SCREENING OF SYNTHETIC DUAL VECTOR SYSTEMS FOR MONITORING E3 LIGASE ACTIVITY IN PLANT PROTOPLASTS

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BACHELOR'S THESIS FOR THE DEGREE OF BIOCHEMISTRY

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1. Institution details

This work is based on research carried out as part of an internship at the *Institute of Molecular Plant Physiology, RWTH Aachen University (Germany)*, in Prof. Marco Trujillo's research group, with Dr. Aravindan Viswanathan as my supervisor. Prof. Trujillo's research focuses on how plants manage environmental stress, with an emphasis on coordinated proteolysis and the role of ubiquitin in cellular responses.

2. Abstract and keywords

Protein homeostasis relies on continuous protein turnover, involving synthesis and degradation. The ubiquitin-proteasome system (UPS) is the primary pathway for protein degradation, where proteins are tagged with the small regulatory protein ubiquitin and directed to the 26S proteasome. The E3 ubiquitin ligases (E3) play a key role in recognizing and recruiting the substrate for ubiquitin attachment and are essential for processes such as cell cycle, DNA repair and hormone signaling. However, determining the stabilizing or destabilizing effects of ubiquitination mediated by E3 ligases is often challenging, as *in vivo* monitoring of protein levels typically depends on easily detectable reporter systems. To overcome this, we developed a synthetic dual-vector reporter system to evaluate E3 ligase activity in *Arabidopsis thaliana* protoplasts. The system was expressed in plant protoplasts but E3 activity monitoring yielded inconclusive results. Nevertheless, this dual-reporter system represents a promising approach for studying E3 ligase function in plants and further optimization is needed to fully validate its utility.

Ubiquitin, ubiquitination, protein turnover, E3 ligase, Arabidopsis thaliana, protoplast transformation.

3. Introduction

3.1. Proteostasis: protein turnover and degradation

Proteostasis refers to the dynamic network of cellular processes that regulate the synthesis, folding, trafficking, modification and degradation of proteins (Hipp et al., 2019). This system ensures that proteins maintain their correct concentration, conformation and function, which is essential for preserving cellular health (Klaips et al., 2018).

Protein turnover consists of the continuous and regulated cycle of protein synthesis and degradation. This is a fundamental component of proteostasis because it enables cells to eliminate damaged, misfolded or obsolete proteins, while adapting protein levels to cell context. This dynamic process helps maintain proteome quality and prevents the toxic accumulation of non-functional or excess proteins (Ross et al., 2020). In eukaryotic cells, **protein degradation** is mainly carried out by two complementary systems: the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway. The UPS is the primary mechanism for the selective degradation of short-lived and regulatory proteins in the cytoplasm and nucleus. It involves the tagging of target proteins with ubiquitin molecules, which marks them for degradation by the 26S proteasome. In contrast, the autophagy-lysosome system targets long-lived proteins, damaged organelles or protein aggregates via the formation of double-membraned autophagosomes that fuse with lysosomes (Feng et al., 2024).

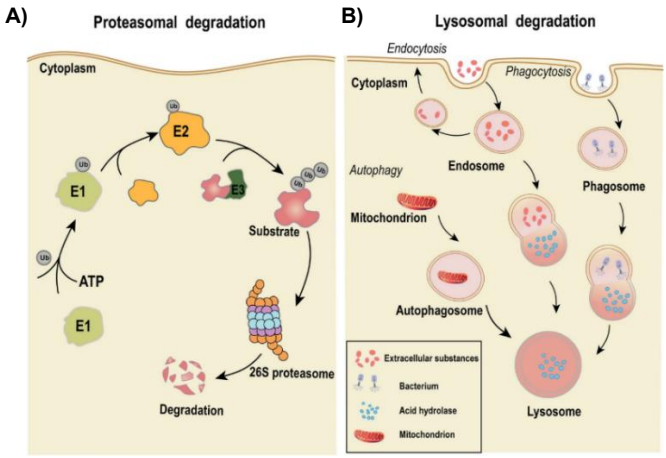

Figure 1. Types of protein degradation.

