UNIVERSITAT ROVIRA i VIRGILI

Institute of Molecular Plant Physiology
iMPP Institute of Biology 3

DEVELOPMENT OF A MODULAR DUAL VECTOR SYSTEM VIA GOLDEN GATE CLONING TO ASSAY E3 LIGASE ACTIVITY IN PLANT PROTOPLASTS

Aida Arjona Martí

BACHELOR'S THESIS FOR THE DEGREE OF BIOTECHNOLOGY

Academic tutor: Mª del Carmen Portillo Guisado. Holder of University. *Biochemistry and Biotechnology Department*. E-mail: carmen.portillo@urv.cat.

In cooperation with *RWTH Aachen University*.

Supervisors: Marco Trujillo Linke. Professor at the *Institute of Molecular Plant Physiology* (*RWTH Aachen University*). E-mail: mtrujillo@bio3.rwth-aachen.de. Aravindan Viswanathan. Postdoctoral researcher at the *Institute of Molecular Plant Physiology* (*RWTH Aachen University*). E-mail: a.viswanathan@bio3.rwth-aachen.de

September 2025, Tarragona (URV)

Confidentiality disclaimer

This bachelor's Thesis is a **reduced version** prepared for public dissemination. **All content of a confidential nature has been removed**. The excluded information is the intellectual property of **Marco Trujillo Linke** (Group Leader at the *Institute of Molecular Plant Physiology, RWTH Aachen University*).
E-mail: mtrujillo@bio3.rwth-aachen.de.

Interested parties who wish to access the complete version or learn more about the confidential aspects of this work may contact the intellectual property holder directly.

© [2025] [Aida Arjona Martí]. All rights reserved.

September 2025

I, *Aida Arjona Martí*, with ID *49648144E*, am aware of the URV's guide for the prevention of plagiarism “*Prevention, detection and handling of plagiarism in teaching: a guide for students”* (approved in July 2017) and I affirm that this Bachelor’s Thesis does not constitute any of the practices considered plagiarism by the URV.

Tarragona, 18 July 2025

Acknowledgments

First of all, I would like to express my deepest gratitude to Prof. Marco Trujillo, leader of the *Institute of Molecular Plant Physiology* at *RWTH University Aachen (Germany)*, for giving me the great opportunity to carry out my internship for three months, which allowed me to develop the entire experimental part of this final degree project.

Secondly, I want to convey my sincere thanks to Dr. Aravindan Viswanathan, a postdoctoral researcher in the *Institute of Molecular Plant Physiology* at *RWTH University Aachen (Germany)*, who was my supervisor during the internship. He helped me in every possible way, corrected me when necessary, treated me with respect and clarified all my doubts to help me improve as a scientist.

En tercer lloc, vull agrair a la meva tutora acadèmica, Carmen Portillo, que m'ha ajudat en tot el seguiment del meu treball de fi de grau, des que vaig començar amb tota la part experimental del projecte fins a les últimes correccions abans de la entrega d'aquest. Gràcies Carmen per sempre estar disposada a ajudar-me en tot el que ha estat necessari.

En quart lloc, però no menys important, vull agrair a la meva família, per respectar sempre qui soc, ajudar-me en tot allò necessari, creure en mi per davant de tot i lluitar juntament amb mi pels meus propòsits. Papa, mama, Sergi i Ainoa: us estimo infinitament.

En quinto lugar, quiero agradecer el apoyo de todos mis amigos y amigas, por ayudarme en todo aquello que ha estado en vuestras manos y animarme cuando todo parecía oscuro. Especialmente, quiero dar las gracias a todas aquellas personas que he conocido durante mi etapa universitaria y durante mi experiencia Erasmus. Sin ellas, todo habría sido más difícil.

I per últim, vull dedicar aquest treball al meu avi, al meu padrí. Sé que sempre estaràs molt orgullós de mi.

