FINAL PROJECT REPORT – DOE Project DE-SC0016589

Title: **MOLECULAR DISSECTION OF THE *ARABIDOPSIS* 26S PROTEASOME**

Principal Investigator: ***Richard D. Vierstra***
Department of Genetics, University of Wisconsin & Department of Biology, Washington University in St. Louis

Project Summary

The 26S proteasome is a 2.5-MDa, ATP-dependent protease complex responsible for degrading many important cell regulators in plants and animals, especially those targeted by ubiquitylation. Its 64 or more subunits generate two subparticles: a 20S core protease (CP) that houses the proteolytic active sites and a 19S regulatory particle (RP) that binds to both ends of the CP and recruits appropriate substrates for breakdown. Despite its importance, we had only a rudimentary appreciation of the structure and functions of the core 26S proteasome from plants, and hints that the previously defined complex actually represents the nucleus of an even more elaborate and dynamic particle harboring multiple routes for substrate recognition and sophisticated mechanisms to control its assembly/location. Genomic analyses also suggested that plants in particular exploit this heterogeneity to generate a wide array of proteasome types, each with distinct compositions, locations, and/or functions/specificities.

The goal of this DOE BES-funded research was to better define the 26S proteasome and its diversity in plants by a thorough analysis of the particle from *Arabidopsis thaliana*. During this completed work, we developed stringent affinity methods to rapidly isolate 26S proteasomes intact from *Arabidopsis* seedlings, and identified by mass spectrometry all the main CP and RP subunits along with a suite of interacting proteins. We also identified a number of post-translational modifications, and detected an alternative proteasome containing the CP capped by the PA200 protein. We then amassed a large collection of mutants eliminating most CP and RP subunits and notable accessory proteins such as PA200. Phenotypic analyses of a few RP mutants support the notion that the *Arabidopsis* 26S proteasome is functionally heterogeneous. Through both genetic and biochemical analyses, we characterized a collection of shuttle proteins that help deliver ubiquitylated substrates, and discovered a subset of accessory proteins that likely act as chaperones that facilitate CP and RP assembly. These chaperone activities were supported by examining 26S proteasome assembly in plants missing these factors, which showed that the mutants accumulated high levels of partially assembled subparticles.

In an effort to understand how the numerous genes encoding the various proteasome subunits are coordinately regulated to assemble the 64-subunit core complex and ultimately maintain adequate proteolytic capacity, we discovered by RNA-seq that most *Arabidopsis* genes required to create the 26S proteasome are coordinately regulated transcriptionally. Through analysis of this 'proteasome-stress' regulon by protein-DNA binding, and reverse genetic methods, we identified the *cis* and *trans*-acting elements involved, that center around a pair of NAC transcription factors (NAC53 and NAC78) binding to a consensus proteasome-related *cis* element (PRCE) upstream of most proteasome-associated genes.

And finally, we discovered that proteasomes are rapidly degraded by autophagy in response to nutrient stress or inactivation via a process we called proteaphagy. Whereas the former turnover is triggered by the ATG1-kinase complex working downstream of stress sensors such as the TOR kinase, the latter turnover is induced by substantial ubiquitylation of the complex followed by recognition of the appended ubiquitin moieties by the ubiquitin-binding autophagy receptor RPN10. Surprisingly, studies with RPN10 revealed that it represents the founding member of a new class of autophagy receptors that exploit a novel way to tether cargo to autophagic membranes. Instead of using the canonical AIM motif to bind ATG8 lining the autophagic membranes, it and relatives exploit a UIM motif to bind ATG8 at an alternative site. Studying the same inhibitor-induced proteaphagy in yeast revealed that an unrelated autophagy receptor Cue5 is required to recognize the bound ubiquitins. Strikingly, we found that carbon starvation also induces the accumulation novel cytoplasmic particles called proteasome storage granules that appear to protect proteasomes from autophagy. Their accumulation requires the CP-binding protein PA200 along with the RP-binding proteins Spg5.