A) Proteasomal degradation. Target proteins are ubiquitinated through the ubiquitination cascade (E1, E2 and E3 enzymes) and subsequently degraded by the 26S proteasome.
B) Lysosomal degradation. Target proteins are enclosed into vesicles (autophagosomes), which then fuse with lysosomes for degradation. Image from Feng et al., 2024.

3.2. Ubiquitination and the ubiquitin code

Ubiquitin is a small regulatory protein found in all eukaryotic cells. It is composed of 76 amino acids, has a molecular weight of approximately 8.6kDa and contains seven lysine residues (K6, K11, K27, K29, K33, K48 and K63), along with an N-terminal methionine (M1). Structurally, ubiquitin adopts a compact β-grasp fold, which contributes to its remarkable thermostability, mechanical strength, resistance to proteolytic degradation and solubility across a wide pH range (Figure 2) (Komander et al., 2012). Ubiquitin functions primarily through covalent attachment to target proteins in a process known as ubiquitination.

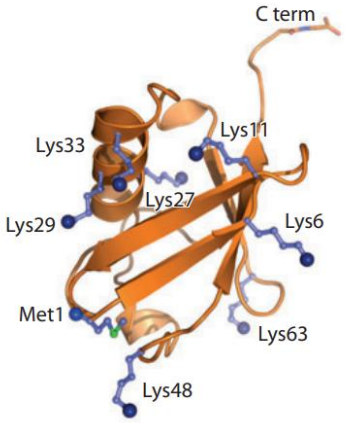

Figure 2. Ubiquitin and its lysine residues.

Schematic representation of the structure of ubiquitin, showing the seven Lys residues and Met1. Blue spheres indicate amino groups used in ubiquitin chain formation. Image from Komander et al., 2012.

Ubiquitination is a highly conserved post-translational modification (PTM) found in all eukaryotic cells, playing a central role in regulation protein turnover, signal transduction, DNA repair, immune responses and cell cycle progression (Damgaard et al., 2021). This process involves the covalent attachment of ubiquitin to target proteins through an isopeptide bond between the C-terminal glycine of ubiquitin and the ε-amino group of lysine residues on the substrate (Trujillo et al., 2017). The ubiquitination cascade consists of three sequential enzymatic steps mediated by ubiquitin-activating enzymes (E1s), ubiquitin-conjugation enzymes (E2s) and ubiquitin ligases (E3s) (Figure 3). First, E1 activates ubiquitin in an ATP-dependent manner, forming a thioester bond with the C-terminal glycine (Figure 3, i). The activated ubiquitin is then transferred to the E2 enzyme (Figure 3, ii). Finally, the E3 ligase catalyzes the transfer of ubiquitin from E2 to a specific lysine on the substrate protein (Figure 3, iii) (Yang et al., 2021).

Ubiquitination can occur in multiple forms, encoding each chain type for a specific cellular outcome (Figure 3, iv). The complex language through which ubiquitin modifications regulate protein function and cellular signaling is called the **ubiquitin code** (Komander et al., 2012). This code defines how proteins are marked, interpreted and processed inside the cell, depending on the type, number and linkage of ubiquitin molecules. In monoubiquitination, a single ubiquitin molecule is added to one lysine residue of a substrate, often influencing processes such as endocytosis, transcriptional regulation or DNA repair. In polyubiquitination, ubiquitin chains are built by linking additional ubiquitin molecules to one of the seven lysine residues or the N-terminal methionine of a previously attached ubiquitin. The choice of linkage determines the chain's three-dimensional structure, binding partners and the cellular fate of the substrate. For instance, K48-linked polyubiquitin chains typically act as a signal for proteasomal degradation, directing misfolded or short-lived proteins to the 26S proteasome. In contrast, K63-linked chains participate in non-proteolytic roles, including the regulation of intracellular trafficking, signal transduction, and DNA damage repair. M1-linked chains play a central role in inflammatory signaling pathways such as NF-κB activation. Additionally, less common linkages like K11, K29 or mixed and branched chains have been implicated in cell cycle regulation and the fine-tuning of specific signaling networks (Akutsu et al., 2016).