Table of contents

1. Institution details 1
2. Abstract and keywords 1
3. Introduction 2
   3.1. Protein homeostasis: the role of the ubiquitin-proteasome system 2
   3.2. The E3 ligase: relevance, families and monitoring challenges 3
   3.3. Dual reporter system for ratiometric analysis of protein stability 6
   3.4. Design of sensors and effectors using the Golden Gate modular tool kit 6
   3.5. Golden Gate cloning 6
4. Objective and hypothesis 8
5. Materials and methods 8
6. Results and discussion 8
7. Conclusions 9
8. Outlook 9
9. References 10
10. Self-evaluation 11

1. Institution details

This project was carried out at the *Institute of Molecular Plant Physiology* at *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

Multiple protein homeostasis networks monitor proteins from their synthesis through their degradation. The steady-state abundance of proteins is thus determined by their relative rates of production and degradation. Ubiquitin modification of proteins dictates their cellular fate, ranging from DNA repair and cell-cycle regulation to proteasome-mediated degradation. Determining the stabilizing or destabilizing effects of ubiquitin modification by E3 ligases is often challenging, as monitoring protein levels *in vivo* frequently relies on easily detectable reporter systems. To address this issue, we developed a dual vector system for ratiometric analysis of E3 ligase activity in plant protoplasts. These constructs were generated using the Golden Gate modular cloning system, which allows the "scarless" assembly of multigene cassettes. Level 0 modules were assembled into Level 1 vectors to form complete transcriptional units. The precise assembly of vector backbones and inserts was confirmed via restriction digestion and sequencing. Overall, we generated seven sensors and two effectors. Moving forward, this system will be tested in plant protoplasts to assess its optimal expression. This platform will facilitate the precise monitoring of E3 ligase effects on reporter protein stability, providing a valuable tool for investigating protein regulation in plants.

Protein degradation, Ubiquitination, E3 ligases, Ratiometric sensor, Golden Gate cloning.

3. Introduction

3.1. Protein homeostasis: the role of the ubiquitin-proteasome system

Protein homeostasis is the process by which cells maintain the concentration, conformation and subcellular localization of proteins. In eukaryotic cells, damaged organelles or proteins are removed via proteasomes or lysosomes (Li et al., 2022). The ubiquitin-proteasome pathway, conserved from yeast to mammals, is required for the targeted degradation of ubiquitinated substrates by the 26S proteasome (Arkinson et al., 2025).

Ubiquitination is a post-translational modification in which the 8.6 kDa ubiquitin protein (Ub) is attached to a target substrate through an isopeptide bond via a sequential reaction involving three enzymes (Trujillo et al., 2017) (Figure 1). These enzymes are known as ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). The E1 enzyme initiates the enzymatic cascade by forming a thioester bond between its active site cysteine and the C-terminal glycine of ubiquitin in an ATP-dependent reaction (Figure 1, i). Next, ubiquitin is transferred from E1 to the active site cysteine of E2, forming an E2–ubiquitin intermediate (Figure 1, ii). Finally, the E3 enzyme mediates the transfer of ubiquitin from E2 to the substrate, creating an isopeptide bond between the C-terminal carboxyl group of ubiquitin and the amino group of a lysine residue in the substrate (Figure 1, iii). This final step can occur in two ways: either ubiquitin is first transferred from E2 to the active site cysteine of E3 before substrate binding, or it is directly transferred from E2 to the substrate (Yang et al., 2021).

This ubiquitination reaction can involve the addition of a single ubiquitin molecule to a substrate (monoubiquitination) or the attachment of multiple ubiquitin molecules linked together in a chain (polyubiquitination). Ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) which act as key sites for building these polyubiquitin chains. The particular lysine residue used to form the chain plays a crucial role in determining the downstream effects and signaling functions of the ubiquitinated protein (Liao et al., 2024) (Figure 1, iv).

The signal for proteasomal degradation is created by the isopeptide bond formed between the C-terminal residue of one Ub and K48 of the previously conjugated Ub. Once this signal is recognized by the specific receptors, the process of protein degradation starts and the deubiquitinases (DUBs) mediate the detachment of Ub chains from the protein to recycle Ub monomers for the next round of ubiquitination (Komander et al., 2021) (Figure 1, v).

