
FROM DEFENSE TO MICROBIOME MODIFICATION: THE TALENTED ROLE OF PROSYSTEMIN AND ITS DERIVED PEPTIDES IN TOMATO PLANTS

Valeria Castaldi

Dottorato in Biotecnologie – XXXVII Ciclo

Università degli Studi di Napoli Federico II



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Dottoranda: Valeria Castaldi

Supervisor: Prof.ssa Rosa Rao

Coordinatore: Prof. Marco Moracci

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Summary

Plants have evolved intricate defense strategies over millions of years to adapt to environmental changes, but the plethora of molecules involved in these processes remains underexplored. A significant area of study focuses on signaling peptides, which hold a great potential for use in crop protection as innovative biotechnological tools. Our contribution to this field of study began with Prosystemin (ProSys), the precursor of the first signaling peptide identified in plants, Systemin (Sys), discovered by Ryan in 1991 in *Solanum lycopersicum*. Since previous research on ProSys revealed its activities were greater than the sole Sys contribution, our work aimed to identify ProSys smallest bioactive sequences responsible for the broader roles it plays. In Chapter 1, we identified new short peptide motifs along ProSys sequence that were tested for their biological activity. Selected peptides successfully protected tomato plants against insect pests and necrotrophic fungi without direct toxicity effects, triggering defense pathways linked to jasmonic acid (JA) and oligogalacturonides (OGs). Given that plants function as holobionts, involving dynamic interactions between the host and its associated microbiome, in Chapter 2 we explored the effects of a selected Prosys-derived peptide on the tomato phyllosphere microbiome. The peptide shaped the leaf microbiome both taxonomically and functionally, with shifts resembling microbial patterns typically associated with JA-mediated stress responses. The enrichment of a targeted microbiome positioned the peptide not only as an effective resistance elicitor, but also as a potential attractant of a specific beneficial microbiota.

In Chapter 3, based on the *in vivo* discovery of some peptide motifs predicted in Chapter 1, it was hypothesized that ProSys might undergo further proteolytic processing to release additional peptides beyond Sys. Revealing the presence of a truncated form of ProSys lacking the Sys sequence (PSDel₁₋₁₇₈) in chloroplasts, we proposed new pathways for ProSys fate inside the plant cell. Overall, our findings provide novel understanding into ProSys multifaceted roles in plant stress responses while underlining its potential as a valuable source of new peptide-based tools for crop protection. This investigation led to an international patent registration involving the discovered ProSys peptide motifs.

Chapter 1

Prosystemin: an Intrinsically Disordered Protein Reveals Functional Repeat Motifs

Valeria Castaldi and Rosa Rao

Work carried out in collaboration with Angela Amoresano^a, Francesco Pennacchio^b, Emma Langella^c and Simona Maria Monti^c

^aDepartment of Chemical Sciences, University of Naples Federico II, Naples and Interuniversity Consortium “Istituto Nazionale Biostrutture e Biosistemi, Rome, Italy

^bDepartment of Agricultural Sciences, University of Naples “Federico II”, Portici, Italy

^cInstitute of Biostructures and Bioimaging, National Research Council (IBB, CNR), Naples, Italy

Abstract

In the history of tomato Prosystemin defense processes, the focus has always been the Systemin peptide, a key player in activating multiple responses to a wide range of stressors. However, the mechanisms that underpin such a broad protection capacity are also possibly linked to the intrinsic disorder of its precursor protein Prosystemin (ProSys). Since recent findings suggest that ProSys has its own biological activity independent from Systemin sequence, the present work aims to demonstrate that this precursor harbours additional peptide motifs capable of activating stress-related pathways. Our *in silico* prediction revealed the presence of repeated peptide motifs along ProSys structure. Selected peptides were synthetically produced to screen their biological activity in protecting tomato plants against different pest organisms. When exogenously supplied in small doses, they induced key defense-related genes associated with jasmonic acid and oligogalacturonides pathways, reduced necrosis caused by *Botrytis cinerea* and *Alternaria alternata* infections, and significantly impaired the survival of *Spodoptera littoralis* larvae. Overall, our study not only advances the understanding of ProSys beyond the well-known Sys peptide but also highlights its potential in plant defense, identifying new derived tools that could be employed as innovative strategies in crop protection.

1.1 Introduction

Agriculture faces significant challenges, many of which are intensified by climate change, ranging from increasing abiotic stresses to pest infestations that threaten crops yield and quality (N'Danikou & Tchokponhoue, 2020). These pressures result in billions of dollars in economic losses annually (Zahid et al., 2024). Abiotic stress alone accounts for up to 70% of global yield reductions (Das & Das, 2019), while the Food and Agriculture Organization (FAO) estimates that around 40% of global crop production is lost each year due to biotic factors, with the greatest impact occurring during the pre-harvest phase (Isman, 2019; Tang et al., 2023). Prospects regarding the survival of staple crops like maize, wheat, and rice under these environmental conditions are expected to deteriorate in the coming years (Tigchelaar et al., 2018). The growing vulnerability within the agricultural systems raises serious concerns regarding the ability to meet the increasing food demands,

highlighting the need for new frontiers in sustainable crop protection strategies that should not rely on chemical pesticides. Therefore, the European Union (EU) has established ambitious goals aimed to preserve crops health and ensure food security, such as the Farm to Fork strategy (Faichuk et al., 2022). Within the European Green Deal, this strategy proposes to reduce pesticide use by 50%, particularly those containing harmful chemicals (Faichuk et al., 2022). These policies emphasize the need for integrated approaches to mitigate environmental stressors without introducing health risks across the entire production chain, from farmers to consumers (Helepciuc & Todor, 2022). Among the alternatives gaining attention there are microbial biological control products (MBCPs) (Helepciuc & Todor, 2022), natural predators like entomopathogenic fungi (Scorsetti et al., 2018), biopesticide molecules derived from animals, plants, or microorganisms, as well as nanotechnologies and plant-based solutions (Constantine et al., 2020; González-García et al., 2021; Zeng et al., 2020). Plant-derived solutions, including signaling peptides or pesticide molecules, may offer sustainable alternatives to chemical pesticides in pest management programs, minimizing environmental and human risks while safeguarding crops health. Signaling peptides, in particular, play a crucial role in plant immunity by inducing resistance to a wide range of stressors (Wiesel et al., 2014; Yamaguchi & Huffaker, 2011). Notably, peptides can act both endogenously and exogenously to modulate plant responses. For instance, their *ex vivo* application can be used to amplify or anticipate plant defense mechanisms. An example is the *Zea mays* peptide ZmPEP3, which has been shown to enhance resilience to herbivorous insects by activating jasmonic acid (JA) and ethylene (ET) pathways, central to herbivore defense (Huffaker et al., 2013). Similarly, *Prunus persica*-derived plant elicitor peptides (PEPs) helped counteract bacterial infections, such as the one caused by *Xanthomonas arboricola* pv. *pruni*, by involving ET, abscisic acid (ABA), and WRKY related genes (Foix et al., 2021). In tomato, the systemin peptide (Sys), derived from the C-terminus of its precursor Prosystemin (ProSys), exemplifies an endogenous signaling peptide, or phyto cytokine, capable of enhancing basal immunity in both tomato and related plant species (Aprile et al., 2022; Coppola et al., 2017, 2019; Gust et al., 2017; Molisso et al., 2020; Pastor-Fernández et al., 2020). Typically associated with JA signaling and the octadecanoid pathway, Sys has long been recognized as a valid weapon against

necrotrophic fungal attacks and chewing insects threats (McGurl et al., 1994; Noval et al., 2007; Zhang et al., 2020), while also playing a role in mitigating salinity stress (Cirillo et al., 2022; Orsini et al., 2010). This defense mechanism relies on JA production and the downstream synthesis of proteinase inhibitors, which deter insect digestion, inhibit fungal colonization, and enhance plant tolerance to drought and salinity (Cirillo et al., 2022).

The potential for integrating peptides into crop protection programs resides in their perception by cell membrane receptors, triggering signaling cascades which can also stimulate the endogenous production of the applied peptides (Coppola et al., 2019). Moreover, the structural homology of leucine rich repeat receptor-like kinases (LRR-RLKs), involved in plant defense and development, broadens the application of peptides, as demonstrated for Sys across multiple plant species (Molisso et al., 2020; Wang et al., 2018). Ongoing research on peptides is not only elucidating their physiological roles but also expanding their potential applications (Olsson et al., 2019), opening avenues for the patenting of innovative crop protection strategies. In this context, we have thoroughly explored new structural elements within the 200-amino acid ProSys protein, aiming to both explain its multifaceted contribution to tomato defense beyond Sys and to identify new exploitable tools for crop protection (Castaldi et al., 2024; Molisso, et al., 2022a). Deletion of Sys sequence from ProSys (PSDel₁₋₁₇₈), revealed additional and non-overlapping defense-related functions of this proteoform compared to Sys, whether overexpressed in transgenic plants or delivered *ex vivo* (Corrado et al., 2016; Molisso et al., 2022b). Notably, the investigated plants produced pectin-derived oligomers known as oligogalacturonides (OGs) with varying degrees of polymerization (DP) (Molisso et al., 2022b). Traditionally, OGs have been described as endogenous damage-associated molecular signals, released upon stress perception, which elicit resistance to fungal and bacterial phytopathogens (Ferrari et al., 2013) but also ET induction, crucial for fruit ripening in tomato (Simpson et al., 1998). Prompted by these results that extended ProSys roles in plant stress-coping mechanisms, Molisso and co-workers (2022a) aimed to functionally dissect and screen ProSys regions, identifying larger biologically active fragments within PSDel₁₋₁₇₈ region, namely PS1-70 (first 70 aa) and PS1-120 (first 120 aa) that we further investigated with the present work, providing a first structural rationale behind their functionality. The formation of repeated

sequence motifs along protein structure belongs to genes encoding intrinsically disordered proteins, such as ProSys (Buonanno et al., 2018; Habchi et al., 2014). Its gene evolutionary history, probably characterized by duplication, module exchange, or expansion events, likely associates with the found repeated motifs (Habchi et al., 2014; McGurl & Ryan, 1992; Tompa, 2003) and aligns with rapidity of evolution, disorder propensity, and functional versatility of IDPs (Habchi et al., 2014). Moreover, disordered regions are believed to be crucial for performing diverse functions, making IDPs indispensable in processes like transcriptional regulation, cell cycle control, and stress adaptation, particularly in eukaryotes (Roey et al., 2014; Theillet et al., 2014). Recently, ProSys has been shown to exhibit interaction promiscuity (Natale et al., 2023), highlighting its versatility under stressful conditions and raising important questions about which structural elements are responsible for such activity. In this study, we aimed to answer this question, performing an *in silico* study of ProSys structure that revealed the presence of repeated peptide motifs along PSDel₁₋₁₇₈ region. Since preliminary studies suggested that PS1–70 fragment is highly disordered compared to PS1–120 fragment predicted to gain some secondary structure (Buonanno et al., 2018; Molisso et al., 2022a), we selected representative peptide motifs located within PS1-70, which already demonstrated a significant activity in protecting plants against both the larvae of the noctuid *Spodoptera littoralis* and the necrotrophic fungus *Botrytis cinerea* (Molisso et al., 2022a). Candidate peptides were synthetically produced and tested for their potential to enhance stress tolerance in tomato plants, placing them as novel immune-modulating agents for plants. Our comprehensive characterization included different tests, from target to non-target organisms, providing additional insights into ProSys functional mechanisms and opening new avenues for the development and future registration of plant-derived, eco-friendly tools for crop protection.

1.2 Results

1.2.1 ProSys displays repeated peptide motifs along its structure

Previously collected data in our lab showed that ProSys truncated form PSDel₁₋₁₇₈, depleted of Sys sequences, exhibited a biological activity in tomato plant protection alone (Corrado et al., 2016; Molisso et al., 2022b). Therefore, we performed a detailed analysis

of ProSys sequence aiming to detect putative other functional motifs beyond Sys. The analysis was carried out using both RADAR algorithm (Heger & Holm, 2000) and multiple sequence alignment method (Notredame et al., 2000). Along ProSys N-terminus, that excludes Sys peptide sequence, we identified and characterized 4 long imperfect repeats of 24–33 residues that were named as “Total 1–4” (T1–4), which contained shorter amino acid repeats assigned to two separate groups, “Green 1–4” (G1–4), and “Red 1–4” (R1–4), of 14–16-residues and 9-residues, respectively (Fig. 1.1). The compositional features of the repeated peptide motifs (RMs) are primarily characterized by disorder-promoting amino acids (Theillet et al., 2013), enriched in polar and highly charged residues in a range of 77,4%-85,7%, as shown in Table 1.1 (in Appendix).

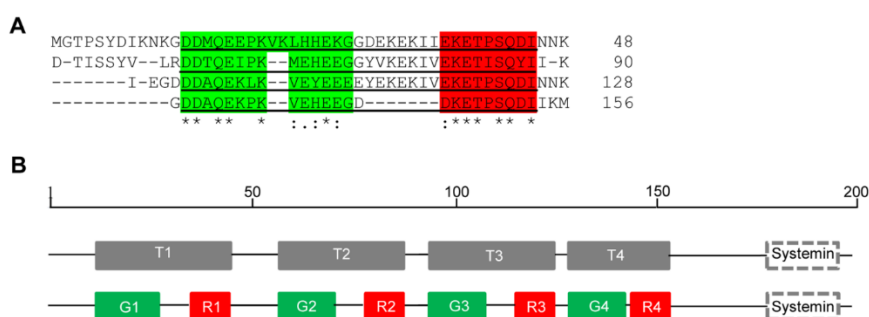


Figure 1.1: Repeated peptide motifs embedded in ProSys N-terminal region. (A) The T1-4 sequences are underlined, while G1-4 motifs are highlighted in green and R1-4 motifs in red. (B) A scaled schematic representation of RMs groups distributed on the 200-amino acids long ProSys sequence. Repeated peptide motifs are illustrated as coloured rectangles, with the Sys sequence represented as a dashed-line rectangle in the C-terminal end.

Among these residues, Aspartic and Glutamic acid appear as the most abundant and confer an overall net negative charge to ProSys protein. In order to screen their ability in counteracting plant biotic stresses, candidate RMs representative of each group, were selected from the first 48 residues of the protein (T1, G1, R1; Fig. 1.1A, B).

1.2.2 Some peptides exhibit an ordered-state tendency

Candidate synthetic peptides exhibited random coil characteristics, with a pronounced minimum around 200 nm and ellipticity near zero

at approximately 220 nm in aqueous solution (Fig. 1.2A, B; in Appendix). To explore their tendency to assume a certain degree of order, we used TFE at different concentrations to mimic hydrophobic conditions (Fig. 1.2C, D; in Appendix), typical of protein-protein interactions (Buonanno et al., 2018; Roccatano et al., 2002). Among the three peptides tested, only T1 and G1 displayed a propensity to adopt an alpha-helical secondary structure, with T1 showing the strongest tendency starting from 15% to reach a peak at 30% TFE. While R1 did not exhibit any ordered conformation even at higher concentrations, T1 behaviour was comparable to ProSys protein as previously described (Buonanno et al., 2018). Moreover, an isodichroic point in the CD spectrum overlay, observed at approximately 206 nm, indicated a transition from a random coil to an alpha-helix, suggesting an equilibrium between these two conformations (Zhou et al., 1993).

1.2.3 Synthetic peptide motifs counteract biotic stressors threats

1.2.3.1 Mortality and lack of development in *Spodoptera littoralis* larvae

Young tomato plants treated with candidate synthetic peptides were able to efficiently contrast *Spodoptera littoralis* larvae attacks. Indeed, plants exogenously supplied with peptides 6 hours before larvae feeding on leaf disks, strongly compromised their survival (Log-rank test: $\chi^2 = 166.7$, $df = 3$, $P < 0.0001$) (Fig. 1.3A). The trend indicated that all the peptides negatively affected the larvae compared to the control group (C-larvae) that showed the 100% of survival (Log-rank test: C-larvae vs T1-larvae $\chi^2 = 126.3$, $df = 1$, $P < 0.0001$; C-larvae vs G1-larvae $\chi^2 = 128.1$, $df = 1$, $P < 0.0001$; C-larvae vs R1-larvae $\chi^2 = 126.5$, $df = 1$, $P < 0.0001$).

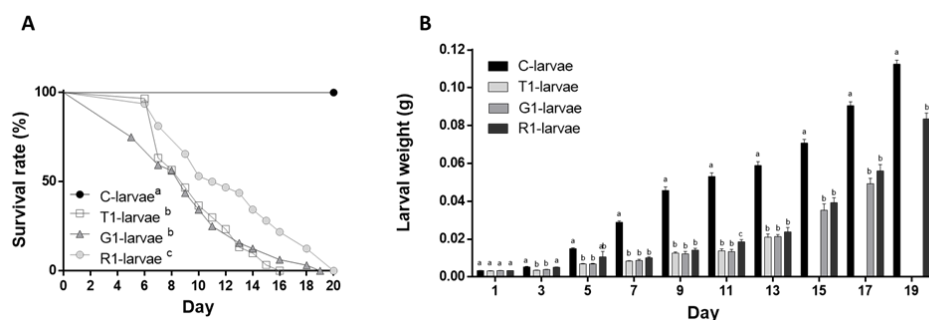


Figure 1.3: Plants treated with 100 fM of T1, G1, and R1 compromise growth and survival of *S. littoralis* larvae. (A) Survival rate of larvae fed on leaf disks from plants treated with 100fM of synthetic peptides or PBS 0.1X as control. Different letters indicate significant differences in the survival curve (Log-Rank test, $P < 0.0001$). (B) Larval weight in grams (g) from 3rd instar until all the larvae per the group died. Synthetic peptides treatments impaired larvae development compared to the control group. Different letters denote significant differences in the larval weight (g) (One Way Anova: $P < 0.001$; day 19 t-test: $P < 0.0001$). Values represent means $\pm$ SE.

Specifically, larvae fed on T1- or G1-treated leaves exhibited a similar mortality trend, showing deaths on the 16th and 18th days of the bioassay, respectively. In contrast, R1-treated leaves appeared less effective compared to both T1 (Log-rank test: $\chi^2 = 8.422$, $df = 1$, $P = 0.0037$) and G1 (Log-rank test: $\chi^2 = 7.017$, $df = 1$, $P = 0.0081$). Larval death was associated with a remarkable impairment of growth and development (Fig. 1.3B), due to a lower body weight (One Way ANOVA: $P < 0.0001$).

1.2.3.2 Reducing necrotrophic infections caused by *Botrytis cinerea* and *Alternaria alternata*

To assess the ability of T1, G1, and R1 to contrast necrotrophic fungal infections, two fungal bioassays with *Botrytis cinerea* and *Alternaria alternata* were carried out. After *B. cinerea* inoculation, tomato leaves treated 6 hours earlier significantly reduced necrotic lesions caused by the fungus (One Way ANOVA: $P < 0.0001$; Fig. 1.4A). A similar trend was observed throughout *A. alternata* assay (Fig. 1.4B). However, while in the first case G1 was the most effective in counteracting the infection among the three peptides, in the second case all of them showed a similar behaviour compared to PBS 0.1X-treated leaves, used as controls (One Way ANOVA: $P < 0.0001$).

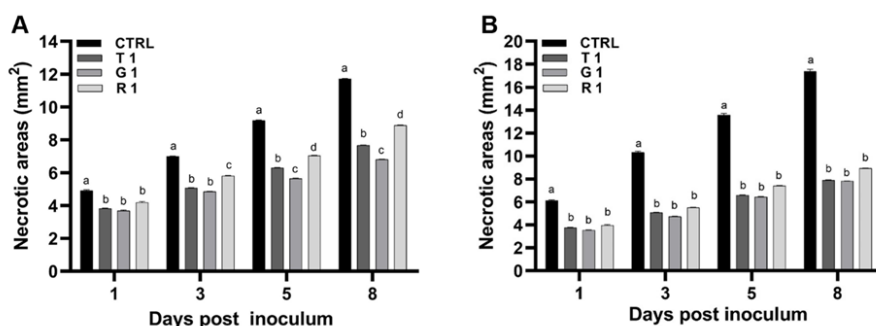


Figure 1.4: Plants treated with T1, G1, and R1 suppress fungal infection. *B. cinerea* (A) and *A. alternata* (B) necrotic lesions progress after leaves treatment with 100 fM of synthetic peptides or PBS 0.1X. Bars represent means of necrotic areas measured 1, 3, 5, 8 days post-inoculum (dpi). Statistically significant differences are indicated with letters (One Way ANOVA: $P < 0.0001$). Error bars indicate standard error.

1.2.4 Peptides effects do not involve direct antimicrobial activity

To assess the potential direct toxicity of synthetic peptides, they were applied *in vitro* to *B. cinerea* and the green microalga *Chlamydomonas reinhardtii*. The viability of *B. cinerea* cultured in media containing T1, G1, and R1 did not significantly differ from controls (Fig. 1.5). Similarly, *C. reinhardtii* displayed similar growth patterns over 7 days when exposed to the experimental peptides (Fig. 1.5B-D). Notably, the presence of the G1 peptide in the culture medium led to a 1.5-fold increase in *C. reinhardtii* growth compared to untreated media (One-Way ANOVA: $P < 0.05$). Therefore, peptides did not show toxicity towards any of the tested microorganisms.