To decode this molecular language, cells rely on a range of specialized proteins that contain ubiquitin-binding domains (UBDs), which specifically recognize and bind to different ubiquitin chain types. These UBDs act as "readers" of the ubiquitin code and determine the downstream outcome of ubiquitin signals (Figure 3, iv) (Husnjak et al., 2012). Additionally, to prevent excessive or inappropriate signaling, the ubiquitin code is regulated by deubiquitinating enzymes (DUBs). These enzymes cleave ubiquitin from substrate proteins or ubiquitin chains, thus editing or erasing the signal. DUBs help maintain ubiquitin homeostasis and control the amplitude and duration of ubiquitin-dependent signaling (Figure 3, v) (Synder et al., 2021).

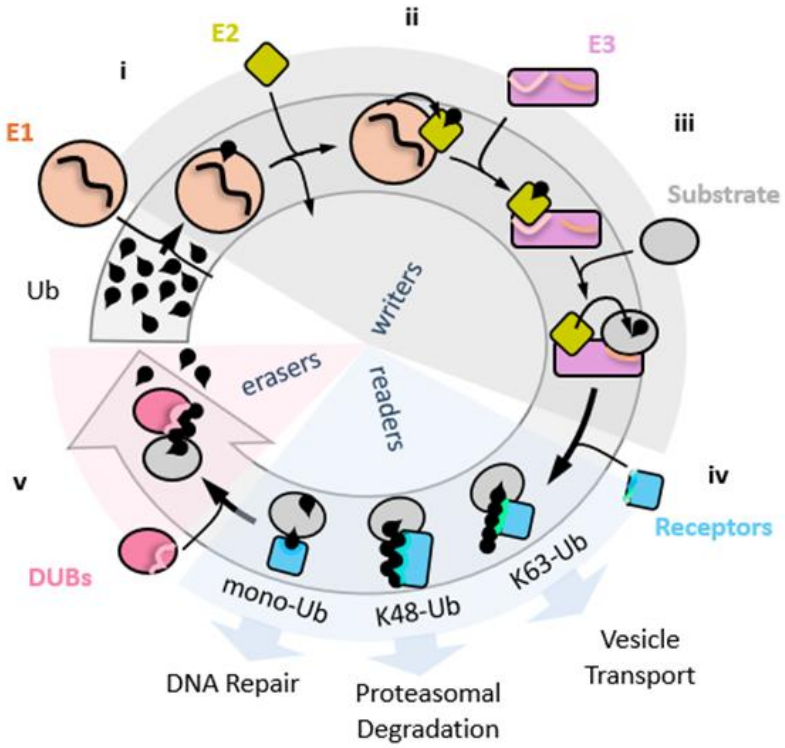

Figure 3. Ubiquitination modification cycle.

Schematic representation of the reactions involved in the ubiquitination modification cycle. **i.** Ubiquitin activation by the E1 enzyme. **ii.** Transfer of ubiquitin from the E1 to the E2 enzyme. **iii.** Ubiquitin conjugation to the substrate via the E3 enzyme. **iv**. Activation of key signaling pathways through the recognition of distinct ubiquitination patterns by specific receptors. **v.** Ubiquitin removal from the substrate by deubiquitinases (DUBs). Image adapted and modified from Trujillo et al., 2017.

3.3. The proteasome: structure, function and inhibition via MG132 treatment

The **26S proteasome** is a multi-subunit protease complex responsible for the selective degradation of intracellular proteins that have been tagged with polyubiquitin chains, specifically via K48 linkages. Proteins that are no longer needed, damaged or misfolded are often targeted for proteasomal degradation by being polyubiquitinated at the lysine-48 residue (Collins et al., 2017). For this reason, it is the central proteolytic machinery of the ubiquitin-proteasome system (UPS).

The 26S proteasome is composed of two main parts: the 20S core particle (CP) and one or two 19S regulatory particles (RP) (Figure 4). The 20S core is a barrel-shaped structure made up of four stacked heptameric rings: two outer α-rings and two inner β-rings. The proteolytic activity resides within the β subunits and includes three major catalytic activities: chymotrypsin-like, trypsin-like and caspase-like (peptidyl-glutamyl peptide-hydrolyzing) activities. The 19S regulatory particle is responsible for recognizing polyubiquitinated proteins, removing ubiquitin chains, unfolding the substrates and translocating them into the 20S core for degradation. It contains multiple subunits with ATPase activity (Rpt proteins) and non-ATPase components (Rpn proteins) that mediate substrate binding, deubiquitination and gate opening (Bard et al., 2019).