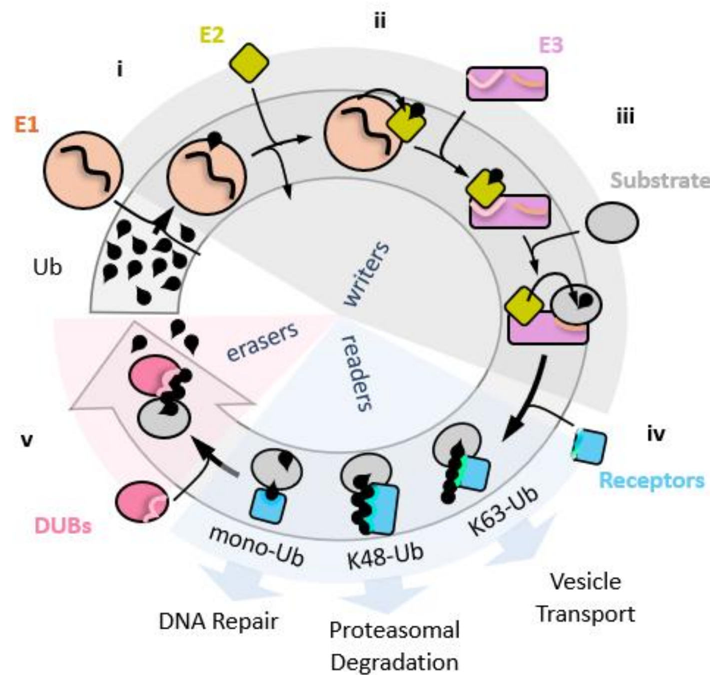

Figure 1. Ubiquitination modification cycle.

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 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.

3.2. The E3 ligase: relevance, families and monitoring challenges

The E3 ligases have an important role in selective protein degradation, acting as a key enzyme in the ubiquitin-proteasome system (UPS). This enzyme is responsible for recognizing specific target proteins and catalyzing the transfer of ubiquitin from E2 conjugating enzymes to the substrates. Based on structural and mechanical differences, the E3 ligases are classified into four different families: the Homologous to E6AP C-Terminus (HECT) type, the Really Interesting New Gene (RING)-finger type, the U-box ligases and the RING-IBR-RING (RBR) family (Li et al., 2021).

In plants, E3 ligases are crucial for proteome remodelling in response to environmental stimuli (Mackinnon et al., 2022). For example, they have been shown to regulate diverse processes related to biotic and abiotic stress, such as drought, salinity and pathogen attack (Su et al., 2024). However, despite their biological importance, determining the stabilizing or destabilizing effects of ubiquitin modification by E3 ligases remains challenging. First, E3 ligases function as a part of a cascade with the E1 and E2 enzymes, so dissecting the specific contribution of E3s within this pathway is difficult. Compounding this, ubiquitination events are transient and reversible, and the detection of specific ubiquitin linkages is often limited by the resolution of current biochemical assays (Hospenthal et al., 2015). *In vitro* systems assaying E3 ligase activity often rely on protein immobilization or artificial tags that can disrupt natural interactions and reduce physiological relevance (Stewart et al., 2016).

With yeast two-hybrid systems (Y2H) systems and biomolecular fluorescence complementation (BiFC) it is possible to assess protein-protein interactions between E3 ligases and substrates. These techniques are useful for detecting interactions, but do not directly demonstrate ubiquitination activity (Lee et al., 2011; Serrano et al., 2018). Additionally, with transient expression in *Nicotiana benthamiana* it is possible to functionally characterize E3s, monitoring ubiquitination levels via western blot. Nonetheless, transient overexpression can lead to non-physiological protein levels (Duplan et al., 2014).