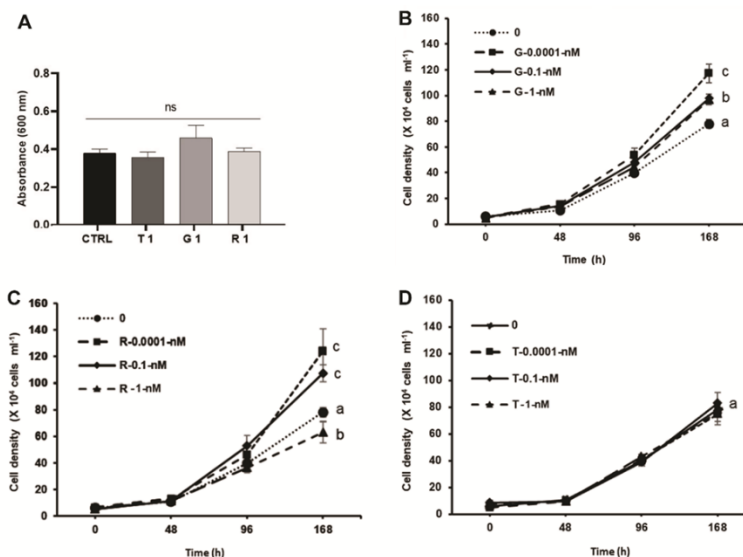


Figure 1.5: *In vitro* antifungal *B. cinerea* vitality assay and growth performance of *C. reinhardtii* exposed to G1, R1, and T1 peptides. (A) Each sterile 12-well plate was filled with 1 mL of PDB 1/2 medium containing synthetic peptides at the final concentration of 100 fM, except for the control wells with growth medium only. Spores of *B. cinerea* were then added to each well and fungal growth was evaluated 24-hpi of the pathogen. The absorbance of the medium was measured at 600 nm (OD_{600}). Statistical significance of data was calculated with One-way ANOVA, Tukey-Kramer Honestly Significant Differences (HSD) test with $P < 0.05$; ns, not significant. Error bars indicate standard error. Microalgae were inoculated in Bold basal medium added with (B) peptide G1 or (C) peptide R1 or with (D) peptide T1 at three different concentrations: 0.0001, 0.1 and 1 nM and incubated at 24 °C up to seven days. The cell density was evaluated every 24 hours for a week.

1.2.5 Peptide motifs trigger defense-related genes

1.2.5.1 The octadecanoid pathway

The phytohormone JA is a key molecule in the octadecanoid pathway for the activation of defense responses, particularly against necrotrophic pathogens and chewing insect pests (Ghorbel et al., 2021). Here we assessed the expression of two early genes involved in this pathway, such as *Allene Oxide Synthase* (AOS; Solyc11g069800) and *Lipoxygenase D* (LoxD; Solyc03g122340), as well as two jasmonate-responsive genes, *Proteinase Inhibitor type I* and *II* (*Pin I* and *Pin II*; Solyc09g084470.2.1 and Solyc09g084450.3). Quantitative Real-Time PCR (qPCR) analysis

revealed that all examined genes were upregulated by peptide motifs application compared to controls, with T1 inducing the most significant response (Fig. 1.6).

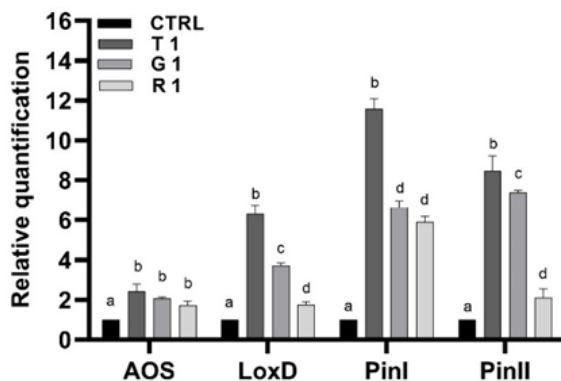


Figure 1.6: Repeat motifs induce defence related genes. Expression analysis of JA-related genes at 6 hours post treatment with 100 fM of T1, G1, R1, or PBS 0.1X as control. Numbers are displayed in relation to the calibrator control condition, which is set to 1 (mock-treated plants). Different statistical groups are denoted by different letters (One-Way ANOVA: $P < 0.05$). Error bars indicate the standard error.

Publications and scientific contributions

Paper: **Castaldi, V.**, Langella, E., Buonanno, M., Di Lelio, I., Aprile, A. M., Molisso, D., Criscuolo, M. C., D'Andrea, L. D., Romanelli, A., Amoresano, A., Pinto, G., Illiano, A., Chiaiese, P., Becchimanzi, A., Pennacchio, F., Rao, R., & Monti, S. M. (2024). Intrinsically disordered Prosystemin discloses biologically active repeat motifs. *Plant Science*, 340, 111969. <https://doi.org/10.1016/j.plantsci.2023.111969>

Paper: Natale, R., Coppola, M., D'Agostino, N., Zhang, Y., Fernie, A. R., **Castaldi, V.**, & Rao, R. (2023). In silico and in vitro approaches allow the identification of the Prosystemin molecular network. *Computational and Structural Biotechnology Journal*, 21, 212–223. <https://doi.org/10.1016/j.csbj.2022.12.006>

Paper: **Castaldi, V.**, Bellino, A., & Baldantoni, D. (2023). The ecology of bladderworts: The unique hunting-gathering-farming strategy in plants. *Food Webs*, 35, e00273. <https://doi.org/10.1016/j.fooweb.2023.e00273>

Paper: Aprile, A. M., Coppola, M., Turrà, D., Vitale, S., Cascone, P., Diretto, G., Fiore, A., **Castaldi, V.**, Romanelli, A., Avitabile, C., Guerrieri, E., Woo, S. L., & Rao, R. (2022). Combination of the Systemin peptide with the beneficial fungus *Trichoderma afroharzianum* T22 improves plant defense responses against pests and diseases. *Journal of Plant Interactions*, 17(1), 569–579. <https://doi.org/10.1080/17429145.2022.2072528>

Talk: **Castaldi, V.**, Criscuolo, M.C., Wicaksono, W.A., Berg, G., Rao, R. Plant peptides as new eco-chemicals: an innovative approach to boost a healthy microbiome. 5th Plant Microbiome Symposium (Microp), Amsterdam, The Netherlands, June 2024

Chair Session: **Castaldi, V.**, and Zlatnar, M. “Plant/Exposome/Food Axis”, 10th Theodor Escherich Symposium, Graz, Austria, January 2024

Poster: **Castaldi, V.**, Criscuolo, M.C., Aprile, A.M., Sequino, G., De Filippis, F., Rao, R. Phyllosphere microbiota composition of tomato

plants treated with Prosystemin-derived peptide (Network Symposium (SMNS), Maribor, Slovenia, November 2023

Poster: Criscuolo, M.C., **Castaldi, V.**, Langella, E., Martina, B., Monti, S.M., Rao, R. Eco-friendly strategies for crop protection: exploring Prosystemin bioactive peptides. IV Memorial Maria Ciaramella Workshop, National Research Council, Naples, Italy, November 2023

Poster: **Castaldi, V.**, Langella, E., Buonanno, M., Aprile, A.M., Criscuolo, M.C., Monti, S.M., Amoresano, A., Pinto, G., Illiano, A., Pennacchio, F., Rao, R. Systemin is not alone: its precursor provides new peptides with still unexplored functions. XII National Meeting of Italian Society of Plant Biology (SIBV), Bari, Italy, September 2023

Poster: Criscuolo, M., **Castaldi, V.**, Aprile, A.M., Langella, E., Buonanno, M., Monti, S.M., Rao, R. Beyond Systemin: An in deep screening of molecular mechanisms underpinning tomato Prosystemin. Meeting of Italian Society of Plant Biology (SIBV), Bari, Italy, September 2023

Poster: **Castaldi, V.**, Di Lelio, I., Langella, E., Buonanno, M., Becchimanzi, A., Monti, S.M., Pennacchio, F., Rao, R. Plant immunity inducers: new peptide motifs from Prosystemin to face plant stressors. CNIE 2023, XXVII Italian National Congress of Entomology, Palermo, Italy, June 2023

Poster: Aprile, A., Cascone, P., Gualtieri, L., **Castaldi, V.**, Criscuolo, M., Ruocco, M., Guerrieri, E., Rao, R. PS1-70 and PS1-120 peptides reduce the damages produced by *Phthorimaea absoluta* (Lepidoptera, Gelechiidae) infestation in tomato plants. CNIE 2023, XXVII Italian National Congress of Entomology, Palermo, Italy, June 2023

Research seminar: **Castaldi, V.** Systemin is not alone: its precursor provides new peptides with still unexplored functions. Youngs for Science meetings “We got this Friday”, Portici, Italy, July 2023

Poster: Aprile, A.M., Coppola, M., Turrà, D., Vitale, S., Cascone, P., Di Lelio, I., Diretto, G., Fiore, A., **Castaldi, V.**, Guerrieri, E., Pennacchio, F., Woo, S.L., Rao, R. Enhancing tomato endogenous

defenses by the combined application of the Systemin peptide and the beneficial fungus *Trichoderma afroharzianum*. (Poster) Priming the future for Healthy Plants (IOBC-WPRS PR-IR 2022), University of Sheffield, United Kingdom, April 2022

Poster: **Castaldi, V.**, Molisso, D., Langella, E., Buonanno, M., Di Lelio, I., Aprile, A.M., Criscuolo, M., Monti, S.M., Pennacchio, F., Rao, R. Prosystemin peptides as novel tools for sustainable tomato crop protection. 65th Congress of Italian Society of Agricultural Genetics (SIGA), Catholic University of Sacred Heart, Piacenza, Italy September 2022

Poster: Magliulo, R., Cirillo, V., Molisso, D., Monti, S.M., Langella, E., Buonanno, M., Aprile, A.M., Criscuolo, M.C., **Castaldi, V.**, Maggio, A., Rao, R. Novel protein fragments for tomato resilience to salt stress. 65th Congress of Italian Society of Agricultural Genetics (SIGA), Catholic University of Sacred Heart, Piacenza, Italy, September 2022

Research seminar: **Castaldi, V.**, Molisso, D., Langella, E., Buonanno, M., Di Lelio, I., Aprile, A.M., Criscuolo, M., Monti, S.M., Pennacchio, F., Rao, R. Novel peptides for tomato pest control. Plant multitrophic interactions for bioinspired Strategies of Pest ConTrol (PROSPECT), PRIN Project, Institute for Sustainable Plant Protection (IPSP), National Research Council of Italy (CNR), Portici, Italy, November 2021

Attachments

Published papers

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Intrinsically disordered Prosystemin discloses biologically active repeat motifs

Valeria Castaldi^{a,1}, Emma Langella^{b,*,1}, Martina Buonanno^b, Ilaria Di Lelio^{a,c}, Anna Maria Aprile^a, Donata Molisso^a, Martina Chiara Criscuolo^a, Luca Domenico D'Andrea^d, Alessandra Romanelli^e, Angela Amoresano^f, Gabriella Pinto^f, Anna Illiano^f, Pasquale Chiaiese^a, Andrea Becchimanzi^{a,c}, Francesco Pennacchio^{a,c}, Rosa Rao^{a,c,**}, Simona Maria Monti^{b,*}

^a Department of Agricultural Sciences, University of Naples Federico II, via Università 100, Portici, Naples 80055, Italy

^b Institute of Biostructures and Bioimaging, National Research Council (IBB, CNR), via Pietro Castellino 111, Naples 80131, Italy

^c Interuniversity Center for Studies on Bioinspired Agro-Environmental Technology (BAT Center), University of Naples Federico II, via Università 100, Portici, 80055 Naples, Italy

^d Istituto di Scienze e Tecnologie Chimiche "Giulio Natta" (SCITEC), Consiglio Nazionale delle Ricerche (CNR), via Alfonso Corti 12, 20131 Milano, Italy

^e University of Milan, via Venetian 21, 20133 Milan, Italy

^f Department of Chemical Sciences, University of Naples Federico II, via Cynthia 8, Napoli and Interuniversity Consortium "Istituto Nazionale Biostrutture e Biosistemi, 80126 Roma, Italy

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In vivo detection

ABSTRACT

The in-depth studies over the years on the defence barriers by tomato plants have shown that the Systemin peptide controls the response to a wealth of environmental stress agents. This multifaceted stress reaction seems to be related to the intrinsic disorder of its precursor protein, Prosystemin (ProSys). Since latest findings show that ProSys has biological functions besides Systemin sequence, here we wanted to assess if this precursor includes peptide motifs able to trigger stress-related pathways. Candidate peptides were identified *in silico* and synthesized to test their capacity to trigger defence responses in tomato plants against different biotic stressors. Our results demonstrated that ProSys harbours several repeat motifs which triggered plant immune reactions against pathogens and pest insects. Three of these peptides were detected by mass spectrometry in plants expressing ProSys, demonstrating their effective presence *in vivo*. These experimental data shed light on unrecognized functions of ProSys, mediated by multiple biologically active sequences which may partly account for the capacity of ProSys to induce defense responses to different stress agents.

1. Introduction

Prosystemin (ProSys) is a 200-amino acid precursor protein involved in plant defense responses in *Solanaceae* (McGurl and Ryan, 1992). Upon wounding or attack by herbivores, this precursor releases an octadecapeptide, named Systemin (Sys), from its carboxy-terminal region (McGurl et al., 1992; McGurl and Ryan, 1992). Sys is the first peptide hormone identified in plants (Pearce et al., 1991; Ryan and Pearce, 1998) and acts by spreading the signal that triggers systemic defense responses by amplifying the jasmonate signaling pathway (Schilmiller

and Howe, 2005). Since its discovery, two decades of research deeply investigated the Sys biological activity in tomato plants, conferring protection against a wide range of stress agents, such as phytophagous insects, phytopathogenic fungi, and salinity (De La Noval et al., 2007; Coppola et al., 2010; Orsini et al., 2010; Pastor et al., 2018; Zhang et al., 2020; Aprile et al., 2022). Recently, it was suggested that Sys is released by a poorly defined pathway, apparently mediated by phytaspases (McGurl and Ryan, 1992; Beloshistov et al., 2018), and it is perceived by a pair of distinct receptors, referred as SYR1 and SYR2, belonging to a leucine-rich repeat receptor kinase (LRR-RK) (Wang et al., 2018). The

* Correspondence to: Institute of Biostructures and Bioimaging, National Research Council, via Pietro Castellino 111, 80131, Naples, Italy.

** Corresponding author at: Department of Agricultural Sciences, University of Naples Federico II, Via Università 100, Portici, Naples 80055, Italy.

E-mail addresses: emma.langella@cnr.it (E. Langella), rao@unina.it (R. Rao), simona.monti@cnr.it (S.M. Monti).

¹ equally contributed

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protective effects of Sys peptide are observed not only on *Solanoideae* subfamily, but also on other plant species where other SYR-like receptors are present (Molisso et al., 2021). Indeed, recent investigations revealed that the direct delivery of Sys to *Solanum melongena* and *Vitis vinifera* protects plants from grey mould (*Botrytis cinerea*) by up-regulating defense-related genes (Molisso et al., 2021).

Corrado and coworkers (Corrado et al., 2016) further investigated this topic, focusing on the possible biological role of Sys precursor. They demonstrated that a mutant of ProSys, irrespective of the presence of the Sys sequence, still promoted defense responses in tobacco plants. These results prompted us to perform a more detailed investigation on the precursor protein. Overexpression of truncated ProSys (ProSys₁₋₁₇₈) in transgenic tomato plants and exogenous treatment of plants with the recombinant truncated protein corroborated previous findings (Molisso et al., 2022b). In both cases the expression of defense-related genes was modulated, and plants were protected against the lepidopteran pest *Spodoptera littoralis* and the fungal pathogen *B. cinerea* (Molisso et al., 2022b). Taken together, these results show that ProSys does not act only as Sys precursor but hides other biologically active regions. This is in line with earlier studies showing that ProSys is an intrinsically disordered protein (IDP), characterized by long disordered regions and missing a unique well-defined fold typical of structured proteins (Buonanno et al., 2018; Langella et al., 2018, 2021). The propensity to interact at a molecular level with a variety of partners is another feature of IDPs (Kragelund et al., 2012; Roey et al., 2014). We recently showed that ProSys has many protein interactors, and this finding well fits with its observed structure (Natale et al., 2023). Indeed, proteins possessing long stretches of intrinsic disorder are often involved in multiple biological pathways, such as transcriptional and translational activation, chromatin remodeling and signal transduction (Dyson and Wright, 2005; Radivojac et al., 2007; Cortese et al., 2008). Although these proteins and their functions have been characterized mostly in mammalian systems, a growing attention is now registered also for plant IDPs (Kragelund et al., 2012; Sun et al., 2013). In a previous work, to correlate the effect of the disorder content with protein functionality, we dissected ProSys and designed fragments starting from its N-terminal region. Two fragments were designed, one containing the first 70 residues (PS1–70), and the second encompassing residues 1–120 (PS1–120), both not including Sys sequence. According to previous modelling studies PS1–70 was predicted to be highly disordered compared to PS1–120, which included the central region 71–120 predicted to gain some secondary structure content (Buonanno et al., 2018; Molisso et al., 2022a). These recombinant fragments induced defense-related genes and protected tomato plants against *B. cinerea* and *S. littoralis* larvae, with a biological activity that was similar but not quite the same with each other and with the Sys peptide used as control (Molisso et al., 2022a). These evidences definitively supported our starting hypothesis that ProSys encompasses, apart Sys, other biologically active sequences which were overlooked so far.

Here we performed a detailed *in silico* analysis of ProSys sequence, identifying 3 classes of repeated motifs (RMs). One member of each class was tested and found able to trigger plant defense responses against biotic stressors. Interestingly, mass spectrometry analyses of peptides extracted from tomato plants over-expressing ProSys revealed the presence of some RMs *in vivo*. This indicates that ProSys scaffold contains, beyond the well-known Sys hormone, other small bioactive peptides released by the precursor which are able to trigger stress-related pathways. These motifs are important component of ProSys defence mechanism.

2. Materials and methods

2.1. *In silico* identification of RMs

In order to detect the presence of repeats into ProSys sequence, the program RADAR was used (Heger and Holm, 2000) which is available at <https://www.ebi.ac.uk/Tools/pfa/radar/> (Madeira et al., 2022).

RADAR identifies gapped approximate repeats and complex repeat architectures in protein sequences. RADAR results were further refined through sequence alignments using T-Coffee program (Notredame et al., 2000) available at EMBL-EBI (<https://www.ebi.ac.uk/Tools/msa/tcoffee/>) (Madeira et al., 2022).

2.2. Chemical synthesis and LC-MS characterization of G1, R1, and T1 repeat motifs

Total1 (T1), Green1 (G1), and Red1 (R1) peptides were obtained by microwave assisted solid-phase synthesis using standard protocols (Sperandeo et al., 2020) on a Rink Amide resin (loading 0.69 mmol/g). Cleavage and deprotection of the peptides were performed treating the resin with a solution of TFA/TIS/H₂O 95/2.5/2.5 v/v/v 90 min. Peptides were precipitated using cold diethyl ether and purified by RP-HPLC on a Phenomenex Jupiter 10 Å Proteo 90 Å Axia 100 × 21.2 mm using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 5 to 50% in 20 min. Synthesized peptides were acetylated and amidated at the N- and C-terminal part, respectively. Peptides were characterized by mass spectrometry by an HPLC system connected on-line with a Q-TOF mass spectrometer (Agilent) equipped with a narrow bore 50 × 2.1-mm C18BioBasic column (ThermoElectron, Milan) with a gradient of solvent B (0.05% trifluoroacetic acid in CH₃CN) to solvent A (0.08% trifluoroacetic acid in H₂O) that ranged from 5 to 70% over a time period of 40 min. Mass spectra were recorded within the *m/z* 400–2000 range in positive mode (Ayoub et al., 2022). Spectra were deconvoluted using the Agilent MassHunter.06.00 program implemented in the Agilent MassHunter workstation package provided by the manufacturer. Sequences and mass spectrometry data as follow:

Total (T1) sequence: Ac-DDMQEEPVKVLHHEKGGDEKEKII EKETPSQDI-NH₂; Calculated mass (Da): 3902.28. Observed mass (Da): 3901.78 [M+H]⁺; 1301.60 [M+ 3 H]³⁺; 976.45 [M+ 4 H]⁴⁺.

Green1 (G1) sequence: Ac-DDMQEEPVKVLHHEKGG-NH₂; Calculated mass (Da): 1961.16 Observed mass (Da): 1960.88 [M+H]⁺; 981.44 [M+ 2 H]²⁺; 654.63 [M+ 3 H]³⁺.

Red1 (R1) sequence: Ac-EKETPSQDI-NH₂; Calculated mass (Da): 1087.13. Observed mass (Da) 1087.48 [M+H]⁺; 544.25 [M+ 2 H]²⁺.

2.3. *In vivo* detection of RMs in tomato leaves

Extraction and desalting step of peptides from leaves was carried out adding 300 µL denaturing buffer (7 M urea, 2 M thiourea, 3.5 mM SDS, 0.01 M TrisHCl, 2 mM EDTA, 10 mM DTT, plus 4 µL of protease inhibitor cocktail) to leaves (100 mg). After cell lysis occurring with a three-repeated cycle of 10 min sonication and 30 min freeze–thaw process, the supernatant was recovered after centrifugation at 10,000 rpm for 10 min. The extracts were obtained after protein precipitation by using four volumes of cold acetone added to the supernatant and extracts recovered after centrifugation at 10,000 rpm for 10 min were dried by SpeedVac concentrator and resuspended in an aqueous solution containing 2% ACN acidified by 0.2 formic acid (HCOOH) for the further desalting step. Homemade C18 StageTip procedure was used as desalting step in agreement to previous papers (Rappilber et al., 2007) before LC-MS/MS in multiple reaction monitoring (MRM) ion mode.