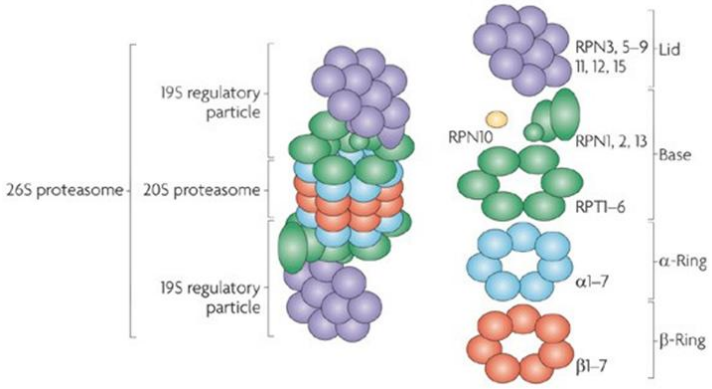

Figure 4. The structure of the 26 proteasome.

Schematic representation of the 26S proteasome. It consists of a cylindrical 20S core particle (CP) with proteolytic activity and one or two 19S regulatory particles (RP) that recognize, unfold and translocate ubiquitinated substrates into the core for degradation. Image from Murata et al., 2009.

Considering the central role of the proteasome in protein turnover, inhibiting its activity is a valuable experimental strategy to study ubiquitination, accumulate unstable proteins or probe protein degradation pathways (Nunes et al., 2017). One of the most widely used chemical inhibitors is **MG132**, a reversible, cell-permeable peptide aldehyde that targets the chymotrypsin-like activity of the proteasome's β5 subunit. This inhibitor blocks substrate degradation by preventing the proteolytic cleavage of polyubiquitinated proteins, leading to their accumulation in cells (Albornoz et al., 2019). MG132 has become a standard tool in plant biology to study protein stability and half-life, regulation of E3 ligases and their substrates, ubiquitin accumulation under stress and proteasome dependency of specific degradation events (Vannini et al., 2014; Karpuz et al., 2022).

3.4. E3 ubiquitin ligase: function and classification

E3 ubiquitin ligase is the enzyme which acts as the ultimate determinant of substrate specificity in the ubiquitin cascade, mediating the transfer of ubiquitin from the E2 enzyme to a lysine residue on a target protein. This step is critical because it determines which substrates are tagged, influencing different cellular processes such as proteostasis, cell cycle regulation, DNA repair, hormone signaling, immune responses and stress adaptation (Yang et al., 2021). There are different families of E3 ligases based on their conserved domains and mechanisms of action: HECT, RING, U-box and RBR (Figure 5) (Wang et al., 2022).

