1 were as described (van Nocker et al. 1996, Smalle et al. 2002, Yang et al. 2004).

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CHAPTER 5

FUTURE DIRECTIONS AND CLOSING REMARKS

INTRODUCTION

The UPS is a crucial eukaryotic regulatory pathway, playing a central role in many cellular responses to environmental cues as well as general growth and development. The post-translational modification of proteins with poly-Ub moieties through the ATP-dependent E1-E2-E3 enzymatic cascade will target them for degradation at the 26S proteasome holoenzyme, which is composed of the CP and RP subparticles. Protein ubiquitylation and poly-Ub chain recognition by the 26S proteasome encapsulates the target specific nature of the UPS to degrade polypeptides, and distinguishes it from other proteolytic mechanisms such as autophagy or other cellular proteases. My research has focused on the regulation and function the *Arabidopsis* UPS, with the bulk of my efforts emphasized toward elucidating the proteasome stress response and regulating *trans*-acting factors.

In Chapter 2, I described the Proteasome Stress-Induced Regulon (PSIR). This in-depth transcriptomic analysis depicted the complex gene expression network that is stimulated upon challenging cellular proteasome capacity either through small-peptide inhibition (MG132) or through genetic crippling of the proteasome itself (*rpn10-1* and *rpn12a-1* mutants). I identified several expected up-regulated genes within the PSIR (*e.g* 26S proteasome subunits), as well as several novel gene expression profiles that can provide clues as to what genes are most crucial for proper UPS response during stress. I also performed an enrichment analysis for all predicted and experimentally verified DNA-binding motifs that exist upstream of the MG132-induced, PSIR, and proteasome subunits genes and found a vast and diverse landscape of sequence elements that can be flexibly recognized by theoretically hundreds of DNA-binding proteins. This analysis, along with a directed Y1H screen, provided few answers regarding the asymmetrical expression of 26S proteasome subunit paralogs and suggests that UPS gene expression could potentially be modulated by multiple *trans*-acting factors.

Despite such a large field of potential candidates, in Chapter 3 I was able to identify that two NAC-family proteins, NAC53 and NAC78, are transcriptional activators of proteasome gene

expression during proteasome inhibitor treatment (MG132 and bortezomib). I established that *Arabidopsis* seedlings need either NAC53 or NAC78, but not both, to adequately up-regulate proteasome gene expression during MG132 treatment. However, seedlings lacking both NAC53 and NAC78 were unable to up-regulate proteasome transcripts and displayed significant growth retardation during MG132 treatment compared to WT.

In Chapter 4 I performed the first-ever molecular characterization of the *Arabidopsis* PA200 protein, showing it to be non-essential for UPS function. Additionally, I determined that *pa200* mutant plants do not display similar sensitivities or phenotypic responses to cellular stressors that have been observed in yeast or mammalian cells, suggesting PA200 has a unique or redundant function in plants. In this final chapter, I will discuss the future directions for these projects and other avenues of investigation that can continue to increase our knowledge of selective proteolysis in plants.

FUTURE DIRECTIONS

PA200 Localization and Associated Proteins

To further characterize its role in UPS, we must determine where and with what PA200 associates in the cell. To do this, we can generate transgenic plant lines that express an affinity-tagged PA200 protein, using such peptide sequences as GFP, FLAG, 6xHIS, *etc*., and then perform a sucrose gradient fractionation followed by immunoblot analysis to identify where in the cell PA200 localizes. This could be done with our existing α-PA200 antibodies, but creating transgenic plant lines with affinity-tagged PA200, driven by either a native or constitutively-expressing promoter, would allow us multiple avenues of investigation into its localization and characterization of associated factors, as well as any effects of *PA200* overexpression on cellular growth and development. All tags would have to be on the exposed N-terminus of PA200, opposite the CP-binding C-terminal portion of the protein. Additionally,

we can transiently express GFP-PA200 in *Nicotiana benthamania* to visualize cellular localization through fluorescent confocal microscopy.