The peptide mixture was analysed by LC-MS/MS analysis using a Xevo TQ-S (Waters) equipped with an ionkey coupled to an Acquity UPLC system (Waters, Milford, MA, USA). For each run, the peptide mixture (2 µL) was injected and separated on a BEH C18 peptide separation device (130 Å, 1.7 µm, 150 µm X 50 mm) at 45 °C with a flow rate of 3 µL/min using an aqueous solution (LC-MS grade) containing 2% ACN as buffer A and 98% ACN as buffer B, both acidified with 0.2% HCOOH. The gradient for the MRM method started with 7% buffer B for 5 min, from 5 to 40 min reached 50% buffer B, ending to 95% buffer B during the next 2 min. The column was finally re-equilibrated to initial conditions for 4 min. The parameters of the MS source were as follows: 3900 V as ion spray voltage, 150 °C interface heater temperature, 150 L/

h gas flow with 7 bar nebulizer pressure.

MRM mass spectrometric analyses were performed in positive ion mode for the run time with 5 points per peak and dwell times of 3 ms. The cone voltage was set to 35 V. A range of 300–1000 m/z was preferentially selected as precursor or product ions. All instrumental parameter of MRM method was included in [supplemental material \(Supplemental Dataset S1\)](#). The latest version of Skyline software (22.2-64 bit version MacCossLab Software, University of Washington, USA) ([Pino et al., 2020](#)) was used for *in silico* selection of peptides with proteotypic sequences for each selected protein. For each peptide, m/z precursor ion, m/z product ion, and relative collision energy were provided by Skyline and included within the [supplemental material \(Supplemental Dataset S1\)](#). Putative peptides were chosen for the development of MRM assays. The six transitions of putative peptides were selected for method development based on the y- and b-fragment ions.

2.4. Circular Dichroism Analysis

The secondary structure of the peptides was investigated by means of Circular Dichroism experiments using a Jasco J-810 spectropolarimeter (Jasco, Essex, UK). Spectra were recorded at room temperature setting a wavelength range of 190–260 nm (50 nm/min scan speed) and three accumulations for each acquisition. Each sample was diluted in ultrapure water in a final volume of 200 μ L in a Hellma quartz cell with 1 nm light path ([D'agostino et al., 2019](#)). The final concentration reached for the experiments was 6.4 μ M, 125 μ M, and up to 367 μ M for T1, G1 and R1, respectively. In order to evaluate the propensity of the synthetic peptides to adopt helical structures, a titration in 2,2,2-Trifluoroethanol (TFE) by adding from 0% to 60% TFE (volume of TFE added/total volume added) was performed. Raw spectra were corrected for buffer contribution, converted to mean molar ellipticity per residue (θ) ($\text{deg cm}^2 \text{ dmol}^{-1}$) as previously described ([Buonanno et al., 2018](#)), and visualized using GraphPad software. The α -helical content was calculated as described by Montserret and coworkers ([Montserret et al., 2000](#)), assuming that the residue molar ellipticity at 222 nm is exclusively due to α -helix and subsequently applying the following equation:

$$\%helix = 100 \frac{[\theta]_{obsd}}{[\theta]_{H_2}}$$

where $[\theta]_{obsd}$ is the observed mean residue ellipticity and $[\theta]_{H_2}$ is the maximum mean residue ellipticity for a helix of finite length. The calculation of $[\theta]_{H_2}$ was based on the empirical equation for helix length dependence ([Chen et al., 1974](#)):

$$[\theta]_{H_2} = [\theta]_{H_{\infty}} (f_H - ik/N)$$

where $[\theta]_{H_{\infty}}$ is the maximum mean residue ellipticity for a helix of infinite length (-39500 for $\lambda = 222$ nm), f_H is the fraction of helix, i is the number of helical segments (1, Green and Red; 3, Total), k is a wavelength-dependent constant (2.57 for $\lambda = 222$ nm) and N is the number of residues. f_H is so defined:

$$f_H = \bar{n}/N$$

where N is the total number of residues and $\bar{n}$ is the average number of residues per segment of the protein molecule.

2.5. Plant material for bioassays

Tomato plants (*Solanum lycopersicum* L. cultivar “Dwarf San Marzano”) were grown as previously reported ([Molisso et al., 2022a](#)). Briefly, sterilized seeds were germinated in Petri dishes and placed in a growth chamber at $24 \pm 1^\circ\text{C}$ and $60 \pm 5\%$ of relative humidity (RH), under dark conditions. Upon root emergence, plantlets were transplanted to a polystyrene plateau and kept in a growth condition of 24

$\pm 1^\circ\text{C}$ and $60 \pm 5\%$ RH, including 18 h light/dark photoperiod and 5000 lux of brightness. After 2 weeks, plants were transferred into pots of 9-cm diameter filled with sterile soil mixture and grown under the same conditions. Healthy and fully expanded leaves of four-week-old plants were treated as previously described ([Molisso et al., 2022a](#)) with 100 fM T1, G1 and R1 by gently placing it on the adaxial surface of intact leaves. A mock treatment with PBS 0.1X (phosphate buffer saline, phosphates 1 mM, NaCl 14 mM, KCl 0.27 mM, pH 7.4, Sigma-Aldrich, Milan, Italy) was used as control. Leaf samples were collected 6 h post treatment (hpt) for bioassays and molecular analyses. The prosystemin over-expressing transgenic plants in the cv. “Castlemart” genetic background were kindly donated by Dr. John P. Delano-Frier (Centro de Investigación y de Estudios Avanzados, Irapuato, Mexico).

2.6. Gene Expression Analysis

Total RNA extraction, synthesis of the first strand cDNA and Quantitative Real Time-PCR (qPCR) were carried out as previously described ([Corrado et al., 2012](#)). qPCR was performed using Rotor Gene 6000 (Corbett Research; Sydney, Australia). Gene expression analysis was carried out using two technical replicates for each of the three biological replicates per samples. EF-1 α gene was used as the endogenous reference gene for the normalization of the expression levels of the target genes ([Marum et al., 2012](#); [Müller et al., 2015](#)). Relative quantification of gene expression was carried out using the $2^{-\Delta\Delta CT}$ method ([Livak and Schmittgen, 2001](#)). Oligonucleotides sequences are reported in [Supplemental Table S6](#).

2.7. Bioassay with *S. littoralis* larvae

S. littoralis (Lepidoptera, Noctuidae) is permanently lab-reared at the Department of Agricultural Sciences and derives from a population collected on flower crops in Agro-Pontino (Latina, Italy). The larvae were reared on an artificial diet (41.4 g/l wheat germ, 59.2 g/l brewer's yeast, 165 g/l corn meal, 5.9 g/l ascorbic acid, 1.53 g/l benzoic acid, 1.8 g/l methyl-4-hydroxybenzoate, and 29.6 g/l agar), as previously described, at $25 \pm 1^\circ\text{C}$, $70 \pm 5\%$ (RH), and photoperiod of 16:8 h light/dark ([Di Lelio et al., 2014](#)).

S. littoralis bioassay was carried out at the same growth condition as previously described ([Molisso et al., 2022a](#)) in 4-well plastic rearing trays (RT32W, Frontier Agricultural Sciences, Pitman, NJ, United States). In each well, 3 ml of 1.5% agar-agar (w/v) were dispensed, to keep the tomato leaves turgid in a moist environment. Briefly, newly molted 3rd instar larvae were weighted and singly isolated in the plastic rearing trays containing in each well tomato leaf disk prepared from leaves harvested 6 h post RMs treatment. Four groups of larvae were created: larvae fed on Control plant leaves (C-larvae), larvae fed on T1 treated plant leaves (T1-larvae), larvae fed on G1 treated plant leaves (G1-larvae), and larvae fed on R1 treated plant leaves (R1-larvae). The rearing wells were closed with perforated plastic lids (RTCV4, Frontier Agricultural Sciences, Pitman, NJ, United State). For each treatment, 32 larvae were used. The leaf disks were daily replaced, adjusting the size (Initially of 2 cm^2 , later of 3, 4 and 5 cm^2) to meet the food needs of growing larvae. Larvae were weighted every 2 days and mortality was daily checked.

2.8. Infection by the necrotrophic fungi *B. cinerea* and *A. alternata*

Fungal bioassay was carried out as previously described ([Molisso et al., 2022a](#)). Briefly conidia of the pathogenic fungi *B. cinerea* and *A. alternata* were collected respectively from malt extract peptone agar (MEP) (Difco, Detroit, MI, United States) or potato dextrose agar (PDA) (Sigma-Aldrich, St. Louis, MO, United States) plates, after 1 week of incubation at 25°C , and filtered through sterile Kimwipes (Kimberly-Clark Dallas, Texas, United States), to remove fragments of hyphae, and

adjusted to a concentration of 1×10^6 conidia/ml). Ten ml drops of spore suspension were put on tomato leaves, 6 hpt with 100 fM peptides, using 10 different inoculation points per detached leaf. The assay was carried out using five leaves per treatment from five different plants. Detached leaves were placed on sponges soaked in sterile water and incubated in a growth chamber at 23°C, under 16:8 h light/dark photoperiod and 90% $\pm$ 5% RH. The size of the lesions was measured at 1, 3, 5, and 8 days after infection using a digital caliper.

2.9. Toxicity assays on Fungi and Microalgae

The antifungal assay was conducted as previously reported (Pastor-Fernández et al., 2020; Molisso et al., 2021). Potato dextrose broth (PDB 1/2) medium containing RMs at the final concentration of 100 fM was placed into a sterile 12-well plate. Upon the addition of *B. cinerea* spore solution to each well until a final concentration of 10^5 spores ml⁻¹, the plate was stirred and incubated for 24 h at 25 °C. On a Bio Photometer Spectrophotometer UV/VIS (Eppendorf, Hamburg, Germany), the value of optical density (OD) at a wavelength of 600 nm (OD600) was recorded in triplicate to evaluate the fungal growth.

Microalgae growth inhibition assay was performed on *C. reinhardtii* (Palomba et al., 2022). Briefly, axenic culture of microalgae grown in flasks, in Bold basal salt medium at 24 °C and subculturing weekly in fresh medium. The assay was performed in 24 well microplates and three replicates for each peptide, at concentration of 1.0×10^{-4} , 1.0×10^{-3} , and 1 nM.

To determine the percentage of growth inhibition (GRI%), for each treatment the specific growth rate (μ) was calculated using the following equation:

$$\mu = \frac{\ln N_2 - \ln N_1}{t_2 - t_1}$$

where N_2 and N_1 are cell densities (cell mL⁻¹) at time t_2 and t_1 in hrs. Then, GRI% at 168hrs was calculated at each treatment level with respect to control using the following equation:

$$\%I = \frac{\mu_c - \mu_t}{\mu_c}$$

Where μ_c is the specific growth rate in the control group and μ_t is the specific growth rate in the DNA treatment. The data were expressed as means and standard deviation for three replicate cultures.

2.10. Statistical analysis

Survival curves of *S. littoralis* were compared by using Kaplan–Meier and Log-rank analysis. One-Way ANOVA test was used to evaluate larval weights, fungal growth and necrotic infection development, microalgae growth inhibition and quantities of transcripts abundance. Normality of data was checked with Shapiro–Wilk test, while homoscedasticity was tested with Bartlett's test. When significant effects were observed ($P < 0.05$), Tukey's post-hoc test was used for pairwise comparisons. Data were analyzed using Prism (GraphPad Software Inc. version 6.0b, San Diego, CA, United States).

3. Results

3.1. ProSys contains repeat motifs

The biological activity observed in portions of ProSys devoid of Sys peptide (Molisso et al., 2022b, 2022a) prompted us to perform a thorough analysis of the amino acid sequence of the prohormone. A preliminary investigation was carried out by RADAR algorithm (Heger and Holm, 2000) to evaluate the presence of repeat units in ProSys. Long non-identical repeats, with a length of about 30–40 amino acids, were recognized (Supplemental Fig. S1). Multiple sequence alignment

(Notredame et al., 2000) allowed the identification of 4 long imperfect repeats of 24–33 residues, named Total 1–4 (hereafter referred as T1–4), which contained shorter amino acid repeats assigned to two separate groups, Green 1–4 (G1–4) and Red 1–4 (R1–4), of 14–16-residues and 9-residues, respectively (Fig. 1). The repeats are widespread along the entire protein sequence, apart from the C-terminal part of the protein where Sys peptide is located (Fig. 1B). Notably, the repeat motifs are mainly constituted by charged and polar residues, as shown in Table 1. In fact, the percentage of combined polar and charged residues in the different repeats is high, ranging between 77.4% and 85.7% (Table 1). In addition, all the repeats have a net negative charge, given the high content of Aspartic and Glutamic acid (Table 1). In order to investigate if the identified repeats could exert a putative biological role in plant defense mechanisms, peptide mimics representative of each repeat motifs (RMs), namely T1, G1, and R1 (Fig. 1) were chemically synthesized, characterized and used for biological and molecular studies (Supplemental Fig. S2).

3.2. RMs show a variable degree of plasticity

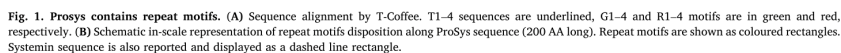
The investigated peptides were characterized by random coil spectra with a single pronounced minimum at about 200 nm and an ellipticity near zero at around 220 nm in aqueous solution (Fig. 2A,B and Supplemental Fig. S3A). The propensity of the RMs to fold at increasing concentrations of TFE was also evaluated (Fig. 2C, D and Supplemental Fig. S3B) (Roccatano et al., 2002). Titration disclosed different behaviors in response to this co-solvent. T1, the longest fragment, showed a high tendency to assume alpha-helical secondary structure, with TFE titration curves very similar to those of the full-length precursor (Buonanno et al., 2018). Indeed, a significant change in the peptide spectrum is already observed at 15% TFE, as in the case of ProSys (Fig. 2C). G1 displayed a slight increase of alpha-helical content, as indicated by the appearance of a positive maximum at near 190 nm and of a negative minimum at 222 nm (Fig. 2D), whereas R1 was not affected by TFE even at high percentages (Supplemental Fig. S3B). At around 30% TFE, the α -helical content of T1 reached values of 39%, showing its propensity to assume a more ordered conformation in presence of TFE (Fig. 2C). An isodichroic point in CD spectra overlay was observed at about 206 nm, indicating that it undergoes a random coil to alpha-helix transition and that an equilibrium exists between the two conformers (Zhou et al., 1993).

3.3. RMs treated plants disrupt insect growth and survival

Treatment of plants with T1, G1 and R1 peptides strongly affected *S. littoralis* larvae survival (Log-rank test: $\chi^2 = 166.7$, df = 3, $P < 0.0001$) (Fig. 3A). All larval groups fed with tomato plant leaves treated with experimental peptides showed 100% mortality, registered between 16 and 20 days from the onset of the feeding bioassay, while controls all survived (Log-rank test: C-larvae vs T1-larvae $\chi^2 = 126.3$, df = 1, $P < 0.0001$; C-larvae vs G1-larvae $\chi^2 = 128.1$, df = 1, $P < 0.0001$; C-larvae vs R1-larvae $\chi^2 = 126.5$, df = 1, $P < 0.0001$). The survival rate of the R1-larvae was significantly different from T1-larvae (Log-rank test: $\chi^2 = 8.422$, df = 1, $P = 0.0037$) and G1-larvae (Log-rank test: $\chi^2 = 7.017$, df = 1, $P = 0.0081$), which did not differ between them (Log-rank test: $\chi^2 = 0.0498$, df = 1, $P = 0.8233$). The observed reduced survival rate was associated with a significantly lower larval weight from day three until larval death (One Way ANOVA: $P < 0.0001$, Fig. 3B and Supplemental Table S1) indicating that *S. littoralis* larvae fed with the treated leaf disks were severely impaired in their growth.

3.4. RMs treated plants show reduced fungal infection

Pre-treated tomato plants were infected with *B. cinerea* spores to test the capacity of RMs to elicit antifungal pathogen responses. As shown in Fig. 4A, RM-treated plants displayed a significant reduction of lesions



* **Hydroph.** stands for hydrophobic residues; * ***pol+char** stands for sum of polar and charged residues

(One Way ANOVA: $P < 0.0001$ and Supplemental Table S3). Nevertheless, there are no appreciable differences among all assayed peptides.

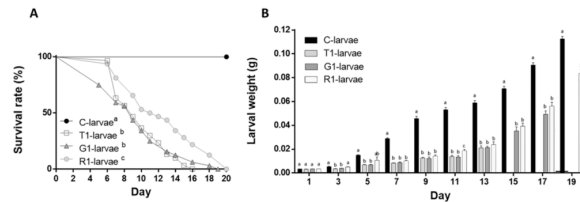


Fig. 3. Plants treated with repeat motifs disrupt insect growth and survival. Effects on survival and development of *S. littoralis* larvae fed on leaves treated with 100 fM of RMs or mock treatment. (A) Survival rate of larvae reared on tomato leaves obtained from plants treated with 100 fM T1, G1 and R1. Feeding on treated plant leaves strongly affected larval survival compared to control. In the graph different letters denote significant differences in the survival curve (Log-Rank test, $P < 0.0001$). (B) Larval weight in grams (g) from 3rd instar until all the larvae in the group died. Larvae fed with the tomato treated leaves showed a reduced larval weight compared to control. In the graph different letters denote significant differences in the larval weight (g) (One Way Anova: $P < 0.001$ and only in the day 19 t-test: $P < 0.0001$). The values are means $\pm$ SE. Punctual statistics is reported in the [Supplemental Table S1](#).

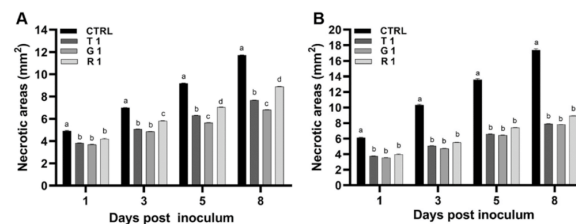


Fig. 4. Plants treated with repeat motifs show reduced fungal infection. *B. cinerea* (A) and *A. alternata* (B) infection progress after RMs or mock treatments. Bars represent means of necrotic areas measured 1, 3, 5, 8 days post-inoculum (dpi). Statistically significant differences are indicated with letters (One Way ANOVA: $P < 0.0001$). Error bars indicate standard error. Punctual statistics is reported in the [Supplemental Tables S2 and S3](#).

3.5. RMs do not have any direct toxic effect both on target and non-target organisms

To investigate whether RMs could exert any direct toxicity, they were directly applied in vitro on *B. cinerea* and green fresh microalgae *Chlamydomonas reinhardtii*. When *B. cinerea* was grown in a culture medium containing T1, G1, and R1 its viability did not differ from controls ([Supplemental Fig. S4A](#)). Similar growth patterns over 7 days in vitro culture were observed when the *C. reinhardtii* was exposed to the experimental peptides ([Supplemental Fig. S4B-D](#) and [Table S4](#)). Interestingly, the presence of G1 peptide in the culture medium resulted in a 1.5-fold growth increase compared the untreated culture medium (One Way ANOVA: $P < 0.05$).

3.6. RMs induce defense related genes

Since the lipid-based octadecanoid pathway leading to jasmonic acid (JA) production is an integral part of the signal transduction pathway guiding the activation of defense responses, we evaluated the expression of two early genes of this pathway, *Allene Oxide Synthase* (AOS; Solyc11g069800) and *Lipoxygenase D* (LoxD; Solyc03g122340), and two genes Jasmonate responsive, *Proteinase Inhibitor type I and II* (*Pin I* and *Pin II*; Solyc09g084470.2.1 and Solyc09g084450.3). Quantitative Real Time-PCR (qPCR) analysis shows that all genes considered were up-regulated by RMs compared to controls, with T1 triggering the most pronounced response ([Fig. 5](#)).

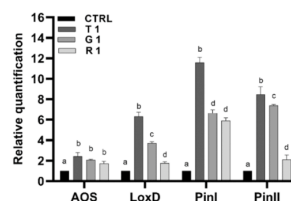


Fig. 5. Repeat motifs induce defence related genes. qPCR expression analysis of defense-related genes at 6 h after treatment with 100 fM RMs. Numbers are displayed in relation to the calibrator control condition, which is set to 1 (mock-treated plants). Different statistical groups are denoted by different letters (One-Way ANOVA: $P < 0.05$). Standard error is shown by error bars.

3.7. In vivo detection of RMs

An LC-MRM/MS method was developed to monitor the putative peptides released from ProSys standard protein (homemade) ([Buonanno et al., 2018](#)). First, a panel of 20 putative peptides was selected, and 5–6 transitions (a combination of each precursor ion to several fragment ions) were chosen for each peptide (4 for y-series and 2 for b-series). A total of 132 transitions were included in the MRM method ([Supplemental Dataset S1](#)) and extracted ion currents of transitions related to

each peptide were monitored. A good instrumental response was assigned whenever the retention time for all transitions describing the amino acid sequence fragmentation was the same and a peak area was higher than 1000.