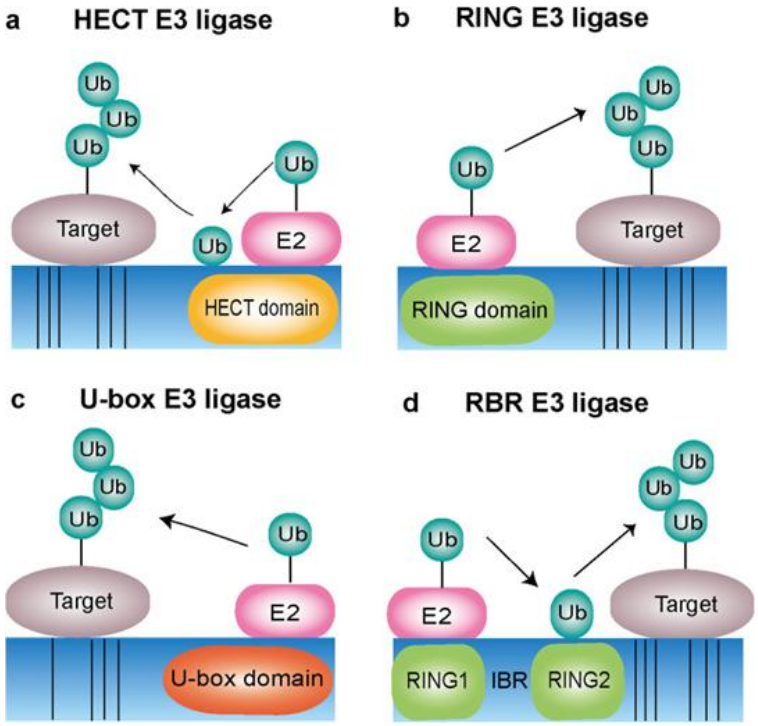

Figure 5. Mechanisms and domains of the four major families of E3 ubiquitin ligases.

(a) HECT-type E3 ligases, contain a HECT domain (C-terminal). They form a thioester intermediate with ubiquitin before transferring it to the substrate (two-step catalytic mechanism).
(b) RING-type E3 ligases, contain a RING domain (N-terminal). They mediate direct ubiquitin transfer from E2 to the substrate.
(c) U-box-type E3 ligases, contain a U-box domain (C-terminal). They mediate direct ubiquitin transfer from E2 to the substrate.
(d) RBR-type E3 ligases, contain RING1, IBR and RING2 domains (N-terminal). They form a thioester intermediate with ubiquitin before transferring it to the substrate (two-step catalytic mechanism).
Image from Yang et al., 2021.

The **HECT (Homologous to E6-AP Carboxyl Terminus) E3s ligases** employ a two-step catalytic mechanism (Figure 5, a): ubiquitin is first transferred from E2 to a conserved cysteine in the HECT domain of the E3, forming a thioester intermediate, and then is conjugated to the substrate. The HECT domain contains a N-lobe for E2 binding and a C-lobe with the catalytic cysteine. Based on their N-terminal structure, the HECT-type E3s include the NEDD4, HERC and other families, with roles in neural development, immunity and tumor suppression (Qian et al., 2020; Weber et al., 2019).

The **RING (Really Interesting New Gene) E3s ligases** employ a one-step catalytic mechanism (Figure 5, b) in which the RING domain recruits E2 and transfers ubiquitin directly to the substrate. They are classified as: monomeric RING E3s, which function as single polypeptides with a RING domain; and multi-subunit RING ligases, which function as modular complexes composed of multiple protein subunits. A major subclass of multi-subunit RING ligases is the Cullin-RING ligase (CRL family), which includes a Cullin scaffold, a RING-finger protein (RBX) and a substrate receptor module (CRBN, SOCS, SPOP, KEAP1) connected via adaptor proteins (DDB1, Elongin, BTB) (Wang et al., 2024; Zhang et al., 2023). Among CRLs, the SCF complex (Skp1-Cullin1-F-Box) is one of the most well-characterized. This complex is composed of Cullin1 (scaffold protein), RBX1, Skp1 (adaptor protein) and F-box protein (substrate receptor: COI, TIR1, FBXW7) (Zheng et al., 2016). They play crucial roles in cell cycle regulation, hormone signaling, stress responses and development. They are the largest and most diverse class of E3s.

The **U-box E3s ligases** also employ a one-step mechanism (Figure 5, c) via a U-box domain, often in association with co-chaperones. A well-characterized member is CHIP (C-terminus of Hsc70-Interacting Protein), which functions as a co-chaperone-associated E3 ligase. They play crucial roles in protein quality control, stress responses and cellular homeostasis (Sharma et al., 2020).

The **RBR (RING-between-RING) E3s ligases** combine RING and HECT features in a two-step mechanism (Figure 5, d). They contain RING1 (E2 binding), IBR (structural stability) and RING2 (catalytic cysteine). Ubiquitin is transferred from E2 to RING2 before substrate conjugation. They are classified based on their functional activity. They play a crucial role in inflammation regulation, immune signaling and organelle quality control (Spratt et al., 2014; Wang et al., 2023).

