
Preface

Plant responses to environmental stimuli and developmental transitions are regulated by complex regulatory networks that deliver the specific physiological outcome to assure plant survival. These networks include transcriptional regulation but also sophisticated posttranslational modifications that aim to regulate protein activity. In contrast to transcriptional regulation, which involves *de novo* protein synthesis, posttranslational modifications modulate protein activity in short time periods facilitating rapid cell responses. The molecular consequences of posttranslational modifications on the protein target are highly variable and include changes in protein structure, subcellular localization, activity, partner interactions, stability, or solubility.

Proteins can be modified by a wide array of compounds that vary in their nature, size, and conjugation mechanism. As such, reactive oxygen species induce protein oxidation independently on enzymatic catalysis, while other posttranslational modifications involving the addition of small organic groups (i.e., phosphate or methyl groups) are regulated by enzymes dedicated to the addition or removal of the specific modifier. Finally, one of the most complex posttranslational modification groups is represented by the ubiquitin (Ub) and ubiquitin-like (Ubl) modifiers, which are small proteins that are conjugated to protein targets through a cascade of three enzymatic steps and deconjugated by specific peptidases.

The branching complexity of post-translational modifications, together with their labile nature and the need of custom-tailored molecular tools, make their analysis really challenging. In plants, the absence of well established commercial tools, the more complex plant cell manipulation required for biochemical studies, and the gene amplification displayed by many members of these regulatory components, result in a higher difficulty degree of biochemical and genetic studies. The analysis of protein homeostasis is even more complex in non-plants models since specific protocols and tools are poorly or not developed.

In this book, we have collected detailed protocols describing state-of-the-art approaches that will facilitate the understanding of protein homeostasis in plant stress responses and development. Some findings made in this area of plant research could become valuable molecular tools in selection processes for improving agronomic performance, but also for contributing to address next challenges in agriculture such as precision horticulture.

Part I contains protocols focusing on the study of ubiquitin-dependent posttranslational modifications. While Chapter 1 describes a protocol for studying a novel ubiquitin conjugation mechanism independent of lysine residues, the other chapters focus on different aspects of the classical ubiquitin-dependent protein degradation system. Chapter 2 provides methods for analyzing the *in vivo* dynamics of cullins, key components of RING E3 ligases catalyzing ubiquitin conjugation to substrates. Chapter 3 describes the study of F-box proteins, another component of RING E3 ligases, as plant hormone receptor, which has become a key step in triggering hormone signaling. As many posttranslational modifications, ubiquitination is a reversible modification and Chapter 4 focuses on approaches for the study of enzymes involved in ubiquitin removal from its substrate. Chapters 5 and 6 address the generation of substrates for analyzing the *in vivo* ubiquitin/proteasome system

and the N-rule pathway for protein degradation, respectively. Finally, Chapter 7 extends the study of the N-rule pathway through methods for identifying E3 ligases.

Part II is dedicated to protocols focused on the study of Ubl posttranslational modifications, including in vitro SUMO chain formation (Chapter 8), the kinetic analysis of SUMO conjugation machinery (Chapter 9), and the in vitro analysis of SUMO proteases involved in SUMO maturation and SUMO removal from substrates (Chapter 10). In addition, Chapter 11 addresses the analysis of cellular distribution of SUMO conjugation machinery members as a strategy to get insights into their in vivo role. Another Ubl modification involved in many aspects of plant stress responses and development is autophagy, and biochemical and cell biology protocols for its study are described in Chaps. 12 and 13.

The study of protein homeostasis requires a broad variety of protocols that go beyond the analysis of enzymatic activities responsible for posttranslational modifications, and some of these protocols are comprised in Part III. A very useful and rapid approach to study protein stability consists in the expression of recombinant protein in plant protoplasts as is described in Chapter 14. Another emerging field in plant protein homeostasis is the study of protein aggregate formation in response to environmental stress, and their purification, described in Chapter 15, is the first step into their analysis. Chapter 16 provides a protocol for the study of another phenomenon occurring in response to stress consisting in protein oxidation under reactive oxygen species generation and the determination of proteasome activity. When the aim is to identify global changes in protein homeostasis during physiological responses, comparative proteomics based on iTRAQ are to be used (Chapter 17). Chapter 18 describes methods for the study of protein binding to phosphatidylinositol as a modulation mechanism of protein homeostasis. Also, organelle purification is recommended in order to reduce the complexity of the sample when performing proteomic studies in cell compartments, and Chapter 19 describes methods for the study of chloroplast proteome. Finally, Chapter 20 focuses on a general but also essential technique when trying to determine fluctuations in protein levels between samples, which is western blotting normalization.

Finally, Part IV encloses protocols for the in silico analysis of different aspects of proteostasis. Chapter 21 describes a protocol for identifying the genes encoding specific protein families and investigating their syntenic relationship. Chapter 22 focuses on methods for performing phylogenetic analysis, as a means of inferring functional conservation in different plant species. The last chapter (Chapter 23) describes the use of bioinformatics tools for data mining, focusing on the SUMO gene network.

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