Out of 20 peptides, four peptides displayed a good instrumental response with 3–4 transitions at the same retention time. As an example, on a total of 6 transitions, 3 extracted ion chromatograms (EIC) ($537.6\text{ m/z} \rightarrow 827.4\text{ m/z}$, $537.6\text{ m/z} \rightarrow 600.2\text{ m/z}$, and $537.6\text{ m/z} \rightarrow 471.2\text{ m/z}$) showed peaks at 16.0 min of retention time suggesting that the revealed peptide displayed the putative amino acid sequence, i.e. DDAQEKPKEHEEG (G4 repeat motif) (Fig. 6). After selecting the best instrumental responses, the relative quantification was performed by comparing the areas underlying the EIC peaks reflecting the differential expression of selected peptides. All values of peak areas averaged for three technical replicates were included in the Supplemental Table S5. The release from the precursor of peptides corresponding to RMs was investigated using transgenic plants overexpressing ProSys in the cv. “Castlemart” genetic background (CM). A quantitative study of the peptide extracts obtained from these plants revealed the presence of a representative member of G, R and T peptides (G4, R4 and T4) beside Sys. The average values from five transgenic plants are shown in Fig. 7, while the value obtained for each single plant is shown in Supplemental Dataset S1. The different amount registered among the transgenic CM

plants is likely the consequence of the variation in transgene expression that frequently occurs in transgenic populations. In wild type (wt) plants, R and T were absent while a low amount of G and Sys were detected. The not significant difference in G4 amount between CM and wt plants is likely due to the large variability registered in the analyzed samples. However, the data here indicate a clear upward trend of G in transgenic CM plants. The low amount of Sys in wt plant is expected since it is known that ProSys has a basal level of expression in plants under physiological conditions (Pearce et al., 1991).

4. Discussion

In recent years, several studies performed on ProSys emphasized its multifunctional nature, since the prohormone does not limit its activity to the mere release of Sys peptide (Molisso et al., 2022b, 2022a). Recent data highlighted the ProSys central role as hub of defense response pathways (Natale et al., 2023), and its capacity to trigger, when devoid of Sys peptide, the production of OGs acting as DAMPs in plant immunity (Molisso et al., 2022b). These multiple behaviors can be in part explained by the disordered nature of ProSys, which allows the interaction of the protein with several partners located in different cellular compartments (Natale et al., 2023). Many functions associated with defense or other physiological events gathered by ProSys have been

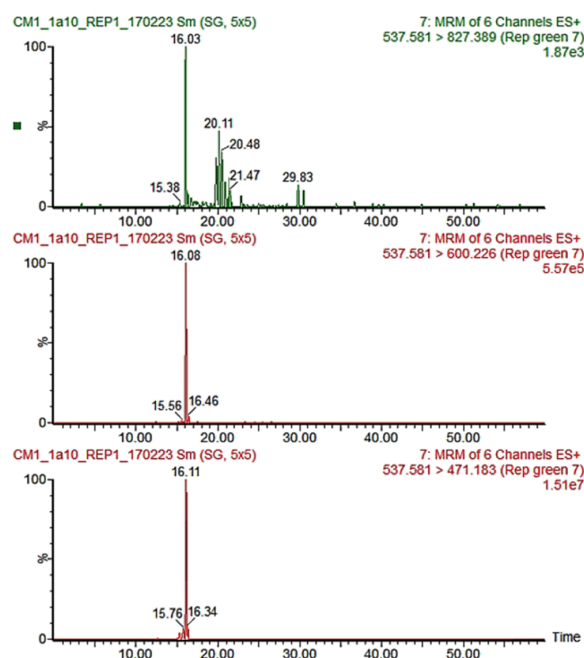


Fig. 6. In vivo detection of repeat motifs. Extracted ion chromatograms of three transitions ($537.6\text{ m/z} \rightarrow 827.4\text{ m/z}$, $537.6\text{ m/z} \rightarrow 600.2\text{ m/z}$, and $537.6\text{ m/z} \rightarrow 471.2\text{ m/z}$) describing the fragmentation of DDAQEKPKEHEEG peptide (G4).

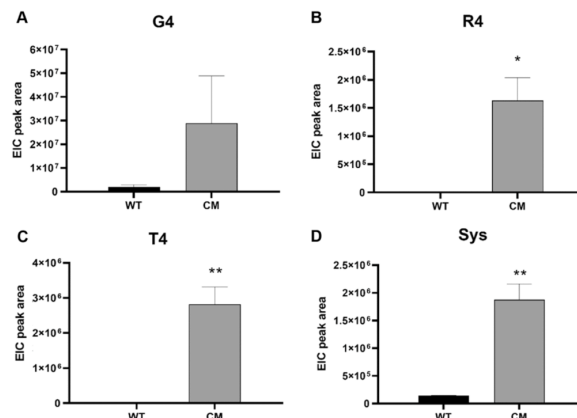


Fig. 7. Relative comparison of peak areas of repeat motifs. Relative comparison of peak areas of extracted ion chromatograms (EIC) detected for each detected peptide, i.e., G4 (panel A), R4 (panel B), T4 (panel C), Sys (panel D), between the wild-type (WT) and transgenic CM plants.

discovered even though it is tempting to speculate that such patterns of interactions may hide novel activities not yet explored.

A deep characterization of ProSys sequence herein reported identified repetitive motifs along the whole sequence. These findings agree with pioneering studies by McGurl and Ryan (McGurl and Ryan, 1992), who described the organization of *prosys* gene and the repeated features within the coding region. Authors hypothesized the presence of duplicated amino acid sequences within ProSys precursor, possibly having biological activity. However, these hypotheses were never proven. Here we identify three main repeat motifs (RMs) based on their different length, varying between 9 and 33 amino acids (Fig. 1, Table 1), occurring 4 times along the whole tomato ProSys sequence (Fig. 1B). The first three putative functional motifs (T1, G1, R1), representing the three varying-length repeats, served as models for the creation of synthetic peptides tested for structural and functional characteristics. From a structural point of view, synthetic RMs revealed a variable degree of plasticity in different environmental conditions. Indeed, these peptides did not show any secondary structure in aqueous solution consistently with our previous investigations carried out on precursor protein and its N-terminal fragments (Buonanno et al., 2018; Molisso et al., 2022a). In presence of TFE, T1 adopted a partial alpha-helical content similarly to the precursor from which it is derived, with a similar shape but a different signal amplitude (Buonanno et al., 2018), suggesting that it preserves the conformational features typical of intrinsically disordered proteins or regions.

Functional tests carried out with synthetic RMs demonstrated that repeat sequences are biologically active as they induce antiherbivore and antipathogen defense responses in tomato plants, while do not exert any direct toxic effects both on target and non-target organisms. These results clearly indicate that the biological activity of the tested RMs is mediated by induced plant changes.

Indeed, the performance of treated plants against insect herbivory and fungal infection was consistent with the result of gene expression study that showed an increased transcript level of *AOS*, *LoxD*, *Pin I* and *Pin II* genes. Since the former are signalling genes associated with JA biosynthesis while the latter are jasmonate responsive (Ryan, 2000), RMs appear to play a role in different steps of the octadecanoid pathway

likely leading to a coordinated induction of JA mediated defense responses and to crosstalk with other plant hormones to regulate the balance between plant growth and defense (Kazan and Manners, 2012). It is worth noting that protease inhibitors were shown to possess antifungal activity against several phytopathogenic fungi besides having anti-proteolytic activity that interferes with insect nutrient uptake, disrupting larval growth and survival (Dunse et al., 2010; Jamal et al., 2013). In addition, JA has a crucial role in plant responses against *B. cinerea*. Therefore, the disruption of JA biosynthesis and signaling pathways greatly affects plant antifungal defense response. For example, several JA biosynthesis and signaling associated mutants, such as *aos*, *opr3*, *jar1*, and *coi1* show compromised resistance to *B. cinerea* (Rowe et al., 2010; Chehab et al., 2011; Mendez-Bravo et al., 2011; Scalschi et al., 2015). We also demonstrate a strong lethal effect of peptide treated plants towards *S. littoralis* larvae, which also showed reduced growth. The best performance was observed with tomato plants treated with T1, which induced 100% mortality after 16 days, followed by G1 and R1 treatment which induced 100% mortality after 19 and 20 days, respectively.

The endogenous accumulation of peptides G4, R4 and T4 in transgenic plants clearly suggests that ProSys is processed to release a pool of peptides, besides Sys, that might accomplish specific defense action leading to the protection against several different stress agents. It was previously demonstrated that the constitutive expression of the *prosys* gene in tomato plants triggers the increase of protease inhibitors (PIs) and other defensive compounds conferring resistance to chewing and sucking insects, phytopathogenic fungi and salt stress (McGurl and Ryan, 1992; McGurl et al., 1994; Ryan, 2000; Dombrowski, 2003; Orsini et al., 2010; Coppola et al., 2015; Zhang et al., 2020). In addition, transgenic plants have a reduced susceptibility to *Cucumber mosaic virus* (Bubici et al., 2017) and stronger indirect defense barriers, compared to untransformed controls, as indicated by the increased attractiveness of natural antagonists of pest insects (Degenhardt et al., 2010; El-Oirdi et al., 2011; Corrado et al., 2007). Why only some of the predicted RMs were endogenously detected is unclear and needs further clarification. We may speculate that not all the peptides corresponding to the RMs are released from the precursor where they could act as molecular

determinants of ProSys interactions with other proteins. Taken together, these results corroborate the multitasking nature of ProSys and open new perspectives on the characterization of its functional motifs.

Interestingly, sequence alignments provided in Supplemental Fig. S6 show that RMs are also preserved in ProSys from other plant species, further supporting their biological significance. Indeed, the presence of repeats possessing a functional role has been reported for other IDPs and precursor proteins belonging to the plant kingdom. An example is given by G3LEA and Dehydrins (Dure, 1993; Sharma and Pandey, 2016), both belonging to the Late Embryogenesis Abundant (LEA) proteins, which possess repeat motifs embedded within their sequence. In particular, G3LEA proteins contain functional regions involving tandem repeats of 11-mer motifs (Wise and Tunnacliffe, 2004; Tunnacliffe et al., 2010) and Dehydrins are characterized by highly conserved stretches of 7 to 17 residues that are repetitively scattered in their sequence (Close, 1996; Mouillon et al., 2006; Wang et al., 2020). Notably, both the groups are water-related stress ID proteins. On the other hand, for what concerns precursor proteins, in plants there are several examples of multiple mature peptides which are released from one single precursor that have no apparent biological function (Tavormina et al., 2015). It is the case of hydroxyproline-rich glycopeptide systemins (HypSys) (Pearce et al., 2001, 2009; Pearce and Ryan, 2003; Pearce et al., 2007; Chen et al., 2008), Cycloptides (Daly et al., 2009; Craik et al., 2010; Craik and Malik, 2013), and *Impatiens balsamina* antimicrobial peptides (Ib-APs) (Lee et al., 1999; Patel et al., 1998). In these 3 reported cases, multiple bioactive peptides are released from a precursor protein and play key functions in plant defense. For example, in tomato, 3 HypSys peptides were isolated (HypSys I: 18 amino acids; HypSys II: 20 amino acids and HypSys III: 15 amino acids). They are all located within a precursor protein of 145 amino acid and induce the synthesis of protease inhibitor II (Pearce and Ryan, 2003). Similarly, two tobacco glycopeptides of 18 amino acids, likely located in a preproprotein of 165 amino acids (Pearce et al., 2001), induce tobacco trypsin inhibitors. The nucleic acid sequences of ProSys suggested that the primary structure of the protein resulted from several gene duplication-elongation events (McGurl and Ryan, 1992). This gene structure led to the prediction that, besides Sys that was experimentally detected in tomato leaf extract (Pearce et al., 1991), other bioactive peptides could be released from ProSys processing, but they were never found until now. Here we demonstrated that the pro-hormone is indeed processed to give rise to several active forms likely playing complementary roles in the induction of plant defense responses, which account for the broad anti-stress capacity of ProSys. The identification of cleavage sites and proteolytic enzyme(s) involved in the *in vivo* release of bioactive RMs is currently under investigation in our laboratories.

Plant peptides have recently proved to be key regulators of stress responses and tolerance (Chen et al., 2020). Several bioactive peptides originate from precursor protein longer than 100 amino acids by proteolytic processing, which occur soon after the challenge to make rapidly available signals that prepare plants to counteract the incoming stress (Stühwoldt and Schaller, 2019). These peptides have also turned out to be a class of hormone molecules (Segonzac and Monaghan, 2019) able to act locally and systemically at extremely low concentrations to regulate plant stress responses and development (Wang and Irving, 2011). Although the number of genes predicted to encode peptides is in the order of thousands (Lease and Walker, 2006), the number of known peptide hormones is still much lower. In the last ten years, many peptides identified by *in silico* studies proved to regulate stress responses being also, in several cases, induced by stresses (Chen et al., 2020). Our results, beside adding novel knowledge to the functional role of ProSys providing insights into the molecular determinants responsible for its wide bioactivity (Molisso et al., 2022a), identified in its scaffold short amino acid chains biologically active, contributing to the more general issue of plant regulatory peptides. In conclusion, we demonstrated that ProSys defence mechanism is much more complex than previously thought providing a new functional framework of peptide precursors in

plants.

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CRediT authorship contribution statement

Rao Rosa: Conceptualization, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing. **Di Lelio Ilaria:** Investigation. **Aprile Anna Maria:** Investigation. **Illiano Anna:** Investigation. **Chiaiese Pasquale:** Investigation. **Castaldi Valeria:** Investigation. **Becchimanzi Andrea:** Investigation. **Langella Emma:** Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing. **Pennacchio Francesco:** Investigation, Writing – original draft. **Buonanno Martina:** Investigation. **D'Andrea Luca Domenico:** Investigation. **Romanelli Alessandra:** Investigation. **Amoresano Angela:** Investigation. **Pinto Gabriella:** Investigation. **Monti Simona Maria:** Conceptualization, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing. **Crisuolo Martina Chiara:** Investigation. **Molisso Donata:** Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2023.111969.

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In silico and *in vitro* approaches allow the identification of the Prosystemin molecular network



Roberto Natale^{a,c}, Mariangela Coppola^a, Nunzio D'Agostino^{a,*}, Youjun Zhang^{b,c}, Alisdair Robert Fernie^{b,c}, Valeria Castaldi^a, Rosa Rao^{a,d,*}

^a Department of Agricultural Sciences, University of Naples Federico II, Portici 80055, Italy

^b Center of Plant Systems Biology and Biotechnology, Plovdiv 4000, Bulgaria

^c Max-Planck-Institut für Molekulare Pflanzenphysiologie, Potsdam-Golm 14476, Germany

^d Interuniversity Center for Studies on Bioinspired Agro-Environmental Technology (BAT Center), University of Naples Federico II, Portici 80055, Italy

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ABSTRACT

Tomato Prosystemin (ProSys), the precursor of Systemin, a small peptidic hormone, is produced at very low concentration in unchallenged plants, while its expression greatly increases in response to several different stressors triggering an array of defence responses. The molecular mechanisms that underpin such a wide array of defence barriers are not fully understood and are likely correlated with the intrinsically disordered (ID) structure of the protein. ID proteins interact with different protein partners forming complexes involved in the modulation of different biological mechanisms. Here we describe the ProSys–protein network that shed light on the molecular mechanisms underpinning ProSys associated defence responses. Three different approaches were used. *In silico* prediction resulted in 98 direct interactors, most clustering in phytohormone biosynthesis, transcription factors and signal transduction gene classes. The network shows the central role of ProSys during defence responses, that reflects its role as central hub. *In vitro* ProSys interactors, identified by Affinity Purification–Mass Spectrometry (AP–MS), revealed over three hundred protein partners, while Bimolecular Fluorescent Complementation (BiFC) experiments validated *in vivo* some interactors predicted *in silico* and *in vitro*. Our results demonstrate that ProSys interacts with several proteins and reveal new key molecular events in the ProSys-dependent defence response of tomato plant.

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1. Introduction

Proteins are the basis of most of the biological processes that determine the functioning of living organisms where they generally act in stable or transient complexes. Thus, large-scale identification of protein–protein interactions (PPIs) provides crucial insights into how biological pathways are structured and coordinated by individual protein functions. PPI networks give essential information on the regulation of plant developmental processes as well as plant responses to environmental stimuli.

The responses to environmental stresses require adaptations at cellular and molecular levels that involve kinase cascades, reactive oxygen species and phytohormones. Such complex signalling net-

works are based on the cooperation of thousands of molecules, including proteins, nucleic acids, lipids, and others [1–2].

PPIs studies have revealed complex networks covering all scientific fields from medicine and pharmacology, such as the mechanism of insulin action [3], to the complex metabolic processes that take place in both animal and plant cells [4–5].

The coverage of PPIs is presently improved by a variety of computational methods [6 and reference therein] also thanks to the development of *in vitro* and *in vivo* techniques, capable of detecting previously uncharacterized or novel PPIs.

In vitro techniques are mainly based on cloning and recombinant expression of proteins and on the identification of physical contact between proteins or between proteins and specific antibodies [7]. These techniques include affinity purification mass spectrometry (AP–MS), yeast two-hybrid (Y2H) and Co-immunoprecipitation (Co–Ip).

The putative interactors detected through these approaches must be validated using *in vivo* techniques, which allow real time

* Corresponding authors.

E-mail addresses: roberto.natale@unifi.it (R. Natale), nunzio.dagostino@unina.it (N. D'Agostino), YOZhang@mpimp-golm.mpg.de (Y. Zhang), Fernie@mpimp-golm.mpg.de (A.R. Fernie), valeria.castaldi@unina.it (V. Castaldi), rao@unina.it (R. Rao).

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monitoring of interactions, as the host organism, in which the PPI takes place, is alive when the analysis is carried out. Because of its specificity and immediacy, bimolecular fluorescent complementation (BiFC) is the most widely used technique for direct visualization of PPIs in living cells. Indeed, the BiFC assay is based on the reconstitution of an intact fluorescent protein when two proteins are brought together due to their interactions [8]. Conversely, *in silico* prediction of PPIs is achieved through computational methods capable of determining reliable potential interactions to be further verified using experimental approaches. Numerous *in silico* methods have been developed to predict new interactions or to confirm already identified interactions [9]. In addition to other databases, PPI studies on tomato (*Solanum lycopersicum*, L.) can make use of the Predicted Tomato Interactome Resource (PTIR) and of the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING). Both resources were used in this study.

PTIR is based on experimentally determined orthologous interactions in six model organisms. It covers more than 300,000 non-redundant PPIs with different confidence levels [10]. More than 30 % of the tomato protein complement is represented in the database. STRING is one of the most comprehensive databases, as it includes information on direct (physical) and indirect (functional) protein associations for over 2000 organisms. PPIs in STRING derive from genomic context predictions, high-throughput experiments, interactions aggregated from other (primary) databases [11]. As for *S. lycopersicum*, the current version (v. 11.5) covers 4,908,531 pairwise interactions.

Additional software allows the analysis of large amounts of protein–protein and protein–nucleic acid data and the visualization of biological networks in a simple way such as graphs, in which the nodes represent biomolecules and the edges the relationships between them. Among these, Cytoscape is an open-source tool for visualizing interaction networks [12], integrating these with attribute data (e.g., expression profiles, phenotypes, etc.), and linking them to functional annotation resources [13].

In tomato plants, after wounding or insect attacks, the octadecapeptide systemin (Sys) is released from its precursor, ProSystemin (ProSys), to trigger defence responses via the octadecanoid pathway [14–15]. Sys was the first plant hormone peptide discovered in 1991 by Ryan's team, following a pioneering study which demonstrated that the peptide is a potent inducer of proteinase inhibitors (PIs) in tomato and potato plants. Several studies have shown that Sys endows tomato plants with resistance against multiple biotic and abiotic stressors. For example, constitutive expression of the *ProSys* gene in tomato plants has been observed to trigger the increase in PIs and other defensive compounds that confer resistance to chewing and sucking insects, phytopathogenic fungi and salt stress [14,16–21]. In addition, transgenic plants have been characterized by an increasing level of indirect defence barriers with a consequent greater attractiveness towards natural enemies of phytophagous insects [22–24]. However, the molecular basis of such a great 'anti-stress' ability has not been fully explained. It has recently been shown that ProSys is an intrinsically disordered (ID) protein [25], a class of proteins with the ability to interact with different molecular partners in many-to-one and one-to-many binding equilibria (i.e., acting as "hub") [26].

The interaction of ProSys with diverse partners might modulate numerous outputs, thus explaining the multiple resistances observed in transgenic plants. Sys is known to interact with 3 different partners: the Sys receptors SYR1 and SYR2 [27], and SR160 [28], a member of the leucine-rich repeat (LRR) receptor kinase family, very similar to the brassinolide receptor kinase BRI1, previously referred to as Sys receptor [29,30]. However, as far as we know, there are no known ProSys interactors. Therefore, in this work we tried to identify ProSys protein partners, using both *in silico* and *in vitro* approaches and validating some interactors by

in vivo studies. The main findings indicate that the strategy adopted was successful, allowing us to describe the ProSys PPI network for the first time.

2. Results

2.1. Description of the ProSys sub-network and *in silico* identification of its main interactors

In a previous work [16], just over 500 differentially expressed genes (DEGs) were identified by comparing wild-type and transgenic tomato plants constitutively expressing *ProSys* (RSYS). These DEGs were used to query PTIR and STRING with the aim of identifying putative interactors. In addition, for each DEG the corresponding ortholog was found in *Arabidopsis thaliana*, in order to take advantage of the wealth of information associated with the protein complement of this model plant. A list of 309 *A. thaliana* proteins was obtained from the over 500 tomato DEGs.

All captured interactions were combined and assessed based on the sharing of gene ontology terms, on co-expression, co-localization, and availability of interacting protein domains. Furthermore, for each interaction the level of confidence was assessed by evaluating the evidence supporting direct contacts [31,10]. A non-directional network was constructed for each dataset obtained by querying the two databases. The STRING and PTIR datasets resulted into a network of 15,642 and 3,334 nodes, respectively. This substantial difference depends on the different sizes of the queried databases.