To overcome some of the limitations regarding the detection of E3 ligase activity, a recent study developed a synthetic proteome-scale platform to functionally identify proteins (including E3 ligases) that can either influence protein degradation or stabilization in a proximity-dependent manner (Poirson et al., 2024) (Figure 2). For this purpose, a dual vector system was designed. A dual vector system is a genetic engineering approach in which two separate vectors are introduced into cells simultaneously. Each vector carries a different genetic construct, one encoding a reporter protein (sensor vector) and the other encoding a protein of interest (POI) (effector vector). This system enables ratiometric analysis, meaning the abundance of the POI can be quantified relative to the reporter protein. This helps control for variability in transfection efficiency and expression levels, leading to more accurate detection of degradation or stabilization effects.

In this study, the sensor vectors are ratiometric dual reporter vectors containing the sequences coding for Enhanced Green Fluorescent Protein (eGFP), ABA Insensitive 1 (ABI1), Internal Ribosome Entry Site (IRES) and Tag Blue Fluorescent Protein (TagBFP). Due to the presence of the IRES sequence, the translation of the sensor vector results in two separate proteins: eGFP-ABI1 and TagBFP (Figure 2, i). The effector vectors contain the sequences for the protein of interest (POI) and GFP nanobody (vhhGFP) or Pyrabactin Resistance 1-Like 1 (PYL1) (Figure 2, ii).

By transforming the cells with both vectors (sensor and effector), the aim was to test the stabilizing or degradative capacity of the proteins under study. If the protein of interest has a stabilizing effect, both eGFP and TagBFP signals are expected to be observed, whereas if it has a degradative effect, only the TagBFP signal is expected to be observed (Figure 2, ii). The interaction between the effector proteins and eGFP-ABI1 can be in a constitutive (vhhGFP) or inducible (PYL1).

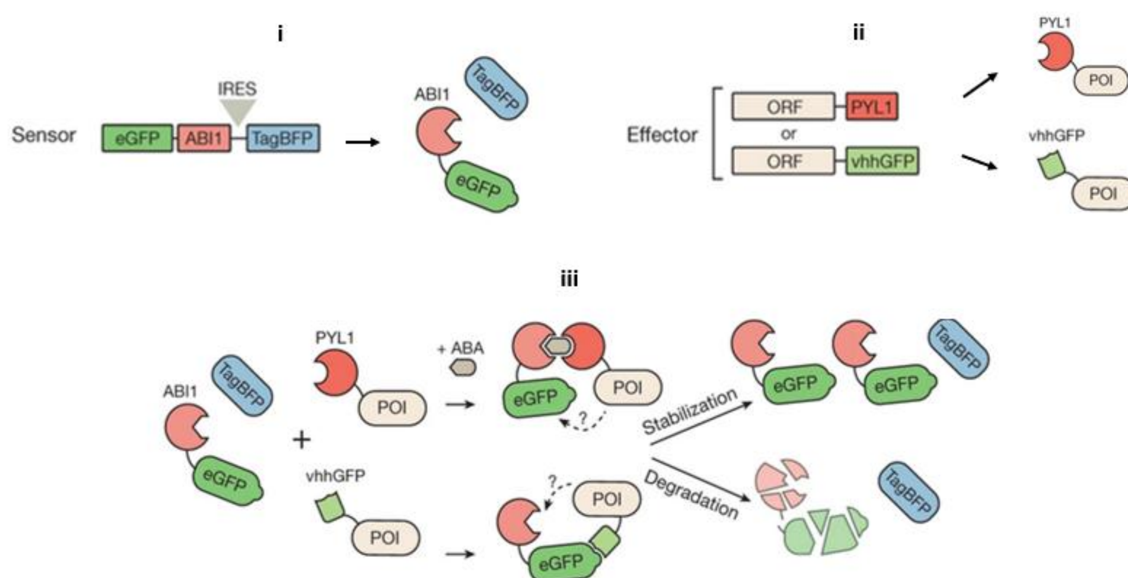

Figure 2. Approach used to discover proximity-dependent effectors of protein stability.