Taken together, this research revealed the composition of plant 26S proteasome, how it is assembled, and how is abundance is regulated transcriptionally and by autophagy. Collectively, this research enhanced our appreciation of the plant 26S proteasome and its roles in ubiquitin-mediated protein turnover, and identified points of opportunity where its abundance or activity can be manipulated to improve food and biofuel crops.

Progress on Specific Aims:

(The list of publications associated with this project are listed after the results descriptions.)

Aim 1: Improved and Deeper MS Analysis of Affinity Purified Proteasomes.

To help define the organization of the 26S proteasome, we developed affinity methods to purify both the CP and RP sub-complexes. For the CP, we engineered transgenic *Arabidopsis thaliana* lines expressing the CP α7 subunit PAG1 as a FLAG tagged version in the *pag1-1* null mutant background, and for the RP, we engineered transgenic lines expressing the base subunit RPT4 encoded by either the a or b isoform in their respective *rpt4a* or *rpt4b* null mutant backgrounds [1,8]. The 26S particle could then be rapidly enriched from either the *PAG1-FLAG pag1-1*, *FLAG-RPT4a rpt4a-1*, or *FLAG-RPT4b rpt4b-1* lines by a single anti-FLAG antibody affinity step if ATP is included in the buffers to retain the 26S complex [14]. However, if ATP is omitted, the CP and RP sub-complexes dissociate and could be enriched selectively. Using tandem mass spectrometry (MS) sequencing approaches, we identified all the expected CP and RP subunits as well as the PA200 protein that often caps the CP [1,8]. Much of these analyses were aided by a new search program written for Morpheus that provides semi-quantitative information of protein abundance based on peptide spectral matches [5]. Interestingly, affinity purification of the RP by either the a or b isoforms of RPT4 enriched for all isoforms of the other CP and RP subunits [14], indicating that *Arabidopsis* and likely other plants do not use isoform diversity to create unique 26S proteasomes assembled with matching pairs of isoforms.

Aim 2: Describe the Assembly Pathway for 26S Proteasomes.

In addition to identifying core subunits of the 26S proteasome, the MS analyses detected a number of accessory proteins, including several that are related to chaperones that help assemble the yeast proteasome [14]. Included are likely orthologs of the UMP1, PBAC1, PBAC2, PBAC3 and PBAC4 proteins that assist in CP assembly, NAS2, NAS6, and HSM3 that assist in RP assembly, and ECM29 that helps dock the CP with the RP. *Arabidopsis* T-DNA insertion mutants compromising the two *UMP1* genes were found to be embryo lethal, indicating that this protein is essential for CP assembly [14]. By contrast, a mutant missing PBAC1 is viable but has an up-regulated proteasome stress regulon, and accumulates partially assembled CP fragments, suggestive of assembly defects [14]. We also identified a novel chaperone designated PBAC5 that is related to PBAC1 and PBAC2; likely orthologs could be found in all land plants and some microorganisms but not present in animals [15]. Both yeast-two-hybrid and bimolecular fluorescence complementation assays indicated that it forms a trimeric complex with PBAC1 and PBAC2. Interestingly, while yeast PBAC1 and PBAC2 are needed for assembly of the CP in yeast, only when *Arabidopsis* PBAC1 and PBAC2 were expressed together with PBAC5 could they complement the yeast *pbac1 pbac2* null mutant, indicating all three work together in assembling the CP in *Arabidopsis* [15].

To help understand how the various chaperones coordinated 26S proteasome assembly, we isolated proteasomes after exposure to the proteasome inhibitor MG132, which stimulates proteaphagy and activates the proteasome stress regulon, thus enhancing the accumulation of partially assembled particles. Upon separation of the 26S, CP, RP, and these partially assembled particles by native PAGE followed by MS analysis, we could assign the individual chaperones to specific assembly intermediates [15].

Aim 3. Defining the Proteasome Stress Regulon.