Then, the individual networks were collapsed into a single and complex network consisting of 16,002 nodes (proteins) and 163,627 edges (PPIs) (supplementary materials, Fig. S1). Finally, a sub-net was extracted by selecting the node corresponding to the ProSys protein (SolyC05g051750) and all direct interactors. The topology of the network was then investigated by evaluating the following parameters: the degree distribution (Fig. 3a) that is the probable distribution of the number of edges that are incident at each node of the network; the betweenness centrality (Fig. 3b) which measures the amount of influence a node has on the flow of information in a graph (i.e., the network); for a node, having a high centrality value, this implies that it is crossed by many paths and becomes an obligatory passage between many nodes; the clustering coefficient distribution (Fig. 3c) that is a measure of the degree to which nodes in a graph tend to cluster together. The values of network parameters are listed in Table C (supplementary materials). The degree distribution reveals a "scale-free" network characterized by the presence of large hubs (i.e., a few nodes strongly connected to other nodes in the network). Indeed, the network includes nodes with scores ranging from 1 to 4,366. This means that nodes with much higher degree distribution scores are essential for maintaining the structure of the network. For example, the highest score (4,366) was assigned to the node corresponding to the RNA polymerase enzyme (SolyC02g083350) (that is not a ProSys direct interactor but, likely a consequence of the plant's need to reprogram the transcriptome in response to the constitutive expression of *ProSys* cDNA). The ProSys's node, indicated in Fig. 3 with blue arrow, is crossed by many paths and this reflects its crucial role for the structure of the entire network. Other nodes as those corresponding to the heat shock protein SolyC06g076020 and the MYB transcription factor SolyC06g053610 are indicated in the Fig. 3 with orange and green arrows because they are crossed by many paths.

The resulting ProSys sub-network is shown in Fig. 1 where the different colours of the nodes indicate the cellular localization of the proteins. Eleven nodes represent proteins encoded by DEGs

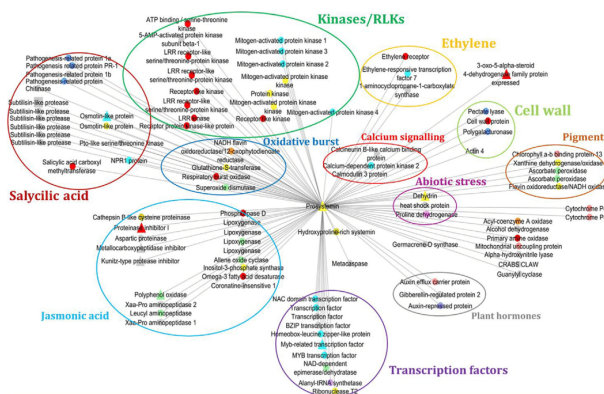


Fig. 1. ProSys sub-network. The nodes were coloured based on the cellular localization of the protein. The up-regulated genes (N = 10) are shown with triangular shape; the square shape is reserved for the down-regulated genes (N = 1) by ProSys over-expression, and the circular shape (N = 88) for putative interactors.

(triangles and squares) while the other putative interactors, indicated with circular shape, come from the queried interactomes.

The ProSys sub-network consists of 99 nodes and 98 interactions (Fig. 1). The size of the nodes is uniform because only the interactions in which ProSys is involved were shown.

For this reason, all the nodes have a degree equal to 1, except for ProSys for which it is 98. The 98 ProSys interactors (listed in Table A, [supplementary materials](#)) were classified according to their role in regulation and participation in defence mechanisms through a careful analysis of the literature. As a result, protein clusters with a specific role in defence were highlighted. The interactors were divided and classified in Gene Ontology (GO) categories (elliptic and circular coloured shape), highlighting several classes of defence-related proteins. Oxidative burst (light blue cluster) and calcium signalling (red cluster) are the first line of defence activated in plants under attack. These clusters included four and three interactors, respectively.

Other additional interactors were several MAPKs (fluorescent green) and proteins related to salicylic acid (SA) and jasmonic acid (JA) pathways (red and blue, respectively). SA is known to mediate host responses to pathogen infection [32]. The SA-related protein cluster included six subtilisin-like proteases, an osmotin-like protease, and the SA receptor NPR1 [33]. JA plays a crucial role in inducing systemic responses to herbivory [21,34–36]. The JA-related protein cluster comprised four lipxygenases and protease inhibitors. A further cluster of defence-related interactors (green) was associated with cell wall, whose reorganization generally follows the attack of a parasite, with the aim of preventing/reducing its penetration into plant tissues. Putative interactions of ProSys with the ethylene pathway (dark blue) have been also found; these include an ethylene receptor and an ethylene-responsive transcription factor, both of which are involved in the plant defence response.

Interestingly, the cluster of transcription factors (pink), included 10 proteins likely involved in the activation of defence-related genes. Other putative interactors were associated with abiotic stress (fuchsia), plant hormones (grey), and pigment metabolic pathways (orange). Fig. 1 also shows a list of coloured squares indicating the different sub-cellular localization of the proteins.

2.2. Experimental identification of ProSys interactors

The experimental identification of ProSys interactors was achieved by AP-MS screening the protein complexes formed *in vitro*. Both mCherry and ProSys-mCherry were expressed and purified from *Escherichia coli*. The recombinant proteins were incubated with total tomato proteins to perform the affinity interaction assay. Subsequently, all interactors were measured by Liquid chromatography–mass spectrometry (LC-MS) with high protein intensity of ProSys (Solyco05g051750). From the interpretation of mass spectra, more than three hundred proteins able to bind ProSys were identified. However, most of these proteins were either not characterized or their function was poorly documented. Given that large amounts of ribosomal proteins and translation-related proteins were detected by the AP-MS, these interactors were filtered out. Normalized signal intensities were processed to determine fold-change abundance (FC-A) scores.

Candidate interactors with a fold change greater than four, around seventy, were selected [37]. Information on functions, role in defence response and subcellular localization of these proteins were retrieved from UniProt, KEGG and Ensemble Plants and are listed in Table B ([supplementary materials](#)).

We next turned our attention to cytoplasmic proteins, as ProSys is a cytosolic protein [38]. Several cytoplasmic interactors showed high scores: the phosphogluconate dehydrogenase 2 (PGD2; Solyco05g010260), the syntaxin-like protein (Solyco12g089150), the heat shock protein (HSP; Solyco07g065840), two oxidoreductase enzymes (Solyco05g010260; Solyco11g010960) and a calcium ion binding protein (Solyco01g099770). Intriguingly, one interactor is involved in the ethylene biosynthesis process (Solyco02g036350).

Four interactors previously identified *in silico* were also found by AP-MS. Two of them were characterized by high scores: NaDED (Solyco09g065180; FC-A = 36.64) and HSP (Solyco06g076020; FC-A = 10.28). Although the proteins interacting with unfolded peptide like heat shock proteins may be artifact of AP-MS [39], the presence of heat shock protein in the network predicted *in silico* encouraged us in considering this protein a good candidate. The remaining two interactors had FC-A scores below the fixed cutoff: the inositol-3-phosphate synthase (Solyco04g054740; FC-

A = 1.38) and the alanine-tRNA ligase synthetase (Soly01g111990; FC-A = 1.79).

2.3. In vivo validation of interactions

Four proteins, identified both *in silico* and *in vitro*, were selected for *in vivo* studies aiming to validate the interactions. In addition, a fifth protein was selected from the *in silico* network, a MAP kinase, due to the essential roles it plays in responses to biotic and abiotic stress. Another of them is NAD-dependent epimerase/dehydratase (Soly09g065180) enzyme. This enzyme uses nucleotide sugar substrates for a variety of chemical reactions including those of the ROS scavenging system to alleviate oxidative damage [40]. In addition, the enzyme activity was associated with cell surface properties, exoenzyme production, and virulence of bacterial diseases [41].

We also selected two additional proteins associated with stress damage/response: a MYB transcription factor (Soly06g053610) involved in the control of various processes, including plant responses to biotic and abiotic stress and defence [42,43] and a MAPK-6 (Soly05g049970) responsible for the regulation of the defence signalling cascade [44,45]. MYB transcription factors represent one of the largest protein families in plants characterized by highly conserved N-terminal MYB DNA-binding domain repeats. MYB proteins have been particularly well studied as regulators of phenylpropanoids, that play key roles in plant defence both as components of chemical barriers and as defence signalling molecules. In addition, MYB proteins also contribute to salt tolerance in plants both regulating ABA synthesis and modulating cuticle formation and antioxidant defence [46,47].

MAP kinases (MAPK) take part in one of the most studied mechanisms that plays a crucial role in eukaryotic systems linking perception of external stimuli with changes in cellular organization or gene expression: phosphorylation has a fundamental role in the progression of the signal through the MAP kinase cascade. A typical MAPK cascade consists of at least three serine/threonine kinases, which act in sequence. MAPKs are clearly involved in JA, SA and ET signalling pathways, the three major phytohormones with essential roles in both biotic and abiotic stress responses [48–50].

Finally, based on the score of AP-MS, we selected an ATP-dependent clp protease (Soly12g042060), an enzyme often expressed as part of the cellular proteostasis, responsible for the breakdown of misfolded or damaged proteins within the cell during various stressful conditions [51].

The results of the infiltration of young leaves of *N. benthamiana* with the recombinant vectors are reported in Fig. 2 and listed in Table 1. For all protein pairs tested, BiFC signals were detected in the cytoplasm and nucleus.

3. Discussion

Protein-protein interactions mediate a wide range of biological processes, including the control of development and metabolism, and cell-cell interactions [9]. At the molecular level, PPIs are involved in post-transcriptional modifications, protein phosphorylation, recruitment of transcriptional co-factors, and are the main actors of many physiological and pathological processes [52,53] such as signal transduction and defence responses [54,39].

The study of protein interactions has undergone a great boost by exploiting omics data and when bioinformatics has become essential to study the biological functioning of PPI networks [55]. By using these tools, we developed the *in silico* ProSys network characterized by the presence of almost 100 ProSys direct interactors. The network shows the central role of ProSys during defence responses, confirmed also by the parameters used to describe the

network (connection degree distribution, betweenness centrality, clustering coefficient distribution; Fig. 3) that reflects its role as central hub in the network. Similarly, the nodes corresponding to HSP and MYB transcription factor have a high degree of connection and can be considered as important hubs for the network.

Notably the ProSys network does not include SYR1 and SYR2, the two LRR-RKs that binds Sys [27]. These receptors likely have affinity only for the Sys peptide; in fact, the three-dimensional folding of ProSys, which exhibits a high index of disorder [25], can hide the Sys peptide located in the C-term region of the pro-hormone.

3.1. ProSys interacts with genes involved in early defence responses

ProSys-mediated defence system begins with the oxidative burst, one of the first defence reactions of plants with consequent rapid and transient production of a massive amount of reactive oxygen species, up to the modulation of phytohormone pathways and defence-related genes through the activation of transcription factors. Over-expression of ProSys produces a cytoplasmic change in calcium ion concentration, which activates calcium-sensitive proteins [20,56]. This observation is in good agreement with the presence in the network of calcineurin B-like (CBL, Soly03g083320), calmodulin (CaM, Soly03g098050) and the calcium-dependent protein kinase 2 (CDPK2, Soly04g009800), highlighted in red ovals in Fig. 1. CBL and CaM are small proteins containing multiple Ca^{2+} binding domains, which, by binding to Ca^{2+} , transduce the signal to target proteins [56–58]. The key role of ProSys in early defence response is also supported by the large number of predicted interactors annotated as kinases (as MAPK6), receptor-like kinases, and LRR-receptors. Mitogen-activated protein kinases (MAPKs) are key components of plant defence pathways, playing critical roles in both basal defence and more specific interactions. These interactions could play a key role in signal transmission with the consequent activation of phytohormone biosynthetic pathways and the subsequent transcription of hormone-activated defence genes. For example, members of the Leucine-Rich Repeat (LRR) Ser/Thr receptor-like kinases (RLKs), generally located on the cell membrane, play an essential role in signalling during pathogen recognition mediated by Pathogen Associated Molecular Patterns (PAMPs) and in subsequent activation of defence mechanisms [59]. These receptors determine the rapid activation of the MAPK signalling cascade and the production of ROS. ROS-related genes (blue cluster in Fig. 1) are induced as a result of the perception of plant stress. ROS are primary defence signals and are released a few seconds after damage is perceived. Superoxide anion ($\text{O}_2^{\cdot-}$), for example, is released locally in the damaged tissue, while hydrogen peroxide (H_2O_2) is produced both locally in the injured area and systemically throughout the plant [60]. The NADPH oxidase enzyme (RBOH1, Soly08g081690) contributes to the production of reactive oxygen species, a critical early signalling event connecting pattern-recognition receptors and intracellularly localized receptors-mediated immunity [61]. Glutathione-S-transferase (GST, Soly01g099590) is a cytosolic enzyme that counteracts the damage caused by oxidative stress to the cell. It is an enzyme that catalyses the conjugation of toxic and hydrophobic chemicals to glutathione, increasing its solubility and promoting its sequestration in the vacuole or its transfer to the apoplast [62].

Several TFs are expected to interact with ProSys. Plant defence responses are triggered by TFs which regulate the transcription of genes involved in plant defence. For example, the MYB Soly06g053610 is known to be involved in the response to biotic and abiotic stress [63]. MYB family members interact with transcription factors belonging to the WRKY family, implicated in plant defence and response to various environmental stresses [64–67].

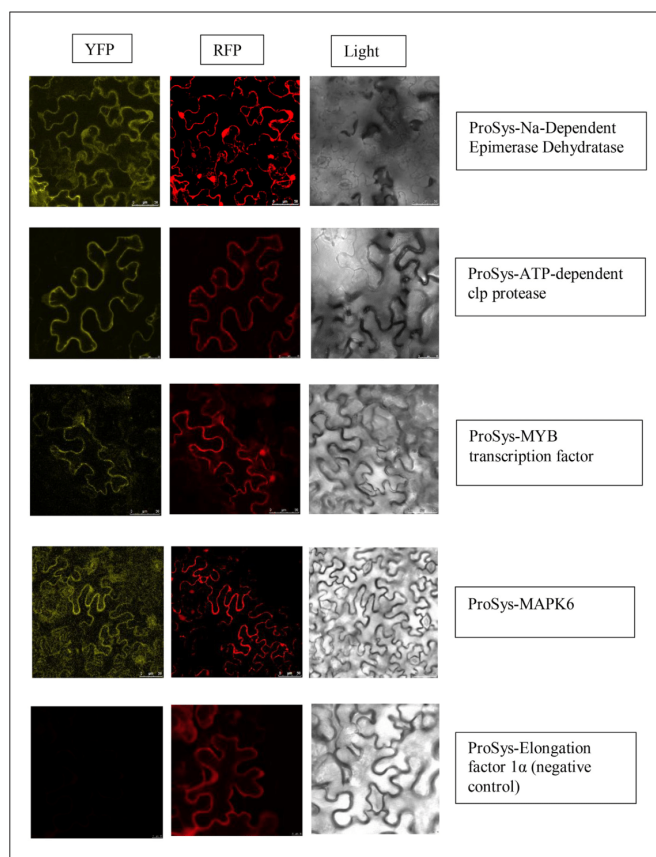


Fig. 2. Confocal microscope images of ProSys interactions. The interaction between the proteins gives a yellow fluorescence signal due to the fusion of two non-fluorescent fragments of yellow fluorescence protein (YFP) (a). The reconstitution of YFP from its fragments (YFP-N, N-terminal fragment [amino acids 1–155]; YFP-C, C-terminal fragment [amino acids 156–239]) is the result of the interaction between the proteins. Furthermore, the vectors also contain a red fluorescent protein (RFP), used as control to verify the expression of the protein inside the cell; the exposure to different wavelengths determined different color emission: a) excitation of YFP (490–515 nm); b) excitation of RFP (555 nm); c) blank: same focal plane without laser excitation.

Notably, WRKY43, (Solyc12g042590) interacted with ProSys *in vitro*. WRKY TFs are involved in the regulation of various physiological programs in plants, including pathogen defence, senescence, trichome development and the biosynthesis of secondary metabolites [68].

The predicted ProSys-Hydroxyproline-rich systemin (HypSys; Solyc06g068520) interaction reinforces previous observations

showing that they work cooperatively to produce a strong systemic response [69].

3.2. ProSys interacts with genes responsible for abiotic stress response

Previously it was observed that ProSys confers resistance to salt stress [19]. This observation correlates well with the predicted

Table 1
List of ProSys interactors detected with different methods.

Detection system	Protein name	Identifiers
AP-MS/BiFC	ATP-dependent clp protease	Solyc12g042060
<i>In silico</i> network/AP-MS/BiFC	NaDED	Solyc09g065180
<i>In silico</i> network/AP-MS	HSP-70	Solyc06g076020
<i>In silico</i> network/BiFC	MYB transcription factor	Solyc06g053610
<i>In silico</i> network/BiFC	MAPK6	Solyc05g049970

interaction with components of the plant responses to abiotic stressors (purple cluster in Fig. 1) such as dehydrin (Solyc02g084850), proline dehydrogenase (ProDH, Solyc02g089620), and heat shock protein 70 (HSP70, Solyc06g076020). Dehydrins are highly hydrophilic and thermostable IDPs, induced by various abiotic factors and phytohormones [70] they are up regulated in RSYS plants [16].

Their main function is to stabilize membranes, enzymes, and nucleotides in cells under abiotic stress [70]. The metabolism and accumulation of proline are associated with abiotic stress prevention mechanisms. For example, in *A. thaliana*, the proline level increases more than fifty folds in response to salt stress [71,72]. The ProDH enzyme is involved in proline catabolism, which helps the plant regulate the proline level. Notably, ProDH is also a defence component against biotic stress, as it contributes to the hypersensitive response and disease resistance [73]. Heat shock protein 70 (HSP), a protein typically induced in response to high temperatures, has a chaperone function as improperly folded proteins accumulate under stressful conditions [74]. The interactors discussed so far support the previous data demonstrating that ProSys can improve tolerance against abiotic and biotic stress and enhances both direct and indirect plant defences [19,22,75].

3.3. Plant late defence response mediated by ProSys interactors

The Osmotin-like protein (Solyc08g080620) in the network is classified as PR-5 protein, which has been found to be up-regulated in RSYS plants [16]. Osmotin is a protein rich in cysteine residues and is involved in osmoregulation. It belongs to the PR protein family and has been used to produce transgenic plants resistant to fungi and tolerant to osmotic stress [76]. Xu and colleagues [77] demonstrated that not all PR proteins are involved in the same signalling cascades in tobacco; in fact, PR-5 was induced by both SA and ET/JA, while other proteins of the family are SA-specific [78]. The up-regulation of PR-5 compared to ProSys can be explained by the need to balance the different hormones and to counteract the attack of necrotrophic microorganisms, since the overexpression of osmotin causes cell death [76].

Since metacaspases are known to promote the induction of programmed cell death during biotic and abiotic stress [79], the ProSys-metacaspase interaction suggest a possible contribution of ProSys in programmed cell death following damage from stress [79]. Previous works have shown the involvement of ProSys in the modification of the Volatile Organic Compounds (VOCs) emitted by tomato plants with the consequent increase in the plant attractiveness of the natural enemies of herbivorous pests [80]. These observations are corroborated by the inclusion in the PPI network of the germacrene-D-synthase (Solyc12g006570.1), an enzyme involved in the biosynthesis of volatile terpenoids released by the plant following the attack of herbivores [81]. Corrado and co-workers [22], highlighted the contribution of ProSys in increasing the indirect defence level of plants by improving the attractiveness of parasitoids of herbivorous insects ascribed to the induction of

germacrene-C-synthase, an enzyme involved in the production of sesquiterpenoids.

3.4. ProSys interactors associated with phytohormone biosynthesis

ProSys also appears to be related to the ET pathway. The ACS interactor (1-Aminocyclopropane-1-Carboxylate Synthase; Solyc01g095080) regulates the synthesis of ET, ensuring the formation of the 1-aminocyclopropan-1-carboxylic acid (ACC) precursor. The ERF7 (Ethylene-responsive transcription factor 7; Solyc06g065820) gene encodes the TF that controls the expression of ET-responsive genes following abiotic and biotic stimuli [82]. Five ERFs have been found to be overexpressed in *Solanum pimpinellifolium* under salt stress, and overexpression of ERF-7 increases salt tolerance [83]. The putative interaction of ProSys with the ACC enzyme, ERF elements and the ethylene receptor (Solyc09g075440) suggests that ProSys contributes to the crosstalk between the JA and ET pathways. As for the JA signalling pathway, several proteins (listed in Table A, supplementary materials) interact with ProSys, most of which are both early and late defence-related enzymes. The early and late wounding-responsive genes are regulated by distinct mechanisms. Local wounding leads to ProSys processing followed by binding of Sys to SYR1 and subsequent activation of genes involved in JA biosynthesis.

Four lipoxygenases (LOXs) were in the JA group. These enzymes are known to be involved in many plants developmental processes and in the regulation of stress responses. Indeed, the LOX pathway plays a key role in local and systemic wound response, releasing free radicals as early signals of defence and toxic metabolites as a preventive measure. In addition, LOXs modulate the plant response against abiotic stresses regulating plant hormones as SA, JA, ABA [84 and reference therein].

The increase in the level of endogenous JA leads to the activation of late JA-responsive genes that play a direct role in defence. Late defence genes include proteinase inhibitors (PIs), induced in wounded or insect-attacked leaves, which directly interfere with the assimilation of nutrients by the larvae. The JA group include polyphenol oxidase (PPO) an enzyme belonging to the jasmonate-dependent inducible defence system. Constabel and colleagues [85] demonstrated the induction of PPO activity in tomato leaves by the overexpression of ProSys and systemin, and the supply of methyl-jasmonate.