Schematic representation of the dual vector system used to discover proximity-dependent effectors of protein stability. **i.** The sensor vector (eGFP-ABI1-IRES-TagBFP) results in two different proteins: eGFP-ABI1 and TagBFP. **ii.** The effector vector contains the open reading frame (ORF) sequence for the protein of interest (POI) and PYL1/or vhhGFP. **iii**. The interaction between the sensor and the effector proteins can be induced by abscisic acid (ABA) (if the interaction occurs between ABI1 and PYL1) or constitutively (if the interaction occurs between eGFP and vhhGFP). If the POI has a stabilizing effect both eGFP and TagBFP are observed, whereas if it has a degradative effect, only the TagBFP signal is expected to be observed. Image adapted and modified from Poirson et al., 2024.

3.3. Dual reporter system for ratiometric analysis of protein stability

Ratiometric reporter systems are tools to dynamically measure the abundance of a protein of interest relative to a reference protein. To achieve a dual-reporter system it is common to perform a co-transformation of separate target and reference-encoding plasmids. However, with this method there is no guarantee that both constructs enter each cell or that they do so with consistent proportions. For that reason, an improvement to this method is to encode the target and reference protein on the same transcript, avoiding the variation in the relative expression of both genes (Khosla et al., 2020).

3.4. Design of sensors and effectors using the Golden Gate modular tool kit

In our work, we developed a dual vector ratiometric system consisting of a sensor and an effector.

In this section, all information related to the sequences contained in our sensor and effector 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.*

To assemble these multigene constructs (sensor and effector) for expression in plant protoplasts, we used the Golden Gate Modular toolkit (Engler et al., 2014).

3.5. Golden Gate cloning

Golden Gate cloning is a DNA assembly method where the Type IIS restriction enzymes cut DNA modules generating four base pair junctions (known as fusion sites) to join them with the DNA ligase (Engler et al., 2014). These DNA modules include promoters (Pro), 5’ untranslated sequences (5U), coding sequences (CDS1/CDS2), 3’ untranslated sequences (3U), transcription terminators (Ter) and N- and C- terminal fusions (NT1/NT2 and CT) and signal peptides (SP) (Figure 3).