To identify associated proteins, we can perform co-immunoprecipitation of FLAG-or 6xHIS-PA200 plants via an affinity column, or using α-PA200 antibodies, followed by tandem mass spectrometry (MS/MS). Y2H and bimolecular fluorescence pairwise interaction analyses can be used to confirm associated proteins from the MS/MS screen.

PA200 and 26S Proteasome Assembly

Since it is likely that PA200 plays some role in 26S proteasome assembly, based on the conservation of other eukaryotic assembly chaperones, we can dissect its position in the pathway through interrogation of genetic mutants. *pa200* mutant lines will be crossed into other CP and RP assembly chaperone TDNA mutants to make multiple homozygous null alleles at various points along the stages of CP and RP assembly. Chaperones will include *UMP1, PIP1, PIP2, PBAC3, NAS2, NAS6,* and *HSM3*. Additionally, RP/PA200 double mutants should also be generated, as the RP could act redundantly in CP maturation. If no TDNA exists for a given allele we can generate RNAi or CRISPER lines against that gene. Embryo or seed lethality will be screened in F1 double heterozygous mutant siliques, since the inability to create proteasomes will result in non-viable plants. Double mutant lines that set seed normally (F2 generation) will then be screened for proper Mendelian segregation (assuming linkage equilibrium between alleles) to identify double homozygous null mutants and any unique phenotype arising from complex genetic backgrounds.

Double homozygous null lines will then be analyzed for proper CP and/or RP assembly and stability through glycerol gradient fractionation, although it is likely that any healthy double mutants will not be significantly deficient in proteasome assembly. For any double mutant lines that display embryonic or seed lethality, we can attempt to generate the double mutant combination using hypomorphic alleles either through a “leaky” TDNA insertion or RNAi that

would still express low levels of transcript. Alternatively, we can also rescue lethal phenotypes by generating transgenic lines that constitutively or inducibly express either PA200 or the other assembly pathway gene in the double mutant. Using inducible promoter-driven expression, such as DEX or XVE promoters, could provide greater investigative value, allowing us to halt expression of one or both alleles and then observing any result on proteasome assembly or structural viability. For any mutant or transgenic line combinations described above, plant fitness would also have to be tested during proteasome stress (MG132, bortezomib, pFP, *etc.*) that would require the creation of more 26S proteasomes. Doing so would emphasize any molecular role PA200 plays in assembly and where in the pathway it might be involved.

Tissue-Specific Proteasome Stress Regulon

Now that I have described an in-depth regulatory gene expression network from young seedlings grown during proteasome stress conditions, focus could be shifted to characterizing the tissue-specific regulation of the UPS upon exposure to MG132 or in proteasome mutant backgrounds *rpn10-1* and *rpn12a-1*. This RNAseq transcriptomic analysis will be performed in an almost identical fashion as described in Chapter 2. To summarize: WT or mutant plants will be spot-treated with MG132 or bortezomib on various tissues, including rosette leafs, inflorescences, flowers, caudate leaves, and siliques; mRNA will be extracted and adaptor libraries will be generated for running on a Illumina HiSeq 2500 using 100 bp single-end reads. Differential expression analysis will be initiated through quality control of the RNAseq reads through the Trimmomatic software package. Alignment will then be done using Tophat/Bowtie2. Read tables will be generated using HTSeq algorithm, and finally, differential expression will be quantified using edgeR. Co-expression/interaction maps, expression heat maps, and Gene Ontology (GO) enrichment analysis can be run on each dataset for better visualization of results. We can them compare the similarities between each tissue type for different conditions and proceed to answer any interesting or novel questions that may arise.