The presence of auxin and gibberellin regulatory enzymes as putative ProSys interactors opens new perspectives about the plastic role of these proteins in plant defence mechanisms. Auxin has long been recognized as a regulator of stress responses, with an interplay with other phytohormones. Indeed, it is well known that it interacts antagonistically with SA (biotrophic resistance) and synergistically with JA (necrotrophic resistance) [86]. Gibberellins are a class of growth hormones with a key role in response to environmental stimuli as light, temperature, salt, and biotic stress. The molecular mechanism is mediated by DELLA proteins under the regulation of gibberellin transduction pathways, reducing the levels of reactive oxygen species [87 and reference therein].

3.5. ProSys affinity with cell wall and pigment proteins

Plant cell wall is composed by a complex network of proteins trapped in a polysaccharide matrix that contributes to signalling events by interacting with both intra- and extra-cellular proteins [88]. Enzymes such as pectate lyases or polygalacturonases, found to be ProSys interactors, influence plant cell wall integrity during stressful events. Indeed, they contribute to pectin hydrolysis, generating molecules that act as plant immunity elicitors known as oligogalacturonides (OGs). Intriguingly, OGs-related pathway has been shown to be up-regulated in transgenic plants that overex-

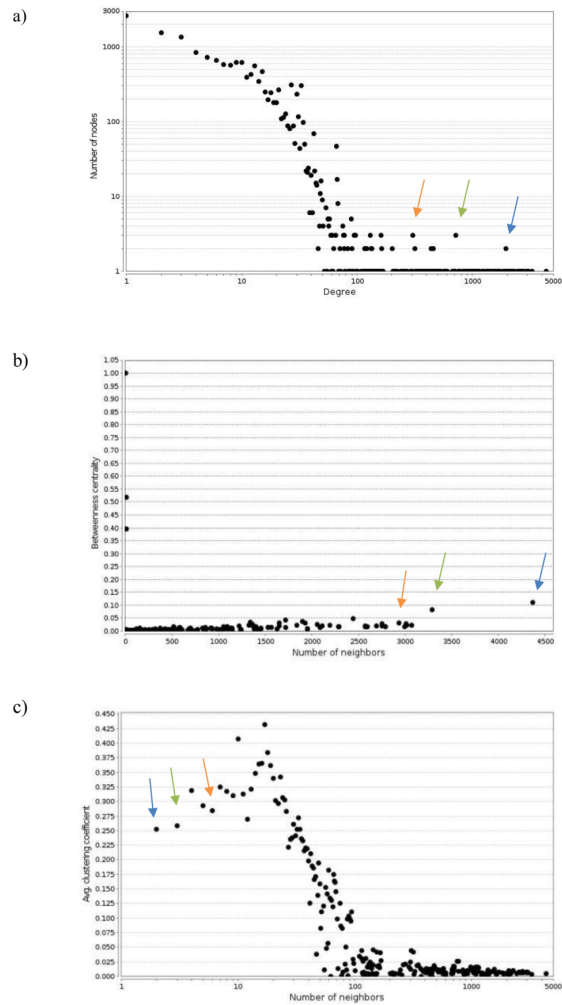


Fig. 3. Network parameters. The image shows the network parameters analysis carried out in Cytoscape: a) Connection degree distribution; b) Betweenness centrality; c) Average Clustering Coefficient distribution. The blue arrows indicate ProSys, orange arrows indicate the Heat Shock Protein (Solyc06g076020) and the green arrows the MYB-related transcription factor (Solyc06g053610).

press the ProSys protein lacking the Sys sequence [89]. We can speculate that ProSys interaction with cell wall proteins can activate several cellular responses in local and distal tissues.

As regards the pigment group affinity with ProSys, is noteworthy the presence of ascorbate peroxidases (APXs), important components of the enzymatic antioxidant defence system used by plants to overcome the oxidative stress. Indeed, as suggested by Dietz in 2016 [90], APXs can interact with different molecular partners, such as proteins involved in the signal transmission after external stimuli. Further efforts are needed to clarify the role of plant APXs and how they interact to integrate stress signals.

Chlorophyll *a/b* binding protein 13 (CAB-13), an additional *in silico* detected ProSys interactor, belongs to the light harvesting complex of the photosystem II and appears to be involved in oxidative stress events and/or continuous light tolerance in tomato plants [91]. Intriguingly, its homolog in *A. thaliana*, named, LHCB3 is linked to the ABA signalling pathway under drought stress and stomatal closure [92]. This suggests that ProSys can act as a mediator also in salt and abiotic stresses [93].

3.6. In vitro and in vivo validation of ProSys interactions

The AP-MS experiments revealed a very large number of interactors confirming the complex scenario drawn by the *in silico* predicted network. Interestingly, the interaction of ProSys with NaDED was confirmed by the three different approaches used. NaDED is a member of a protein family with catalytic activity, involved in various biological processes such as rRNA processing, and positive regulation of translation and transcription. In addition, it is involved in the formation of carbohydrate derivatives by adding a carbohydrate residue to other molecules [94]. Sugars can stimulate plant immunity and promote the expression of defence genes [95]. For example, a high level of sugars in plant tissues improves the resistance of plants against pathogenic fungi [40]. This mechanism has been termed “high-sugar resistance”. It is important to note that sugars are the primary substrate that provides energy and structural material for defence responses in plants. Sugars trigger an oxidative burst early in the infection, inducing certain PR proteins. In addition, some sugars act as triggers by inducing greater resistance of plants to pathogens. Sugars can also act as intermediates, interacting with the hormone signalling network that regulates the plant immune system [40]. For example, several phytohormones including ET and JA, influence the sucrose signalling pathway [96]. For all these reasons, the ProSys-NaDED interaction could be associated with both sugar and plant defence signalling hormones. Among the possible interactors identified by AP-MS, several HSPs were found. Several interactors have catalytic activity, for example the enzymes encoded by Solyc05g010260 and Solyc11g010960 are oxidoreductases involved in redox processes during ET biosynthesis [97–100] and under abiotic stresses such as floods, salinity, and drought. The crosstalk between JA, ET and SA signalling is thought to act as a mechanism for fine-tuning induced defence that is activated in response to multiple attackers. JA and ET interdependently and synergistically induce the expression of pathogen-responsive genes, such as defensins, to support plant tolerance against infections [101]. The latest ProSys interactor validated with BiFC was the ATP-dependent clp protease (Solyc12g042060). In the cytosol and nucleus of plant cells the damaged proteins are degraded by 26S proteasomes, whereas in chloroplasts and mitochondria this function is performed by proteases.

The main protease in chloroplasts is the ATP-dependent stromal Clp [102] which is overexpressed under (a)biotic stress and contributes to the digestion of misfolded proteins [103]. It is tempting to hypothesize that ProSys recruits the ATP-dependent clp pro-

tease which, thanks to its proteolytic activity, counteracts the toxic effects of damaged proteins.

SIMYB14 functions as a JA-responsive TF that plays a role in flavonoid accumulation and oxidative stress tolerance [104]. Flavonoids are secondary metabolites that could act as phytoalexins, compounds released by plants to ward off diseases and pathogens [105]. At the same time, ROS have additional signalling roles in plant adaptation to stress [106]. However, plants reduce ROS accumulation by altering the expression of ROS scavenging enzymes such as catalases, Cu-Zn-superoxide dismutases and peroxidases [107]. Therefore, the ProSys-MYB interaction could be responsible for activating the transcription of JA-responsive defence genes, reducing ROS accumulation, and promoting flavonoid biosynthesis.

4. Conclusion

Our results demonstrate that ProSys interacts with multiple proteins and reveal new key molecular events in the ProSys-dependent defence response of tomato such as the involvement in the metabolism of carbohydrates, in the adaptive plasticity of the plant under stress and in the promotion of the biosynthesis of flavonoids. Proteins are usually involved in interactions with an estimated average of 5–10 protein partners [108]. Our results suggest that ProSys is involved in a far greater number of interactions likely due to its ID structure. Understanding the functional role of the interacting complexes shown here will provide crucial insights into the ProSys-dependent defence mechanisms.

5. Materials and methods

5.1. In silico prediction of protein interactions

The 695 previously identified tomato DEGs [16] were used to query several plant PPI databases [10–11]. BLASTx was performed (e-value 10^{-5}) to search the DEGs against the *A. thaliana* RefSeq database. The resulting 309 *A. thaliana* proteins were used to predict PPI networks by querying the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; <https://string-db.org>). The list of tomato proteins was used to retrieve the PPI networks from the Predicted Tomato Interactome Resource (PTIR; <https://bdg.hfut.edu.cn/ptir/index.html>). The ProSys sub-network was selected and imported in Cytoscape 3.8.2 (<https://www.cytoscape.org>) to study the network topology. Each node of the network was described with several attributes (cellular localization, biological function and belonging to metabolic pathways) retrieved from the attribute files downloaded from the Ensembl Plant (<https://plants.ensembl.org/index.html>). After removing any duplicates and self-loops, the Cytoscape Network Analyzer Tool function has allowed the analysis of the graph through the automatic calculation of a series of parameters such as betweenness centrality; clustering coefficient; connections degree. The schematic representation of the procedure is represented in [supplementary materials, Fig. S2](#).

5.2. CDNA cloning and vector construction

The ProSys gene was amplified from the pMZ vector [109] using specific primers with attB recombination sequence (Table 2), based on Gateway BP cloning system, and cloned in the pDONR221 Donor vector (Thermo Fisher Scientific, Waltham, MA). The gene-specific primers used did not include a stop codon to ensure C-terminal fusion of tags. Expression vector for AP–MS was constructed using the Gateway LR reaction with pET301 and pET300 vectors generating the expression cassette: pET301-mCherry-ProSys-HisTag and pET300-HisTag-ProSys-mCherry.

Table 2
List of primers used for ProSys cloning. The *italic style* represent the attB adapter sequence.

Primer name	Sequence
ProsysFw	AAAAAGCAGGCTCCACatgggaactcttcattatgatac
ProsysRv	CAAGAAAGCTGGGTcatagccagattattgtctgtttgcat
attB1 adapter	5-GGGGACAACTTTGTACAAAAGCAGGCTccacc-3
attB2 adapter	5-GGGGACCACTTTGTACAAAAGCTGGGTcatagcc-3
M13 Fw	GTAAAGCAGCGCCAG
M13 Rv	CAGGAACAGCTATGAC

The Destination vectors for BiFC-2in1 [110] were constructed using specific primers including sequences for the B1 and B4 regions, and B3 and B2 regions, respectively (*italic style* in Table 3). The destination vectors used were pBiFCt-2in1-NN or pBiFCt-2in1-NC. *Agrobacterium tumefaciens* strain AGL1 was used for vectors expression.

5.3. Protein extraction for AP-MS

ProSys proteins were extracted from cells by Ultra-sonication and using a lysis buffer containing 20 mM of Phosphate-Buffered Saline (PBS) at pH 7.4, 20 mM NaCl, 5 % glycerol and 20 mM Imidazole and Phenylmethylsulfonyl fluoride (PMSF) 0.1 M. Three-weeks-old tomato plants (cultivar MoneyMaker) were wounded on the upper side of the leaves, to simulate stressful condition, and harvested after 9 hours. Then, total proteins were extracted from wounded leaves using an extraction buffer, consisting of Tris HCl pH 7.5, 25 mM, MgCl₂ 15 mM, EGTA 5 mM, DTT 1 mM, PMSF 1 mM, NaCl 150 mM.

5.4. Sample preparation for AP-MS

The detailed materials and procedure were published by Zhang and co-workers in 2019 [37]. The total protein extracted from the leaves and the mCherry-ProSys complex were mixed in a 1.5 ml tubes with GFP-Trap[®] and gently mixed at 4 °C for 1 hour, to allow the formation of protein complexes.

Tubes were centrifuged to precipitate the beads coupled with the protein complexes and the supernatant was removed. The beads were recovered and washed with a specific buffer. The samples were ready for on-beads enzymatic digestion. The samples were dissolved in a small volume of 6 M urea/2 M thiourea pH 8, then 1 µl trypsin/LysC (0.4 µg/µl) was added. C18 Stage-SepPak[®] columns were used for desalination and peptide concentration, along with the Visiprep[™] 12-Port Vacuum Manifolds and the vacuum pump. The peptides were dried in a SpeedVac[™] evaporator and then they were resuspended in a final volume of 40 µl of resuspension solution (0.2 % TFA/5% acetonitrile) and transferred to a microtiter plate for mass spectrometric analysis. For this step, A

Nano LC 1000 liquid chromatograph with a reversed-phase C18 column (Acclaim PepMap RSLC, 75 µm × 150 mm, C18, 2 µm, 100 Å²) was used for this step.

5.5. LC-MS data analysis

LC-MS/MS analysis was performed on a Q Exactive Plus instrument (Thermo Fisher Scientific). Quantitative analysis of MS/MS measurements was performed with Proteogenis Q1 software (Non-linear Dynamics, Newcastle, UK). The proteins were identified from the spectra using Mascot (Matrix Science, London, UK) with the following parameters: TAIR10 protein annotation, requirement for tryptic ends, one missed cleavage allowed; fixed modification: carbamidomethylation (cysteine); variable modification: oxidation (methionine), peptide mass tolerance = ±10 p.p.m., MS/MS tolerance = ±0.6 Da, allowed peptide charges + 2 and + 3. A decoy database search was used to limit false discovery rates to 1 % at the protein level. Identifications of peptides below rank one or with a Mascot ion score less than 25 were filtered out. Mascot results were imported into Proteogenis Q1, the quantitative information about the peak area was extracted, and the results were exported for data plotting and statistical analysis.

For each protein, the corresponding Solyc identifier was retrieved by querying the UniProt database (<https://www.uniprot.org>). All entries were associated to GO terms (<https://www.geneontology.org>) and were assigned to KEGG pathways (<https://www.genome.jp>).

Ribosomal proteins and the translation-related proteins were filtered out at this stage. Normalized signal intensities were processed to determine the fold-change abundance (FC-A) scores, using the SAINT algorithm embedded in the CRAPome software [111–113]. Compared with the GFP control, background proteins were eliminated in the case of FC-A values of at least four within at least three replicates [114]. Compared with intensity of bait, only the proteins for which the intensity score was greater than 2 % should be considered as positive interactors.

Statistical analysis for this dataset was performed using the Student's T-test.

5.6. BiFC analysis

Young leaves of four-weeks-old *N. benthamiana* plants were infiltrated with *A. tumefaciens*, transformed with BiFC vectors grown on selective medium, and protein expression visualized with DM6000B/SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). Laser excitation was 490–515 nm for YFP and 555 nm for RFP; emission fluorescence was captured by 500 to 520 nm and 555 to 580 nm band-pass emission filters, respectively.

Table 3
List of primers used for BiFC 2in1 cloning system.

Primer names	Sequence
Prosys Fw B1	GGGGACAACTTTGTACAAAAGCAGGCTTaatgggaactcttcattatgatac
Prosys Rv B4	GGGGACAACCTTTGTATAGAAAAGTTGGGTGagtttattattgtctgtttgcat
EF1α Fw B3	GGGGACAACCTTTGTATATAAAGTTGGGAatgggtaaggaaagattccac
EF 1α Rv B2	GGGGACCACTTTGTACAGAAGCTGGGTGcttccctctttctggcgaac
NAD-dependent epim/dehyd Fw B3	GGGGACAACCTTTGTATAATAAAGTTGGGAatggtaactctcttctcttc
NAD-dependent epim/dehyd Rv B2	GGGGACCACTTTGTACAGAAGCTGGGTGcactttcaggtctccaga
ATP-dependent clp protease Fw B3	GGGGACAACCTTTGTATAATAAAGTTGGGAatggtaactcaagcatccatcg
ATP-dependent clp protease Rv B2	GGGGACCACTTTGTACAGAAGCTGGGTGaaatccaactcccaaaaaga
MAP kinase Fw B3	GGGGACAACCTTTGTATAATAAAGTTGGGAatgaagaagatctttgcaac
MAP kinase Rv B2	GGGGACCACTTTGTACAGAAGCTGGGTGtagctcagtaagttgtgccaatgg
MYB-related protein Fw B3	GGGGACAACCTTTGTATAATAAAGTTGGGAatgggttagagctcttggg
MYB-related protein Rv B2	GGGGACCACTTTGTACAGAAGCTGGGTGaaattctggaattctggca

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6. Authors statement

R.R. and M.C. designed the study, planned the experiments and supervised the work.

R.N. carried out the experiment with the supervision of R.R., M. C., Y.Z. and A.R.F.

R.R., N.D. and R.N. wrote the manuscript with the support of Y. Z., A.R.F. and V.C.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.csbj.2022.12.006>.

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Review

The ecology of bladderworts: The unique hunting-gathering-farming strategy in plants

Valeria Castaldi¹, Alessandro Bellino^{*}, Daniela Baldantoni

Dipartimento di Chimica e Biologia "Adolfo Zambelli", Università degli Studi di Salerno, Via Giovanni Paolo II, 132, Fisciano 84084, SA, Italy



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ABSTRACT

Carnivorous plants typify a mixotrophic strategy where autotrophy is supported by predation on animals, achieved through fascinating morpho-physiological adaptations and unique mutualisms. Exploring such symbiotic interactions is pivotal to understand how carnivorous plants feed upon wide ranges of resources, by relying on symbiont-mediated digestion. This concept finds its extreme realization in *Utricularia* (bladderwort), the largest genus of carnivorous plants, where the formation of a symbiotic microbiota within the traps drives its trophic resources further, including algae and complex organic matter. The present review synthesizes the key aspects of *Utricularia* ecology, from evolution to habitat selection, looking into the fascinating complexity of the structure and the microbiome of their traps. It highlights the gaps and gaps in the understanding of bladderworts, including promising applications based on models of the *Utricularia*-microbiome system and the possible development of biomimetic devices. From the environmental drivers through the solutions developed by these plants, the review offers a comprehensive view of their unique ecology, which could be factually defined a "mixotrophic omnivory".

1. Various shades of mixotrophy

Because of their unusual and efficient adaptations to trap and digest small preys, providing access to multiple food sources at once, plants that feed on animals have always captivated scientists and curious people (Darwin, 1875; Lloyd, 1942).

The co-occurrence of photosynthesis and predatory behaviour represents a peculiar expression along a broad spectrum of evolutionary strategies mixing autotrophy and heterotrophy, collectively referred as mixotrophy. Based on the nutrition mechanism and the source of organic carbon, such a strategy can be divided into three classes, namely i) *absorbotrophic* mixotrophy, where organic C is obtained from non-living sources, ii) *biotrophic* mixotrophy, where organic C is obtained from living organisms through biotrophic parasitic or mutualistic interactions, and iii) *necrotrophic* mixotrophy, where organic C is obtained from other organisms through their killing and digestion - the main strategy to be found in carnivorous plants (Selosse et al., 2017; Firmin et al., 2022). According to the most comprehensive analysis of the evolutionary ecology of mixotrophy (Selosse et al., 2017), such a strategy allows the adaptation of organisms to the ubiquitous environmental

gradients of light and nutrients (the so-called "grand écart" hypothesis – Selosse et al., 2017) and provides for sufficient plasticity to modulate energy fluxes in response to alterations in the availability of resources (Mansour and Anestis, 2021; Gonzalez et al., 2022; Lepori-Bui et al., 2022). Indeed, the "carnivorous syndrome", i.e., the complex of traits allowing plants feeding on preys, is positively selected in nutrient-poor substrates, where it allows overcoming the competition for the limiting resources (Adamec, 1997; Klink et al., 2019). Mixotrophy, however, is a remarkably flexible strategy, allowing balancing the different sources in relation to the environmental fluctuations (Bellino et al., 2014; Selosse et al., 2017). When light becomes limiting, preys can be a source of carbon compounds as well (Adamec, 1997; Klink et al., 2019), with the balance between autotrophy and heterotrophy tipping toward the latter.

Currently more than 800 species of carnivorous plants are known, with *Lentibulariaceae* (order Lamiales) being the largest and most widespread family, comprising more than 360 species in three genera: *Pinguicula*, *Genlisea* and *Utricularia* (Darwin, 1875; Lloyd, 1942; Fleischmann and Rocca, 2018). The latter, small rootless aquatic plants commonly known as bladderworts (Fig. 1), stand out for their complex suction traps (Lloyd, 1942; Taylor, 1989) called utricles. Utricles are not

^{*} Corresponding author.

E-mail addresses: valeria.castaldi@unisa.it (V. Castaldi), abellino@unisa.it (A. Bellino), dbaldantoni@unisa.it (D. Baldantoni).

¹ Present address: Dipartimento di Agraria, Università degli Studi di Napoli Federico II, Via Università, 100, 80,055 Portici, NA, Italy

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only of fascinating complexity from a biomechanical point of view, but they attracted researchers' attention also because of the microbial community colonizing their lumen, able to digest complex organic matter. By widening the resources accessible by bladderworts, trap microbiome on the one hand guarantees a competitive advantage in oligotrophic environments and, on the other hand represents a powerful evolutionary force. The understanding of the structure and the role of trap microbiome, as well as the influence of microbial activity on the growth of bladderworts, represents, therefore, a crucial step toward the elucidation of *Utricularia* ecology (Širová et al., 2018b).