Proteotoxic stress is mitigated by a variety of mechanisms, including activation of the unfolded protein response and coordinated increases in protein chaperones and activities that direct proteolysis such as the 26S proteasome. Using RNA-seq analyses combined with either chemical inhibitors or mutants that induce proteotoxic stress by impairing 26S proteasome capacity, we in the Gladman *et al.* (2016) paper defined the transcriptional network that responds to this stress in *Arabidopsis* [6]. Included are genes encoding core and assembly factors needed to build the complete 26S particle, alternative proteasome capping factors, enzymes involved in protein ubiquitylation/deubiquitylation and cellular detoxification, protein chaperones, autophagy components, and various transcriptional regulators. Many loci in this proteasome-stress regulon contain a consensus *cis* element upstream of the transcription start site which we designated as the Proteasome-Related *Cis* Element (PRCE) [6]. Notably, this sequence was previously identified as a binding site for the NAM/ATAF1/CUC2 (NAC)-78 transcription factor. Double mutants disrupting *NAC78* and its closest relative *NAC53* are compromised in the activation of this regulon, and notably are strongly hypersensitive to the proteasome inhibitors MG132 and bortezomib, indicating that this two factors help control this regulon [6]. Given that NAC53 and NAC78 homo- and hetero-dimerize, we propose that they

work as a pair in activating the expression of numerous factors that help plants survive proteotoxic stress, and thus play a central regulatory role in maintaining protein homeostasis.

Aim 4. Discovery of Proteaphagy in *Arabidopsis*

Autophagic turnover of intracellular constituents is critical for cellular housekeeping, nutrient recycling, and various aspects of growth and development in eukaryotes. During the investigation of proteasome levels in various autophagy mutants, we found in the Marshall et al. (2005) paper that autophagy also impacts the ubiquitin-proteasome system by eliminating 26S proteasomes in a process we termed proteaphagy [3,4]. Using *Arabidopsis* proteasomes tagged with GFP, we observed their deposition into vacuoles via a route requiring components of the autophagy machinery. Deposition and degradation could be observed by the confocal microscopic observation of proteasomes moving from the nucleus (where most proteasomes are located) to the vacuole, and by SDS-PAGE analysis of the GFP-fusion, which releases free GFP when the fusion protein is deposited inside the vacuole and then degraded by autophagy. This transport could be separately induced by nitrogen starvation and requires that upstream ATG1 kinase or by chemical or genetic inhibition of the proteasome through an ATG1-independent route [3,4]. Remarkably, we found that proteasome inhibition induced comprehensive ubiquitylation of the complex, which then directed recognition of the ubiquitin moieties by the ubiquitin-binding protein RPN10. RPN10 then binds the core autophagy component ATG8 embedded in the autophagy membranes thus allowing the proteasome cargo to be trapped inside and transported to vacuoles [3]. This proteaphagy appears to be important for disease resistance, as the plant pathogen *Pseudomonas syringae* uses proteasome turnover by autophagy to enhance its virulence [10].

Aim 5. Characterizations of Proteaphagy in Yeast.

Our striking discovery that dysfunctional plant 26S proteasomes are cleared by autophagy identified a novel route for particle regulation, which involves ubiquitylation of inactivated proteasomes followed by their recognition by the autophagic receptor RPN10. Given the potential importance of proteaphagy to human health, especially as it relates to the now widespread use of proteasome inhibitors for cancer treatment, we sought to determine if proteaphagy is common outside of plants, using yeast as the model. In the Marshall et al. (2016) paper incorporating the same approaches perfected with *Arabidopsis*, we indeed found similar proteaphagic pathways, one sensitive to nutrient demand and regulated by the Atg1 kinase and a second the removes inactivated proteasomes independent of Atg1 [7]. The second route employs a completely different autophagic receptor Cue5, one of a family of proteins with a signature ubiquitin-binding CUE domain that also uniquely binds Atg8 lining the autophagic membranes. Intriguingly, we also found that proteaphagy of inactive proteasomes first involves their cytoplasmic aggregation [7]. This sequestration occurs within insoluble protein deposits (or IPODs), identified by the IPOD marker Rnq1, and like other IPOD-destined cargo requires the Hsp42 chaperone for its aggregation [7]. Together, ubiquitylation, Cue5, Hsp42, and IPODs provide a quality control checkpoint in yeast directed at recycling inactive 26S proteasomes.