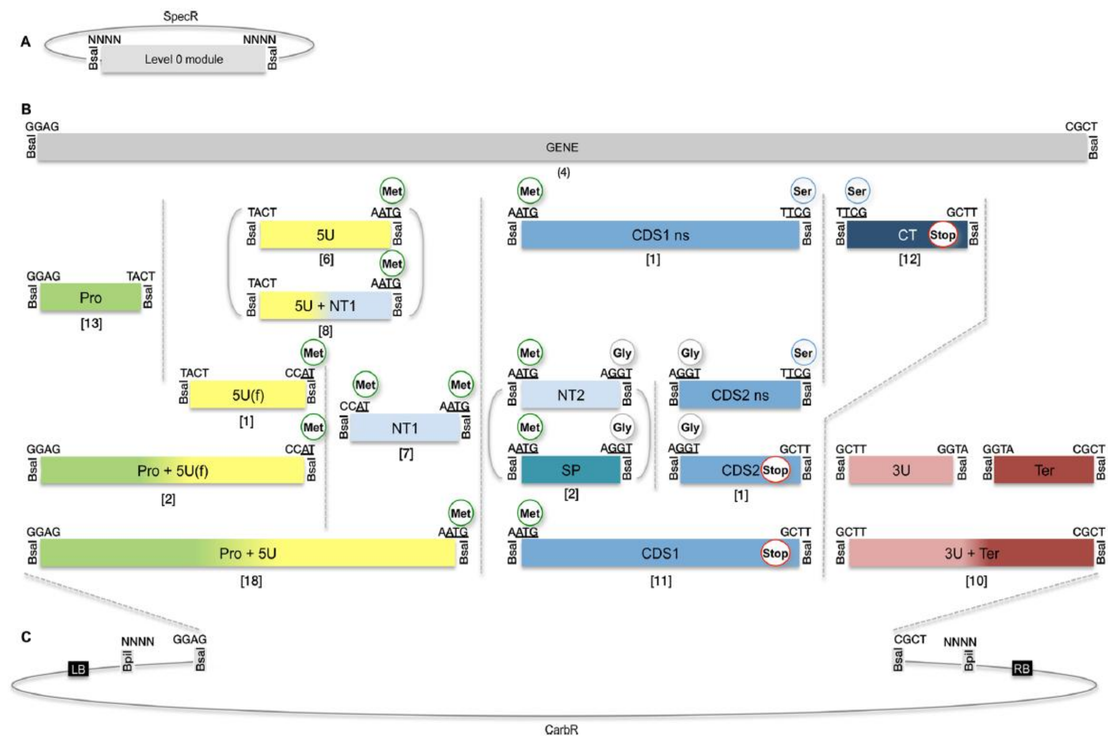

Figure 3. Level zero and level one modules for Golden Gate cloning.

A. Level 0 vector for Golden Gate cloning. This vector includes one DNA module flanked by two BsaI restriction sites in opposite orientations and a spectinomycin resistance gene.

B. Representation of all the module types possible to assemble via Golden Gate cloning reaction into a complete gene. These DNA modules include promoters (Pro), 5' untranslated sequences (5U), coding sequences (CDS1/CDS2), 3' untranslated sequences (3U), transcription terminators (Ter) and N- and C- terminal fusions (NT1/NT2 and CT) and signal peptides (SP). The specific four base pairs junctions allow the assembly between modules.

C. Level 1 vector for Golden Gate cloning. This vector includes the assembled DNA modules, and they are flanked by two BsaI restriction sites in opposite orientations. It also includes a spectinomycin resistance gene. Image from Engler et al., 2014.

The Golden Gate cloning process is hierarchically organized into Level 0 and Level 1 vectors. Level 0 vectors contain the individual DNA modules, a spectinomycin resistance gene and they are flanked by two BsaI restriction sites in opposite orientations. Their internal sequences are free of recognition sites for the restriction endonucleases BsaI and BpiI. Level 1 vectors, on the other hand, contain complete transcriptional units, assembled from multiple DNA modules. These vectors include a carbenicillin resistance gene and are flanked by two BpiI sites in opposite orientations. Like Level 0 vectors, their internal sequences are devoid of recognition sites for BsaI and BpiI.

The advantage of using this method rather than the conventional methods for cloning, is that transcriptional units can be assembled from standard modular parts using a one-step Golden Gate cloning reaction. In this approach, all plasmid donor constructs, the recipient vector, a Type IIS restriction enzyme and DNA ligase are combined in a single reaction tube and subjected to a defined thermocycling program to enable simultaneous digestion and ligation. Nevertheless, one important prerequisite of Golden Gate cloning is the domestication process, where the internal recognition sequences for the type IIs enzymes used are modified in the DNA fragments that will be assembled together (Engler et al., 2009).

4. Objective and hypothesis

The goal of this project is to develop a dual vector ratiometric system using the Golden Gate modular cloning technique, generating a set sensors and effectors. This dual vector ratiometric system would later be tested in plant protoplasts for their efficiency in assaying E3 ligase activity.

The hypothesis of this project is that with Golden Gate modular cloning it is possible to generate a “scarless” assembly of multigene transcriptional units, resulting in a functional dual vector ratiometric system.

5. Materials and methods

In this section, all information related to the materials and methos used to develop our dual vector ratiometric 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.*

6. Results and discussion

In this section, all information related to the results and discussion of the development of our dual vector ratiometric 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.*

7. Conclusions

The aim of this project — the development and generation of a dual vector ratiometric system using Golden Gate cloning for monitoring E3 ligase enzyme activity in plant protoplasts— were successfully achieved. The necessary Level 0 and Level 1 constructs for the generation of these sensors and effectors were generated and their sequences were confirmed by Sanger sequencing.