2. Systematics and phylogeny

The genus *Utricularia* currently counts over 250 species (Baleeiro et al., 2022), up to 274 (Cheng et al., 2021), including several taxa deriving from hybridization (Astuti et al., 2019; Bobrov et al., 2022), which are divided into three subgenera, *Polypompholyx*, *Utricularia* and *Bivalvaria*, and 35 sections (Taylor, 1989; Müller and Borsch, 2005). Its phenotypic and evolutionary plasticity resulted in a broad differentiation and a nearly worldwide distribution compared to its sister genera *Genlisea* and *Pinguicula* (Darwin, 1875; Müller and Borsch, 2005). Cladistic studies conducted by Jobson et al. (2003) using the chloroplastial markers *rps16* and *trnL-F* define the 35 sections identified by Taylor (1989) as clearly monophyletic and, in agreement with Müller et al. (2006), the subgenera *Polypompholyx* and *Utricularia* as sharing a common ancestor. The group constituted by *Bivalvaria* and *Utricularia*, instead, has been proved (Jobson et al., 2003) to be paraphyletic.

The clade *Genlisea-Utricularia* exhibits the widest diversity within the *Lentibulariaceae* family (Silva et al., 2018), a trait attributed to the fast evolution of their very small and dynamic genomes (Greilhuber et al., 2006; Albert et al., 2010; Fleischmann et al., 2014; Silva et al., 2020). The genomic diversity translates into appreciable morphological

differences among the three subgenera of *Utricularia* (Rutishauser, 2015), especially in their bladder-trap shapes and functional traits (Lloyd and Goebel, 1931; Lloyd, 1932b; Jobson et al., 2003; Albert et al., 2010; Westermeier et al., 2017). In this context, the morpho-functional adaptations of the traps, from the passive behaviour of the ancestral species like *U. multifida* and *U. westonii* (subg. *Polypompholyx*) to active sucking mechanisms, are thought to have played a decisive role in the diversification of the genus across multiple environments (Westermeier et al., 2017; Plachno et al., 2019a), and the presence of active suction episodes in *U. westonii*, recently discovered by Poppinga et al. (2020), do not substantially change such an evolutionary scenario.

A South American lineage, emerged 39 million years ago, is probably the last common ancestor of the *Utricularia-Genlisea* clade, from which only 9 million years later *Utricularia* diverged with subgenus *Polypompholyx* across Australia at first and then Africa (Silva et al., 2018). However, there is considerable uncertainty in the dating, with Jobson et al. (2017) collocating the split of subgenus *Polypompholyx* from *Utricularia* + *Bivalvaria* around 31 Mya and advancing the hypothesis of an Australian appearance of *Polypompholyx* around 46 Mya.

Utricularia ancestral forms were probably terrestrial (Silva et al., 2018) and such a plesiomorphic trait has been preserved in *Genlisea* and *Pinguicula*, whereas *Utricularia* mainly colonizes aquatic habitats (Lloyd, 1942; Taylor, 1989). Recent studies reveal that this transition is associated to extensive adaptations, like the loss of root hairs and cotyledons, the development of extensive aerenchyma, the preferential localization of chloroplasts in the parenchyma of floating organs and the changes in the morphology of leaves and traps (Hepler et al., 2019; Reut et al., 2021; Reut and Plachno, 2022). Interestingly, in *U. gibba* this is consistent with a reduction in the number of genes coding for the plant cell wall protein superfamily called expansin, because of an evolutionary contraction in genome size. In particular, the loss concerns the α -expansin family (EXPA-X) involved in root hair development and

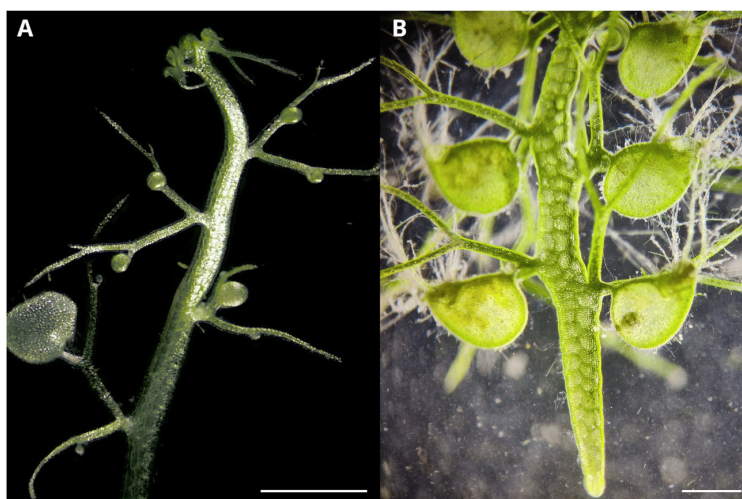


Fig. 1. Apical (A) and basal (B) portions of *U. gibba* shoots from the marshes along the Alento River (Campania region, Southern Italy), the former with traps in various stages of their early development and the latter with fully developed traps, in some cases digesting preys. Scale bar lengths are equal to 1.00 mm.

EXPA-VI genes that regulate lateral roots growth (Hepler et al., 2019). However, it must be considered that the rheophytic species in *Utricularia* genus, such as the South American *U. neottioides* (sez. *Avesicaria*), are able to develop anchor stolons (claw-like rhizoids) that allow them colonizing flowing waters, as compared to the others mostly occupying stagnant or slowly moving waters (Rutishauser, 2015; Plachno et al., 2020). The understanding of bladderwort adaptations to the aquatic lifestyle calls thus for broad-spectrum evolutionary analyses of root-associated genes in different species: terrestrial, aquatic and rheophytic ones (Bárta et al., 2015).

3. Distribution and habitat

Carnivorous plants mainly develop in habitats with a high availability of water and light, but which are extremely poor in nutrients, such as swamps or peat bogs. Indeed, the energetic costs associated to the mixotrophic behaviour are not rewarded in nutrient-rich habitats, where non-carnivorous plants are thought to have a competitive advantage (Ellison, 2006; Selosse et al., 2017; Klink et al., 2019; Firmin et al., 2022). *Utricularia* spp. are no exception to the rule and are found in shallow, acid and dystrophic waters (Ceschin et al., 2020), where the slow decomposition of organic matter limits the growth of potential competitors, and the suspended organic matter may constitute an accessible source of nutrients (Adamec, 2012). In this context, the abundance of organic matter allows aquatic carnivorous plants growing also in light limiting environments due to a heterotrophic carbon nutrition (Adamec, 1997; Firmin et al., 2022), whereas terrestrial plants mostly rely on photosynthesis. The association to acid waters, however, finds exceptions in several widespread species, such as *U. x neglecta/U. x japonica* (ex *U. australis* – Bobrov et al., 2022) and *U. minor*, tolerating wide ranges of pH, and in a few species of temperate zones, living in alkaline (pH 8.0–9.3) waters (Adamec, 2020; Ceschin et al., 2020).

The Southern Hemisphere, in particular the Neotropical zone and Australia, hosts the highest number of bladderwort species, especially terrestrial forms (Guisande et al., 2007; Müller and Borsch, 2005). Some of them have a wide distribution, such as *U. subulata*, found in tropical regions of both hemispheres, and *U. gibba*, with a cosmopolitan distribution. Several endemic species are found in the Indomalayan realm, Afrotropics and Neotropics (Jobson et al., 2018). The Northern Hemisphere, instead, is rich in aquatic species (Guisande et al., 2007) such as *U. breinii*, *U. intermedia*, *U. minor*, *U. macrorrhiza*, *U. rigida*, *U. vulgaris*, in addition to widely distributed species such as *U. gibba* (Taylor, 1989). In aquatic settings where sexual recruitment is difficult, species including *U. vulgaris*, *U. breinii* and *U. intermedia* reproduce asexually by clonal propagation, as do the sterile hybrids such as *U. x neglecta* or *U. x japonica* (Bobrov et al., 2022), which results in limited genetic variability (Kameyama and Ohara, 2006; Silva et al., 2018). Nevertheless, common bladderworts like *U. vulgaris* can produce minute seeds, mainly by self-pollination, that rely on light to effectively germinate on the surface of warm and slightly alkaline water (Prausová et al., 2022). *Utricularia* distribution pattern supports the hypothesis of its early evolutionary radiation in the Neotropical realm, where most of the terrestrial species are present, whereas the appearance of the aquatic life-form is a derived condition (Guisande et al., 2007) that developed repeatedly within the clade. In this context, the ongoing elucidation of the phylogeography of the genus is progressively shedding light on the appearance of the different living forms of *Utricularia* and their distribution all over the world (Jobson et al., 2018). For instance, (Silva et al., 2020), highlighting genome differences between the terrestrial, endemic Brazilian, *U. reiniformis*, and the aquatic cosmopolitan *U. gibba*, indicate Whole Genome Duplication (Lan et al., 2017) and expansion or loss of introns (Ma et al., 2018) and transposable elements like LTR-retrotransposons (Silva et al., 2020) as evolutionary driving forces leading to speciation, typical distribution patterns and genome size changes in *Utricularia* genus. Indeed, notwithstanding the 60% shared genes, *U. gibba* has undergone a genome contraction (101 Mb) compared

to the highly repetitive one of *U. reiniformis* (317 Mb), which translates into a series of phenotypic features: asexual reproduction by stolon fragmentation, reduced vegetative organs, peculiar trap size dependent on different preys and also the microbiome (Silva et al., 2020). Interestingly, the contraction in genome size is accompanied by dramatic alterations in DNA methylation, with unusual distribution of small RNAs and reduced global DNA methylation levels (Cervantes-Pérez et al., 2021).

4. The bladder trap: An ingenious eating machine

The suction traps (Figs. 1 and 2), exclusively belonging to the *Utricularia* genus, are epiphyllous leaves (Darwin, 1875; Taylor, 1989; Rutishauser, 2001; Miranda et al., 2021) that look like little egg-shaped or spherical empty vesicles, representing remarkably fast and biomechanically complex organs employed to feed on preys (Joyeux et al., 2011; Poppinga et al., 2013; Plachno and Muravnik, 2018). The origin of traps from leaves is supported by the local changes in gene expression during trap ontogeny in *U. gibba* (Whitewoods et al., 2020), accompanied by anisotropic growth rates (Lee et al., 2019) producing oblate spheroid traps (Figs. 1B) from near spheric primordia (Figs. 1A). Indeed, the expression of UgPHV1 gene, that controls leaf adaxial polarity, is bound into a region of primordium developing into the trap interior, an occurrence consistent with the transition from a flat leaf to a bladder-shaped leaf.

Schematically, the trap (Figs. 2A–C), attached to the plant through a stalk, is constituted by a cavity ranging from 0.2 to 12 mm in length and from 0.5 to 6 mm in diameter, opening on the outside through a mouth surrounded by secretive hairs and appendages (“antennae”), the latter occurring in some species also in other positions on the external surface of the utricle (Taylor, 1989; Reifenrath et al., 2006). The mouth is closed by a moving trapdoor, which seals the opening by resting on a structure called “threshold”, in cooperation with a cuticular membranous structure covered by mucilage called “velum” (Lloyd, 1942; Plachno et al., 2019b). Trap opening is triggered by specialized appendages (“trigger hairs”), in response to the mechanical stimulation by preys. A dense layer of glandular cells (“monofid” and “bifid” glands) involved in trap mechanical functioning covers the opening, especially the threshold, whereas the inner surface of the cavity is layered with secretive cells (“quadrid” glands) involved in prey digestion (Reifenrath et al., 2006; Plachno et al., 2015). Such a structure appears to be in common between species relying on passive collection behaviour, such as *U. westonii* (showing also active suction events – Poppinga et al., 2020) or *U. multifida*, and the others relying on an active suction mechanism (Plachno et al., 2019a). However, in spite of the common general structure, trap morphology exhibits a remarkable diversification, especially in trapdoor structure and biomechanics, thought to be responsible for the evolutionary radiation of the genus (Westemeier et al., 2017). Particularly intriguing is also the trap polymorphism in shape and size observed in several *Utricularia* species (Lloyd and Goebel, 1931; Lloyd, 1932b, 1942; Taylor, 1989) that may actually allow them expanding their feeding resources (Friday, 1991; Guisande et al., 2004; Guiral and Rougier, 2007; Adamec and Poppinga, 2016).

Adamec (2011b) measured trap efficiency in catching preys of 13 different and widespread *Utricularia* species in terms of firing and resetting rates, using trap length and thickness as proxies of trap size. The results showed that size and efficiency are inversely related, i.e., the smaller and narrower the traps the higher the firing and resetting rates, as confirmed by other, more recent researches on *U. gibba* (Berg et al., 2020).

The suction functional mechanism is probably one of the most sophisticated prey-hunting system among carnivorous plants (Poppinga et al., 2015; Gowri and Rathipriya, 2018) and includes two steps.

1. Water from the trap lumen is actively pumped out by specialized bifid glands through the trapdoor (Fineran and Lee, 1975),



Fig. 2. Details of *U. gibba* traps from the marshes along the Alento River (Campania region, Southern Italy). Young (A) and old (B) utricles are distinguished by a thick layer of periphyton colonizing the latter, preferentially developing over the trap external surface, rather than the plant shoot. The section (C), stained with 1% aq. aniline blue (shown with inverted colors to enhance details), highlights the main structures of the trap: appendages (a), trapdoor (d), stalk (s), threshold (t), monofid and bifid glands (m/b), quadrifid glands (q) and secretive hairs (sh). The trap readiness to capture preys are clearly recognizable by the collapsed lateral walls (D), storing the energy needed for the active suction process. Scale bar lengths are equal to 1.00 mm in A, B and D, and to 250 μ m in C.

generating an internal negative hydrostatic pressure of $-12/-16$ kPa (Singh et al., 2011; Vincent and Marmottant, 2011). The energetic costs of this process are covered by the activity of Cytochrome-c oxidases (Laakkonen et al., 2006) or alternative pathway enzymes (AOX), recently discovered to be more important than the former in *U. reflexa* (Pavlović et al., 2022), in spite of their lower ATP yield. The differential pressure across the trap walls leads, within 15 min, to the collapse of the flexible lateral walls (Fig. 2D) and to the storage of elastic energy within the trap structure (Lloyd, 1942; Westemeier et al., 2017; Poppinga et al., 2018; Müller et al., 2020). In this process, a key role is played by the trapdoor ($\sim 20-40$ μ m thick in *U. vulgaris*) and the velum, closing the trap watertight and making it ready to catch small preys (Lloyd, 1932a, 1942; Poppinga et al., 2017; Westemeier et al., 2017; Plachno et al., 2019b).

2. When the sensitive hairs on trapdoor are triggered by small crustaceans, rotifers, ciliates etc., the trapdoor opens in less than 0.5 ms with a concomitant conversion of elastic into kinetic energy (Lloyd, 1932a; Poppinga et al., 2017; Müller et al., 2020). During the process, trap lateral walls turn from a concave to a convex shape, with a concomitant suction of water and preys (Vincent and Marmottant, 2011; Poppinga et al., 2017). With a speed of 4 m/s, the utricle can swallow the prey, which will perform a whirling pattern inside the trap with no chance to escape (Poppinga et al., 2017).

Once in the trap, the prey is digested by enzymes secreted by the quadrifid glands covering the inner walls (Fineran, 1985; Plachno et al., 2006). As soon as the digestion ends, the trap restores its concave shape within a lapse of $\sim 25-30$ min, by the gradual spillage of 40% of the water, before being able to be triggered again (Sydenham and Findlay, 1973; Sasago and Sibaoka, 1985). This time is part of the so called "reset period", i.e., the time needed to fully restore the trap functioning, the completion of which has a natural duration of 24–48 h in *U. vulgaris*, *U. inflata* and *U. reflexa* (Adamec, 2011a).

It is interesting to note that *Utricularia* traps can fire spontaneously (Vincent et al., 2011; Poppinga et al., 2017) 0.3–2.4 times a day during the reset period (Adamec, 2011a) and the time between one shot and another is on average 5–20 h (Adamec, 2011b, 2018; Gowri and Rathipriya, 2018). This firing can happen either randomly or regularly (Adamec, 2011a; Vincent et al., 2011) but its function is likely related to a relaxation of trap walls and trapdoor, avoiding tissue stresses, and preventing trap damages (Poppinga et al., 2015; Adamec, 2018). Curiously, the trapdoor size of several species, most notably *U. gibba*, ~ 1 mm in length, is similar to the mouth size of other suction-feeders floating organisms like fish larvae and tadpoles (Berg et al., 2020), further evidence of the feeding on similar resources. In respect to the latter, however, the traps of *Utricularia* are able to generate more efficient suction flows that are inertia-dominated rather than viscosity-dominated and akin to the flows generated by adult fishes (Müller et al., 2020; Singh

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et al., 2020).

Among the main resources for bladderworts there are bacteria, protozoa, annelids, copepods and insects, that are captured with an efficiency depending on the availability, size, morphology and motility of preys (Horstmann et al., 2021; Ceschin et al., 2022). The age of the trap (Fig. 2A,B) also affects predation, both in terms of efficiency, with younger traps able to catch more preys than older ones (Friday, 1989), and of the spectrum of preys, with older traps preferentially capturing grazers feeding on the periphyton growing on them (Ceschin et al., 2022). Incidentally, predation is one of the processes through which bladderworts are able to directly and indirectly shape ecological dynamics in aquatic ecosystems, with effects on morphological traits, life history and behaviour of preys (Kruppert et al., 2022), population dynamics (Harms, 2002; Larson et al., 2022) and community structure (Mentes et al., 2018; dos Santos and Ferragut, 2018; Miyashita et al., 2019; Zuardi and Wardhana, 2021; de Matos Costa et al., 2022; dos Santos et al., 2022).

During firings, however, either spontaneous or induced by preys, it is common for *Utricularia* species to catch also epiphyte algae, phytoplankton and organic detritus, expanding the feeding horizons of these peculiar, not-only-carnivorous plants.

5. "Mixotrophic omnivory"

The digestion of such a wide variety of trophic resources, ranging from animals to bacteria and algae, can be hardly completed using plant enzymes only. Indeed, it is usual for carnivorous plants to digest preys with the contribution of symbiotic partners enriching the enzymatic profile of the host, and potentially acting as growth promoters and abiotic and biotic stress mitigators (Berg et al., 2014; Alcaraz et al., 2021). In this context, cysteine proteases are among the main digestive enzymes of carnivorous plants, which appear to be constitutively produced in *Utricularia* rather than being synthesized in response to the triggering of traps through jasmonic acid signalling, as occurs in several other genera such as *Aldrovanda*, *Dionaea* and *Drosera* (Jakšová et al., 2021). Reportedly, the complex communities living inside the lumen of *Utricularia* traps actively participate in the digestion of the organic matter entering the traps through their own enzymes, since algae, pollen and protozoa are consumed within the utricles, questioning the carnivorous attitude of these plants (Richards, 2001; Peroutka et al., 2008; Alkhalaf et al., 2009; Koller-Peroutka et al., 2014). In some cases, the capability of feeding upon resources different from the usual preys may actually shift the trophic preferences of *Utricularia* toward such non-conventional "preys". For instance, common freshwater species like *U. x neglecta* and *U. vulgaris* appear "greedier" of algae than animal preys (Fleischmann et al., 2018), and almost on the edge of phytophagy. A similar strategy is also observed in other necrotrophic mixotrophs such as *Nepenthes ampullaria* which feeds on leaf litter collected through its traps (Moran et al., 2003; Pavlović et al., 2011), or several *Pinguicula* species that catch and digest pollen granules (Karlsson et al., 1994; Adlassnig et al., 2005).

Defining *Utricularia* diet involves a deep understanding of the composition of trap microbial communities and their role in the digestion process and growth promotion (Alcaraz et al., 2016; Širová et al., 2018b). The trap lumen is an aquatic micro-digestive-ecosystem, where microorganisms have to face challenging living conditions (Adamec, 2007; Širová et al., 2018a, 2018b). Indeed, the communities within the utricles are subjected to wide variations in the environmental conditions, during the different phases of the trap functioning or the circadian cycles, like the concentration of dissolved oxygen and pH.

Širová et al. (2018b) deeply explored the microbiome structure of *U. australis* and *U. vulgaris* traps, highlighting a dynamic but rather preserved presence of both prokaryotes and eukaryotes, independent of plants provenances. The strong connection between periphyton and trap microorganisms observed, suggests that the first likely represents a source for trap microbial colonization (Širová et al., 2018b). Such a

hypothesis is supported also by the strict dependence of trap community composition on trap age: young utricles show internal sterile condition at first, and a progressive colonization as long as the organic carbon from photosynthesis is gradually stored, allowing the settling of microorganisms during trap openings (Plachno et al., 2012; Širová et al., 2018b) (Fig. 2B).

Proteobacteria dominate the prokaryote communities, both in the trap and in the periphyton on its external surfaces (Širová et al., 2011; Alcaraz et al., 2016; Širová et al., 2018b). Among these, especially represented are the aerobic *Comamonadaceae*, competitive bacteria that are also found in animal gut, and *Bacteriovoraceae*, predators of other bacteria that probably regulate the composition of the microbial community (Širová et al., 2018b). *Actinomycetes*, involved in plant organic matter digestion, are also abundant, followed by *Cyanobacteria*, *Acidobacteria*, *Chloroflexi*, *Firmicutes*, *Spirochaetes*, *Verrucomicrobia*, and many cellulolytic bacteria (Lima et al., 2018; Širová et al., 2018a, 2018b). Among *Firmicutes*, *Peptostreptococcaceae* stand out as anaerobic fermenting bacteria often associated with animal gut, where they produce large amounts of ammonia, found also in trap lumen (Širová et al., 2018b).

Eukaryotes found in the trap lumen involve fungi, protozoa and algae. Most of the fungi are commensals, but also saprophytes like *Basidiobolus* sp., that appear to supply *Utricularia* with nutrients derived from the decomposition of organic detritus (Širová et al., 2018b). Other represented groups include *Fusarium*, *Arcaeorhizomycetes*, *Epicoccum*, *Phialocephala* that utilize plant photosynthetic products as source of organic carbon, especially in younger traps of *U. vulgaris* (Širová et al., 2018a, 2018b).