Importantly, IPODs have recently emerged as key protein graveyards essential for protecting us against various aggregation-prone neurodegenerative disorders, including Alzheimer's and Huntington's. Thus, inhibitor-induced proteaphagy offers a facile model to understand the connections between damaged proteins, aggregation, and final autophagic clearance as related to these debilitation pathologies, as well as their likely roles in maintaining protein homeostasis in plants.

Aim 6. Roles of Proteasome Storage Granules in Protecting Proteasomes from Autophagy

Surprisingly, while nitrogen starvation rapidly induces proteaphagy in both *Arabidopsis* and yeast, the lack of fixed carbon does not. Instead in the Marshall and Vierstra (2018) paper, we discovered that fixed-carbon starvation stimulates the aggregation of proteasomes into cytoplasmic foci previously called proteasome storage granules (PSGs) [11]. Both the CP and RP sub-complexes are sequestered separately into PSGs via pathways dependent on the accessory proteins Blm10 and Spg5, respectively. Modulating PSG formation, either by perturbing cellular energy status or pH, or by genetically eliminating factors required for granule assembly inversely influences the rate of proteasome degradation, indicating that PSGs represent a protective response from proteaphagy [11]. PSG assembly also enhances cell viability upon recovery from carbon starvation, implying that PSGs represent an evolutionarily conserved cache of proteasomes that can be rapidly re-mobilized based on energy availability [11,12]. The discovery of PSGs and IPODs adds to the list of dynamic cytoplasmic puncta collectively now referred to as biomolecular condensates that are emerging as critical effectors of cellular metabolism.

Aim 7. Defining a New Class of Autophagy Receptors Bearing Ubiquitin-Interacting Motifs.

During autophagy, cargo recruitment and vesicle dynamics are driven by numerous receptors/adaptors that become tethered to the autophagic vesicles through simultaneous interactions with their respective cargo/partners and lipidated ATG8 decorating the expanding membrane. Most currently described ATG8-binding proteins exploit a well-defined ATG8-interacting motif (AIM, or LC3-interacting region (LIR)) that contacts a hydrophobic patch on ATG8 known as the LIR/AIM-docking site (LDS). In a Marshall et al. (2019) manuscript currently under review by *Cell*, we described a new class of ATG8 interactors that exploit ubiquitin-interacting motif (UIM)-like sequences for high-affinity binding to an alternative ATG8 interaction site [13]. Assays with candidate UIM-containing proteins together with unbiased screens identified a large collection of UIM-based ATG8 interactors in plants, yeast, and humans. Analysis of a subset also harboring UBX domains revealed a role for UIM-directed autophagy in clearing CDC48/p97 complexes that become non-functional or impaired in several human neurological diseases [13]. CDC48/p97 is a AAA-ATPase that assembles into homo-hexamers with important disaggregase activities crucial for mitigating proteotoxic stress and for removing misfolded ER proteins after retrograde transport back to the cytoplasm. With this new class of adaptors/receptors, we greatly extended the reach of selective autophagy and potentially identify new factors regulating autophagic vesicle dynamics.

The sum total of this DOE BES-funded research also led to the preparation of an extensive review of autophagy in plants that was published by the Annual Reviews of Plant Biology in 2018 [9].

Publications Derived from the DOE Project (DE-FG02-ER1396)

b-1. Journal Articles and Accepted Manuscripts:

1. Russell, J.D., M. Scalf, A.J. Book, D.T. Ladror, R.D. Vierstra, L.M. Smith, and J.J. Coon (2013) Characterization and quantification of intact 26S proteasome proteins by real-time measurement of intrinsic fluorescence prior to top-down mass spectrometry. *PLoS One* 8: e58157.
2. Hua, Z., and R.D. Vierstra (2016) Spotlight: Ubiquitin goes green. *Trends Cell Biol.* 26: 3-5.
3. Marshall, R.S., F. Li, D.C. Gemperline, A.J. Book, and R.D. Vierstra (2015) Autophagic degradation of the 26S proteasome is mediated by the dual ATG8/ubiquitin receptor RPN10 in *Arabidopsis*. *Mol. Cell* 58: 1053-1066 *(highlighted in* Mol. Cell *by Bonnie Bartel and in* Autophagy *by Daniel Klionsky; selected by Faculty of 1000; blogspot.com/2016/06/what-is-morpheus-up-to-these-days.html*)
4. Marshall, R.S., and R.D. Vierstra (2015) Eat or be eaten: the autophagic plight of inactive 26S proteasomes (Commentary). *Autophagy* 11: 1927-1928. doi 10.1080/15548627. 2015.1078961.
5. Gemperline, D.C., M. Scalf, L.M. Smith, and R.D. Vierstra (2016) Morpheus Spectral Counter: a computational tool for quantitative mass spectrometry using the Morpheus search engine. *Proteomics* 16: 920-924 (doi:10.1002/pmic.201500420). (*highlighted in* Proteomics News*, http://proteomicsnews*).
6. Gladman, N.P., R.S. Marshall, K.-H. Lee, and R.D. Vierstra (2016) The proteasome stress regulon is controlled by a pair of NAC transcription factors in *Arabidopsis*. *Plant Cell* 28: 1279-1296.
7. Marshall, R.S., F. McLoughlin, and R.D. Vierstra (2016) Autophagic turnover of inactive 26S proteasomes in yeast is directed by the ubiquitin receptor Cue5 and the Hsp42 chaperone. *Cell Rep*. 16: 1717-1732 (*highlighted in https://source.wustl.edu/2016/08/big-trash-pickup/ and the DOE site http://science.energy.gov/*)
8. Marshall, R.S., D.C. Gemperline, and R.D. Vierstra (2017) Purification of 26S proteasomes and their subcomplexes from plants (Chapter 24). In: *The Isolation of Plant Organelles and Structures: Methods and Protocols* (Methods in Molecular Biology series). (N. Taylor and H. Miller eds) Springer Science, Berlin. 1511: 301-334.
9. Marshall, R.S., and R.D. Vierstra (2018) Autophagy; the master of selective recycling. *Annu. Rev. Plant Biol.* 69: 173-208. doi:10.1146/annurev-arplant-042817-040606.
10. Ustun, S, A. Hafren, Q. Liu, R.S. Marshall, E.A. Minina, P.V. Boshkov, R.D. Vierstra, and D. Hofius. (2018) Bacteria exploit autophagy for proteasome degradation and enhanced virulence in plants. *Plant Cell* 30: 668-685. (doi:10.1105/tpc.17.00815) (*paper highlighted in Plant Cell by J Gallegos*)
11. Marshall, R.S., and R.D. Vierstra (2018) Proteasome storage granules protect 26S proteasomes from autophagic clearance during carbon starvation. *eLife* 2018;7:e34532.doi:10.7554/eLife.34532. (*highlighted by eLife as important read*)
12. Marshall, R.S., and R.D. Vierstra (2018) To save or degrade: balancing proteasome homeostasis to maximize cell survival (Commentary). *Autophagy* doi.org/10.1080/15548627.2018. 1515531.

b-2. Journal Articles Submitted or In Preparation

13. Marshall, R.S., Z. Hua, S. Mali, F. McLoughlin, and R.D. Vierstra (2019) ATG8-binding UIM proteins define a new class of autophagy receptors and adaptors. *Cell* (*under review*).
14. Gemperline, D.C, R.S. Marshall, A.J. Book, M. Scalf, L.M. Smith, and R.D. Vierstra (2019) Mass spectrometric analysis of 26S proteasomes from *Arabidopsis* under proteotoxic stress identify a collection of proteasome assembly chaperones. *J. Biol. Chem.* (*in preparation*).
15. Marshall, R.S., D.C. Gemperline, A.J. Book, and R.D. Vierstra (2019) Plants and microorganisms engage a novel proteasome chaperone to assemble the core protease subcomplex. *J. Biol. Chem.* (*in preparation*).