Targets of NAC53/NAC78 Regulation

Since I have demonstrated that plants lacking both NAC53 and NAC78 are unable to up-regulate proteasome subunits and some associated factors, it would be interesting to see the scope of genes that these two proteins are responsible for regulating via transcriptomic analysis (RNAseq) and/or DNA-binding (ChIpSeq) analysis. The RNAseq analysis would be performed on the *nac53-1xnac78-1* and *nac53-2xnac78-2* double mutant, as well as the *nac53-1* and *nac78-1* mutants, and compared to WT seedlings (+/-MG132). This would provide an unbiased look at which genes are acted upon when either or both NAC53 and NAC78 are present and when they are both lacking. Genes that fail to be up-regulated in the *nac53-1xnac78-1* background during short MG132 treatment (< 5-6 hrs) are likely direct targets of the transcription factor homo- and hetero-dimers. Additionally, ChIpSeq could identify the NAC53/NAC78 binding sites within target genes, although ChIpSeq does not always yield reliable results based on the low efficacy of trapping the protein-DNA interaction through chemical fixing. It would be better to perform the RNAseq analysis first, and then continue with ChIpseq if deemed necessary.

In addition to MG132 treatment, I could expose *nac53-1xnac78-1* plants to a variety of other stress conditions and observe for any similar hypersensitive phenotype, then also use those treatment conditions as a part of the RNAseq analysis to further interrogate the NAC53/NAC78 regulatory network. Some examples of other stress conditions to be evaluated would be oxidative stress (methyl viologen), heat shock, ionizing radiation, osmotic stress (NaCl, mannitol), DNA-damage (bleomycin), and the host-triggered immune response (*Psuedomonas* or flg22 incubation).

Gene candidates from RNAseq can be confirmed as direct targets of NAC53/NAC78 regulation through Y1H: using the upstream DNA sequences of the target gene as bait and either NAC53 or NAC78 as prey. To determine what DNA-binding sequence motifs

NAC53/NAC78 recognize I can perform a MEME analysis on all candidate gene upstream sequences. If there are any significantly enriched motifs in the promoter sequences, they can then be confirmed in multiple ways: (i) through promoter truncation analysis, using fragments of the upstream region that either contain or lack the motif of interest as bait and either NAC53 or NAC78 as prey, and/or (ii) through electrophoretic mobility shift assays (EMSA). EMSA would be performed by incubating the promoter fragments that contain or lack the motif with exogenously-expressed NAC53 and/or NAC78 proteins and visualizing any size shift on an PAGE gel. The benefit of EMSA is that it could show if only NAC53, NAC78, or both are required for motif binding *in vitro*, whereas the Y1H analysis can only show if the homo-dimer, but not the hetero-dimer, can bind. The downside of EMSA is it requires soluble exogenous protein expression, but the transmembrane domain-containing NAC proteins are not always easy to express in bacteria, and may not be soluble once produced.

Alternative Proteolytic Pathways That Do Not Require NAC53 and NAC78 Regulation

While I did determine that the cells lacking both *NAC53* and *NAC78* are unable to up-regulate UPS genes during proteasome inhibition with MG132 or bortezomib, I discovered that *nac53-1xnac78-1* seedling growth was not significantly affected during pFP treatment; meaning that challenging cellular proteasome capacity in a non-proteasome specific manner is insufficient to cause growth defects in seedlings that are unable to up-regulate 26S proteasome expression. This suggests that other proteolytic pathways can compensate for diminished proteasome gene expression in the *nac53-1xnac78-1* background during certain stress conditions. The two major possibilities are either autophagy or other cellular proteases. Autophagic compensation in the *nac53-1xnac78-1* mutant can be tested through simultaneous inhibition of both the UPS and autophagy pathways. The UPS will be specifically inhibited with MG132 or bortezomib and the autophagy pathway can be inhibited either chemically with Concanamycin A or through genetic mutants, such as *atg7-1*. Seedlings deficient in both

autophagy and NAC53 and NAC78 will show significant growth sensitivity to pFP or other more mild forms of proteolytic stress compared to WT and other controls. However, if autophagy does indeed compensate for the lack of UPS up-regulation during certain proteasome-stress conditions, then the combination of an autophagy mutant in the *nac53-1xnac78-1* background will likely be embryonically lethal, so chemical inhibition of autophagy should be prioritized.