3.5. E3 ubiquitin ligase: monitoring challenges and emerging tools

Considering all the biological functions in which the E3 ligases are implicated, they play an important role in maintaining proteostasis within the cell (Mackinnon et al., 2022). In plants, they are also crucial for proteome remodeling in response to environmental stimuli, where they regulate processes related to biotic and abiotic stress (Su et al., 2024). However, determining the stabilizing or destabilizing effects of ubiquitin modification by E3 ligases remains challenging. First, E3 ligases operate within a hierarchical cascade involving E1 and E2 enzymes, making it challenging to isolate and attribute their specific functional contributions. This complexity is further compounded by the transient and reversible nature of ubiquitination events, while the ability to detect distinct ubiquitin linkages is frequently constrained by the limited resolution of current biochemical techniques (Hospenthal et al., 2015). Moreover, i*n vitro* assays used to evaluate E3 ligase activity often depend on protein immobilization or artificial tagging, which may interfere with native protein–protein interactions and diminish the physiological relevance of the results (Stewart et al., 2016).

Protein-protein interactions between E3 ligases and their substrates can be investigated using yeast two-hybrid (Y2H) assays and biomolecular fluorescence complementation (BiFC). While these methods are effective for detecting physical interactions, they do not provide direct evidence of ubiquitination activity (Lee et al., 2011; Serrano et al., 2018). Alternatively, transient expression in *Nicotiana benthamiana* allows for functional characterization of E3 ligases by monitoring ubiquitination levels through Western blot analysis. However, this approach often involves protein overexpression, which can result in non-physiological levels and compromise the biological relevance of the observations (Duplan et al., 2014).

In this section, all information related to the dual vector system has been removed due to its confidential nature. Interested parties may contact the intellectual property owner: ***Marco Trujillo Linke*** *(Group Leader at the Institute of Molecular Plant Physiology, RWTH Aachen University). E-mail: mtrujillo@bio3.rwth-aachen.de*

3.6. The dual vector system: design and expression

In this section, all information related to the dual vector system has been removed due to its confidential nature. Interested parties may contact the intellectual property owner: ***Marco Trujillo Linke*** *(Group Leader at the Institute of Molecular Plant Physiology, RWTH Aachen University). E-mail: mtrujillo@bio3.rwth-aachen.de*

4. Objective and hypothesis

The goal of this project is to screen synthetic dual vector systems generated by Trujillo's group for monitoring E3 ligase activity in plant protoplasts. To achieve this, the study aims to confirm the expression of the dual vector system and determine whether E3 ligase activity can be effectively monitored through this system.

The hypothesis of this project is that the expression of this dual vector reporter system in plant protoplasts will enable the monitoring of E3 ligase activity in a plant cellular context.

5. Materials and methods

In this section, all information related to the materials and methods of this study has been removed due to its confidential nature. Interested parties may contact the intellectual property owner: ***Marco Trujillo Linke*** *(Group Leader at the Institute of Molecular Plant Physiology, RWTH Aachen University). E-mail: mtrujillo@bio3.rwth-aachen.de*

6. Results and discussion

In this section, all information related to the results and the discussion of the dual vector system functional screening has been removed due to its confidential nature. Interested parties may contact the intellectual property owner: ***Marco Trujillo Linke*** *(Group Leader at the Institute of Molecular Plant Physiology, RWTH Aachen University). E-mail: mtrujillo@bio3.rwth-aachen.de.*

7. Conclusions

The dual vector system tested in this study was successfully expressed in *Arabidopsis thaliana protoplasts:* both effectors and sensors that individually constitute the system were detected, despite variations in their expression levels.

Regarding the functional screening of the dual vector system in monitoring E3 ligase activity in *Arabidopsis thaliana* protoplasts, the results obtained were inconclusive. Nevertheless, despite the limitations observed, it can be concluded that the dual vector system has the potential to serve as a useful tool for monitoring E3 ligase activity.

8. Outlook

In this section, all information related to the outlook of this project has been removed due to its confidential nature. Interested parties may contact the intellectual property owner: ***Marco Trujillo Linke*** *(Group Leader at the Institute of Molecular Plant Physiology, RWTH Aachen University). E-mail: mtrujillo@bio3.rwth-aachen.de*

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