However, an important limitation of the project is that the system was not functionally tested in protoplasts. As a result, the actual performance of the sensors and effectors in reporting E3 ligase activity remains unknown.

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.*

9. References

- Agrata, R., & Komander, D. (2025). Ubiquitin-A structural perspective. *Molecular cell*, 85(2), 323–346. https://doi.org/10.1016/j.molcel.2024.12.015
- Arkinson, C., Dong, K. C., et al. (2025). Mechanisms and regulation of substrate degradation by the 26S proteasome. *Nature Reviews Molecular Cell Biology*, 26(2), 104–122. https://doi.org/10.1038/s41580-024-00778-0
- Biró, J. B., Kecskés, K., et al. (2024). Golden EGG, a simplified Golden Gate cloning system to assemble multiple fragments. *Scientific Reports*, 14, 25288. https://doi.org/10.1038/s41598-024-77327-4
- Engler, C., Youles, M., et al. (2014). A Golden Gate modular cloning toolbox for plants. *ACS Synthetic Biology*, 3(11), 839–843. https://doi.org/10.1021/sb4001504
- Iconomou, M., Saunders, D. N., et al. (2016). Systematic approaches to identify E3 ligase substrates. *Biochemical Journal*, 473(22), 4083–4101. https://doi.org/10.1042/BCJ20160719
- Khosla, A., Rodriguez-Furlan, C., et al. (2020). A series of dual-reporter vectors for ratiometric analysis of protein abundance in plants. *Plant Direct*, 4(6), e00231. https://doi.org/10.1002/pld3.231
- Li, K., Zhang, Y., et al. (2022). Targeted protein degradation: Mechanisms, strategies and application. *Signal Transduction and Targeted Therapy*, 7, 113. https://doi.org/10.1038/s41392-022-00966-4
- Liao, Y., Wang, X., et al. (2024). The role of ubiquitination in health and disease. *MedComm*, 5(1), e736. https://doi.org/10.1002/mco2.736
- Mackinnon, E., Stone, S. L., et al. (2022). The ubiquitin proteasome system and nutrient stress response. *Frontiers in Plant Science*, 13, 867419. https://doi.org/10.3389/fpls.2022.867419
- Potapov, V., Ong, J. L., et al. (2022). A user's guide to Golden Gate cloning methods and standards. ACS Synthetic Biology, 11(8), 2773–2788. https://doi.org/10.1021/acssynbio.2c00355
- Tracz, M., & Bialek, W. (2021). Beyond K48 and K63: non-canonical protein ubiquitination. *Cellular & molecular biology letters*, *26*(1), 1. https://doi.org/10.1186/s11658-020-00245-6

10. Self-evaluation

The project on which my bachelor’s thesis is based has met all the expected goals. After completing the entire experimental process and writing the report, I feel that I have been able to demonstrate everything I have learned during my five years of studying the Double Degree in Biotechnology and Biochemistry and Molecular Biology.

Firstly, carrying out this project in a German laboratory allowed me to learn how to work in an international environment, where the only common language was English. In this way, I can confidently say that I am capable of working in such an environment in my future professional career and presenting my experimental work in this language.

Secondly, the project that formed the basis of my thesis has been of great interest to me. I learned to use the Golden Gate cloning technique and performed procedures such as DNA gels and restriction assays, which provided me with valuable experience in the DNA cloning field. Without a doubt, I would like to continue training in this area in both my academic and professional future.

Thirdly, developing the experimental part of this final thesis has taught me that science requires a great deal of discipline and that results are not always as expected. Therefore, it is essential to always be aware of what is being tested in each experiment in order to identify the root cause of any issues and to critically evaluate all results.

Fourthly, completing this final degree thesis allowed me to test my organizational skills. Managing both the experimental work and the writing process required a significant amount of time, so being well-organized was key to delivering a quality project.

And finally, but no less importantly, this thesis has once again shown me that being a biochemist and biotechnologist is truly my passion.