The NAC53/NAC78 Regulatory Feedback Mechanism

I established that NAC53 and NAC78 are regulators of UPS gene expression during proteasome-specific stress. The next step in understanding this regulation is to clarify the feedback mechanism that results in NAC53 and NAC78 activation and translocation into the nucleus. Both NAC proteins contain a trans-membrane motif-1-like domain. It is unknown which membrane the NAC proteins dock to, but it is likely the ER membrane based on other proteins that contain the similar domains. During stress conditions, the NAC proteins are proteolytically cleaved away from the membrane they are bound to, generating a mature DNA-binding domain-containing protein that can then activate gene expression (Lee et al. 2012). It is possible that NAC53 and NAC78 share a similar regulatory scheme to that of Nrf1 in mammals, which is also a trans-membrane domain-containing UPS transcription factor: Nrf1 is translocated into the ER membrane in a cytosolic-facing orientation; degraded constantly by the 26S proteasome via ERAD; and proteolytically cleaved away from the ER membrane during proteasome stress and then transported into the nucleus to activate gene expression (Radhakrishnan et al. 2010). For Nrf1, all of these steps, except for nuclear import, are dependent upon p97 (CDC48 in plants) (Radhakrishnan et al. 2014). CDC48-dependence would have to be evaluated genetically through *cdc48a* and *cdc48b* mutants and potentially other ERAD pathway mutants. Additionally, chemical inhibition of CDC48 through DBeQ could be used to interrogate CDC48 function in NAC protein regulation.

To determine if NAC53 and NAC78 are also ER-bound and substrates of the 26S proteasome via ERAD, I could generate antibodies against full-length NAC53 and NAC78 proteins or transgenic plant lines containing full-length NAC proteins with different affinity tags (*e.g.* FLAG and 6xHIS) translationally fused to the N- and C-terminus of the transcription factor. Both the anti-NAC antibodies and transgenic lines would allow me to verify if the NAC proteins are cleaved away from their bound membrane during stress. They would then generate two protein products that can be visualized through immunoblot: (i) a higher molecular weight band that represents the full-length, inactive transcription factor bound to the membrane, and (ii) a lower molecular weight product that represents the cleaved protein not associated with the membrane. To determine if the NAC proteins are membrane bound, I can perform sucrose gradients on plant tissue treated with or without MG132 and then immunoblot against either the NAC proteins or the affinity tags in the sedimentation profile to determine if the full-length or processed NAC products localize to the nucleus, cytosol, or organellar membrane-containing areas of the gradient. If the above model is correct, I would expect only the mature, processed form of the NAC proteins would be in the cytosol and nucleus, and only the full-length protein to be localized to the ER or other membrane containing regions. If this processing model holds true, the dual affinity-tagged NAC protein transgenic lines would allow me to determine the orientation of the transcription factor as it is bound in the ER: only one affinity tag would be cytosolically exposed and processed and could be detected in the cytosol and nucleus through immunoblots of sucrose gradients.

CLOSING REMARKS

My thesis has contributed to the understanding of the regulation and function of the 26S proteasome and UPS during stress conditions. I am the first to genetically and molecularly characterize *Arabidopsis* PA200, showing its role in the UPS may be different in plants as compared to yeast and other organisms. I have also performed the most detailed transcriptomic analysis of the proteasome stress gene regulatory network to date in any organism, shedding light on the numerous developmental and environmental response pathways that share similar gene regulation with the PSIR. Finally, I have established that NAC53 and NAC78 are transcriptional activators of proteasome subunits and some associated factors, and that plants lacking both these crucial *trans*-acting factors are severely deficient in situations that specifically inhibit proteasome activity. Additionally, due to the close relationship between NAC53 and NAC78 across plant evolution, it is likely they are both a part of a conserved feedback mechanism that is responsible for regulating UPS activity across multiple lineages.

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