Protozoa are largely represented by ciliates, active as commensals or trophic resource suppliers since they represent potential preys. Among ciliates, the *Tetrahymena* genus appears to be especially associated to *Utricularia*, with several species living in the traps of many different Taiwanese *Utricularia* (Cheng et al., 2019), questioning a possible host specificity. However, to date, only one peculiar species endemic to the utricles of the African species *U. reflexa*, has been found: *Tetrahymena utriculariae* (Alcaraz et al., 2016; Pitsch et al., 2016; Šimek et al., 2017; Širová et al., 2018b; Cheng et al., 2019; Širová et al., 2020). This species is both an active bacterivore and mixotroph, through a facultative endosymbiotic relationship with green algae of the *Micractinium* genus (Pitsch et al., 2016; Šimek et al., 2017; Pröschold et al., 2020), recently recognized as a new and unique species called *Micractinium tetrahymenae* (Pröschold et al., 2020). The latter seems to guarantee oxygen to *T. utriculariae* in challenging conditions of anoxia such as those inside the trap (Pitsch et al., 2016; Šimek et al., 2017; Pröschold et al., 2020). In fact, the ciliate, placed in opposite conditions, in an environment rich in trophic resources and oxygen, tends to lose endosymbiosis to go back to a heterotrophic behaviour (Pitsch et al., 2016; Pröschold et al., 2020).

Despite a recent greenhouse experiment of Širová et al. (2020) demonstrated that mixotrophic *T. utriculariae* can also colonize different *Utricularia* species (*U. ochroleuca*, *U. aurea* and *U. inflata*), the affinity with *U. reflexa* remains the highest, in terms of abundance of ciliates per trap and residence time. Overall, *T. utriculariae* appears contributing, with other ciliates, to nutrient recycling of bacterial origin and its acquisition by the plant (Šimek et al., 2017; Širová et al., 2018a, 2018b; Cheng et al., 2019).

Regarding the phytoplankton, the high concentration of soluble nutrients inside the trap lumen allows different species of algae to settle and grow, forming communities depending on provenance, trap age and *Utricularia* species (Širová et al., 2018a). Especially osmotrophic genera such as *Euglena*, *Phacus* and *Scenedesmus* can live in the trap lumen without being digested, like other algae, by microbial community (Plachno et al., 2012; Širová et al., 2018b). For instance, the green alga *S. alternans* and the euglenoid *E. agilis* are more abundant in *U. reflexa* traps than in the surrounding environment, increasing their biomass as the trap ages (Šimek et al., 2017). There seems to be a mutual exchange: on the one hand *Utricularia* traps offer a complete micro-environment to

colonize, on the other hand algae favour the acquisition of limiting nutrients, such as phosphorous, trace elements and organic growth factors (Koller-Peroutka et al., 2014). A similar mutualism appears to take place between the plant and the microbial community digesting the trophic resources entering the traps, where the photosynthetic products of the former support the metabolism of the latter and the costs associated to the production of hydrolytic enzymes, obtaining limiting nutrients in exchange (Širová et al., 2018a, 2018b). In this context, hydrolytic enzymes, such as α - and β -galactosidase, play a key role especially in the degradation of algal dead mass, rich in cellulose, hemicellulose, and D-galactose. Other enzymes such as UDP-glucose-4-epimerase, involved in galactose metabolism, and Propionyl-CoA, catalysing propionate probably derived from the fermentation of plant material, are abundant in *U. vulgaris* traps (Širová et al., 2018b). Curiously, these enzymes are also found in herbivore gut, suggesting a parallelism between the metabolism of such an environment and the one of the trap lumen.

Overall, a “vegetarian diet” seems to be a much more advantageous nutritional strategy in aquatic species of *Utricularia* living in nutrient and animal poor habitats (Ellwood et al., 2019; Širová et al., 2018b). Here, a sophisticated and miniaturized trap ecosystem allowing a “mixotrophic omnivory”, rather than a strictly carnivorous diet, ensures *Utricularia* efficiently accessing resources and competing (Raven, 1997; Koller-Peroutka et al., 2014; Ellwood et al., 2019; Klink et al., 2019).

6. Opportunities and challenges

As sometimes occurs, the recent advancements in the study of *Utricularia* ecology contributed more to the raise of new questions than to the filling of the gaps. The idea of these plants as exceedingly complex ecological systems hosting an own microbiome, acting like a sort of auxiliary genome guiding numerous physiological functions and the adaptation and evolution of species, puts *Utricularia* in a domain thought to pertain exclusively to humans and other higher organisms, and opens new horizons to be explored.

Novel questions span from the molecular level to the development of technological devices based on the *Utricularia* biomechanics. For instance, what are the mechanisms allowing some algae to settle and be almost “cultivated” within the utricles whereas others are digested? What are the compartmentalisation or protection mechanisms avoiding the utricles being digested as well? What are the dynamics, evolution, and self-organization mechanisms of the microbial community?

At a higher level of organization, utricles represent micro-ecosystems with remarkable similarities at a functional level with the rhizosphere and ruminant gut, but with important differences (Širová et al., 2018b). Under these premises, it is straightforward the idea to approach the study of the energy fluxes and biogeochemical cycles in these systems using *Utricularia* traps as model systems, as they are easy to manipulate (Širová et al., 2018a). The use of *Utricularia* as model systems, however, is not limited to these topics and, moving to higher levels of complexity, the elucidation of the plant-microbiome interactions and their changes through plant ontogeny and in response to environmental factors have crucial implications in plant eco-physiology, evolution and conservation. It would pave the way to the understanding of the control mechanisms granting plants to shape the properties of the interacting microbial communities, how such systems can co-evolve and adapt to environmental variations, how the microbiome affects plant evolutionary radiation, to name but a few cutting-edge topics. For example, dramatic alterations of trap microbiome in response to anthropogenic stressors such as warming (Mieczan and Bartkowska, 2022) and microplastic pollution (Yu et al., 2022), have been recently documented and are concerning in terms of long-term conservation of threatened species.

As model systems, bladderworts offer also interesting research prospects both in the field of biomimicry and biocontrol of insects. Biomimicry is an innovative emerging research area, inspired by nature

dynamics, seeking to create structural models with the aim of solving human problems (Aziz, 2016). Plants that show ultra-fast and thigmotactic movements like *Utricularia* suction traps could be an exceptional source of inspiration for engineering applications (Guo et al., 2015).

As a result of exacerbated climate changes, mosquitoes—vectors of dangerous diseases like Dengue, Chikungunya, and Malaria—are spreading and becoming invasive in urban environments (Courret et al., 2020). Several *Utricularia* species, collected from field and placed in a domestic setting, can counteract the growth of *Aedes* mosquito larvae, reducing their population (Courret et al., 2020; Perera et al., 2021). In this challenging perspective, *U. vulgaris* and its biomechanics in capturing preys have already inspired an eco-friendly solution to handle the problem. The device behind the project is called “UPod” and is able to float, gain solar energy, and pull water and mosquito larvae into a tightly sealed water chamber through a trap door that functions with a smart sensor mechanism (<https://news.cornell.edu/stories/2018/07/carnivorous-plant-inspires-sustainable-mosquito-control-device>). Once the chamber is filled up with water, the trap door closes bringing the larvae to death by drowning, and then pumps them out to restart the cycle. It can be used in a wide range of habitats and represents a sustainable solution to lower larvae population density, reducing the use of pesticides compromising, in the long run, biosphere dynamics. A better understanding of the biophysics and the molecular mechanisms involved in trap functioning, with the aid of computational models (Joyeux et al., 2011), is crucial for the further development of such devices and the related materials. In terms of materials science, the cyclic deflation and expansion of *Utricularia* traps to capture preys has recently inspired original and efficient ways to create yolk-shell nanoparticles (Ji et al., 2022). In particular, through the use of ultrasound waves providing energy to the process, cyclic contractions and expansions are induced in hollow porous carbon shells, which capture nanoparticles of vanadium oxide in the process, and thus form their core though progressive accretion. The resulting biomimetically created VO_x/C yolk-shell spherical particles have exceptional properties in energy storage and have shown promising applications in the production of Li–S batteries (Ji et al., 2022).

To conclude, *Utricularia* offers numerous research cues, with several conceptual and technical challenges and the opportunity to provide breakthrough discoveries and innovations, not only from a theoretical point of view, but also in applied sciences. The limit is the researcher’s imagination!

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Combination of the Systemin peptide with the beneficial fungus *Trichoderma afroharzianum* T22 improves plant defense responses against pests and diseases

Anna Maria Aprile^a, Mariangela Coppola^a, David Turrà^{a,g}, Stefania Vitale^{a,b}, Pasquale Cascone^b, Gianfranco Diretto^c, Alessia Fiore^c, Valeria Castaldi^d, Alessandra Romanelli^d, Concetta Avitabile^{e*}, Emilio Guerrieri^b, Sheridan Lois Woo^{f,g} and Rosa Rao^{a,g}

^aDepartment of Agricultural Sciences, University of Naples "Federico II", Portici, Italy; ^bInstitute for Sustainable Plant Protection, National Research Council of Italy, Portici, Italy; ^cItalian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), Casaccia Research Centre, Rome, Italy; ^dDepartment of Pharmaceutical Sciences, University of Milan, Milan, Italy; ^eInstitute of Biostructures and Bioimaging, National Research Council of Italy, Naples, Italy; ^fDepartment of Pharmacy, University of Naples "Federico II", Naples, Italy; ^gInteruniversity Center for Studies on Bioinspired Agro-Environmental Technology (BAT Center), University of Naples Federico II, Portici, Naples, Italy

ABSTRACT

Trichoderma spp. are among the most widely used plant beneficial fungi in agriculture. Its interaction with the plant triggers resistance responses by the activation of Induced Systemic Resistance mediated by Jasmonic acid and Ethylene and/or Systemic Acquired Resistance, which involves Salicylic acid, with the consequent control of a wide range of plant parasites. However, the benefit they can confer to plants may be reduced or nullified by environmental conditions or fungal ecological fitness. A novel approach to enhance their effectiveness in plant defense is to combine them with bioactive molecules including plant-derived compounds. Here, we show that plant treatment with *Trichoderma afroharzianum* (strain T22) and Systemin, a tomato peptide active in triggering plant defense, confers protection against the fungal pathogens *Fusarium oxysporum*, *Botrytis cinerea* and the insect pest *Tuta absoluta*. The observed defensive response was associated with an increase of Jasmonic acid and related metabolites and a decrease of Salicylic acid.

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1. Introduction

Tomato (*Solanum lycopersicum* L.) is the second most important economic crop in the world after potato (*Solanum tuberosum* L.), with cultivation covering 4.85 million ha and fruit production totaling about 182.3 million tons every year (FAOSTAT 2019). In pre-Covid-19 pandemic era, global tomato market income amounted to \$190.4 billion (Research and Markets 2020). Regardless of the type of cultivation or the plant life-cycle, increasing amounts of pesticides are required to protect tomato plants from pathogens and pests worldwide (Almeida et al. 2019; Savary et al. 2019; Abbas et al. 2020) with well-known undesirable effects on the environment (pollution, soil depletion, biodiversity loss) and human health (Rani et al. 2021). Public concerns prompted the research for alternative strategies of plant protection that are effective, reliable, and sustainable (Iriti and Vitalini 2020). In this context, biological control of plant diseases through the application of bioagents is a highly promising approach (Vinale et al. 2008). Among biological control agents (BCA), beneficial fungi belonging to *Trichoderma* spp. represent the most widespread soil microbes used in agriculture and a major constituent of commercially available biological products (i.e. biopesticides, biostimulants, biofertilizers) (Woo et al. 2014). Different *Trichoderma* species/strains and their metabolites act against plant pathogens

through several mechanisms, including direct antagonism of disease agents (mycoparasitism) (Poveda 2021), antibiotic-mediated suppression, secretion of lytic enzymes and other byproducts, competition for nutrients (Al-Ani 2018). Additionally, *Trichoderma* root colonization induces a state of alert (defense priming) in plants leading to a faster and stronger defense response against future pathogen attacks, this is achieved by reducing the effector-triggered susceptibility (ETS) and concurrently enhancing the effector-triggered immunity (ETI) (Hermosa et al. 2012; Martínez-Medina et al. 2017). Within this scenario, the use of inducers of plant endogenous defenses is considered one of the most promising approaches for the promotion of sustainable agricultural practices that may reduce synthetic chemical inputs (Burketova et al. 2015).

A pioneering study showed that plant root colonization by *T. afroharzianum* is associated with a reduction of the symptoms produced by the necrotrophic fungus *Botrytis cinerea* as a consequence of systemic defense responses elicitation (De Meyer et al. 1998). More recent studies demonstrated that different *Trichoderma* species can control several diseases deriving from bacterial, oomycete, fungal, or nematode infections (Poveda et al. 2020; Sood et al. 2020). Other studies showed that *Trichoderma* interaction with plants is able to induce a set of defense-related genes associated with the Jasmonic acid (JA) and Ethylene (ET)

CONTACT Rao Rosa  rao@unina.it  Interuniversity Center for Studies on Bioinspired Agro-Environmental Technology (BAT Center), University of Naples Federico II, Via Università 100, 80055, Portici, Naples, Italy

*Present address: Institute of Crystallography (CNR), Bari, Italy.

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pathways which may explain at molecular level the observed broad spectrum biocontrol activity of this fungus (Shore et al. 2005; Korolev et al. 2008). Notably, *Trichoderma* spp. do not only protect against parasitic microorganisms or nematodes but also against different insect pests (Muvea et al. 2014; Contreras-Cornejo et al. 2018). Indeed, it was previously demonstrated (Coppola et al. 2019a) that *Trichoderma atroviride* induced defenses are effective against two tomato pests with distinct eating habits: the piercing-sucking aphid *Macrosiphum euphorbiae* and the chewing noctuid moth *Spodoptera littoralis*. Moreover, plant colonization by the fungus activated indirect defenses via the production of volatile organic compounds (VOCs) that attracted the pest parasitoid *Aphidius ervi*.

It is well known that plant protection mediated by *Trichoderma* spp. can be deeply influenced by environmental conditions and ecological fitness of biocontrol agents hence being poorly predictable *a priori* (Howell 2003; Alabouvette et al. 2009; Lecomte et al. 2016; Malik et al. 2018). Consequently, the sole use of plant beneficial microorganism may not be always sufficient to fully protect plants against biotic stresses. To overcome such limitations, new approaches to increase plant resistance may focus on the combined application of different resistance inducers (*i.e.* chemical and/or biological) (Zehra et al. 2017; Zhou et al. 2021).

Among plants-derived molecules, the tomato peptide Systemin (Sys) has proved to be a very efficient elicitor of plant defense in tomato, grapevine, and eggplant (Coppola et al. 2019c; Molisso et al. 2021). Sys was the first peptide signal to be discovered in plants (Pearce et al. 1991). It is an octadecapeptide proteolytically released, upon wounding and herbivore attack, from the carboxy terminus of its 200 amino-acid precursor ProSystemin (ProSys) (Ryan and Pearce 2003). In plants, the autocrine interaction of Sys with its receptor is followed by the activation of the octadecanoid pathway and the production of JA and its derivatives (JAs) (Pearce et al. 1991). The role of Sys in the defense response against wounding or herbivory was evaluated in several studies exploiting transgenic plants overexpressing or downregulating ProSys. Its overexpression increased not only the resistance of plants against lepidopteran larvae, fungi, and aphids, but also the tolerance to salt stress (McGurl et al. 1994; Orsini et al. 2010; Coppola et al. 2015), while its downregulation strongly increased plant susceptibility to herbivores (Orozco-Cardenas et al. 1993).

Recently, it was demonstrated that exogenous supply of Sys induces an array of defense-related responses leading to the final accumulation of proteinase inhibitors (PIs) and other molecules able to interfere with the colonization of fungal pathogens or the growth and survival of herbivores (Coppola et al. 2019c; Molisso et al. 2021). Also, Sys-treated plants proved to be more attractive for pest natural enemies as a consequence of the modified blend of volatile compounds emitted, which is known to be used by predators and parasitoids of phytophagous insects to locate their preys and hosts.

Given that both *Trichoderma* and Sys activate the JA pathway, a key hub for plant defense and resistance towards insects and fungi, we hypothesized that their combined treatment on plants could result in an amplified disease and pest control outcome. In addition, considering that the endophytic root colonization by the beneficial fungus is limited by Salicylic Acid (SA) pathway, while JA triggered defenses have positive effect on it, (Alonso-Ramirez et al. 2014; Martinez-Medina et al. 2017) Sys application on the plant could

define a more efficient root colonization of T22 due to the negative crosstalk between the JA- and the SA-regulated pathways (Pieterse et al. 2012).

Accordingly, here we show that their combination confers resistance to tomato plants towards both the necrotrophic *B. cinerea* and hemibiotrophic *Fusarium oxysporum* fungal pathogens, and the insect pest *Tuta absoluta*. Our data provides evidence that plant treatments in a combination of beneficial fungi and plant-derived resistance inducers might represent an effective tool for sustainable pest management.

2. Materials and methods

2.1. Preparation of fungal cultures and inoculum and insects rearing

Fungal cultures were obtained from the collection available at the Department of Agricultural Sciences of the University of Naples Federico II. *T. afroharzianum* (formerly-*T. harzianum*) strain T22 (T22), was isolated from the commercial product Triunfo-P (Koppert Biological Systems, Rotterdam, the Netherlands), cultured on potato dextrose agar (PDA; HiMedia) and grown at 25°C until complete sporulation. Conidia were collected in sterile distilled water by scraping the surface of sporulating fungal cultures and recovering the spores by centrifugation at 2700 g for 10 min. Conidia concentration was adjusted to 10⁷ spores/ml for subsequent bioassays.

F. oxysporum f.sp. *lycopersici* (Fol) race 2 isolate 4287, and the *Fol* knockout mutant *fmk1Δ* were grown in potato dextrose broth (PDB) at 28°C with orbital shaking at 170 rpm. Phleomycin (5.5 µg/ml) was added to the culture medium when required. Fresh conidia were separated from the mycelium by filtering 5 d-old cultures through a nylon filter membrane (mesh size 10 µm) and collected after centrifugation at 2700 g for 10 min. Storage of the obtained conidia was performed as previously described (Di Pietro et al. 2001).

B. cinerea was grown on MEP (Malt extract with peptone) solid medium at 22°C. Conidia were collected by washing the agar surface with sterile distilled water containing 0.1% Tween 20, filtered, and adjusted to a concentration of 1 × 10⁶ conidia/ml with sterile distilled water.

The original strain of *T. absoluta* was collected in 2019 in tomato greenhouses located in Battipaglia (Salerno, Italy) (Gontijo et al. 2019). The insect was continuously reared at the National Research Council, Institute for Sustainable Plant Protection (CNR-IPSP, Portici), inside aerated cages (Vermandel[†], The Netherlands) at 22 ± 5°C, 65 ± 5% relative humidity (RH) and 16L:8D photoperiod.

2.2. Peptide synthesis

The Sys peptide synthesis and purification were obtained as previously described (Coppola et al. 2019c). In brief, Sys was obtained by solid phase synthesis following standard protocols (Romanelli et al. 2011). Purification of the peptide was carried out by RP-HPLC on a semipreparative column (Jupiter 10 µ, Proteo 90 Å, 250 × 10 mm) using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 5 to 50% in 30 min at 5 ml/min. Sys was characterized by mass spectrometry (LC-MS ESI-TOF 6230 Agilent Technologies, Milan, Italy). Sequences and mass spectrometry data follow. Sys sequence: AVQSKPPSKRDPKMQTD. Mass calculated (Da): 2009.3 Mass spectrum fragmentation data (Da): 670.94

[M + 3 H]³⁺; 1005.60 [M + 2 H]²⁺. Analysis of the HPLC (Shimadzu LC-8A, equipped with a SPD-M10 AV) profiles and of the mass spectra collected indicates the peptide stability in all the tested conditions.

2.3. In Vitro *Trichoderma*-Sys compatibility assay

To assess if Sys had a direct antimicrobial effect on *T. afroharzianum* T22, an *in vitro* assay was carried out to measure fungal growth in the presence or absence of different concentrations of the peptide. Antifungal assay was conducted as previously described (Pastor-Fernández et al. 2020). Wells of a sterile 12-well plate were filled with 1 ml of half-strength potato dextrose broth (PDB 1/2) medium containing Sys, at a final concentration of 100 pM or 100 fM except for the negative control wells. The positive control was filled with 200 µg/ml Switch[®] fungicide (Syngenta, 37.5% w/w cyprodinil and 25% w/w fludioxonil). Conidia of *T. afroharzianum* T22 were then added to each well to reach a final concentration of 10⁵ conidia/ml. Each plate was then placed in a shaker and incubated for 24 h at 25 ± 1°C. End-point fungal growth was assessed by measuring the medium turbidity at a wavelength of 600 nm (OD₆₀₀) on BioPhotometer Spectrophotometer UV/VIS (Eppendorf, Hamburg, Germany).

2.4. Seed treatment and plant growth

Seeds of *S. lycopersicum* cultivar 'San Marzano Nano' were surface sterilized with 2% sodium hypochlorite for 10 min, then fully rinsed in sterile distilled water. For the seed treatments, washed tomato seeds were either coated by immersion in a fresh spore suspension (final concentration of 10⁷ conidia/mL) of *T. afroharzianum* T22, or treated with water (Ctrl), stirred frequently to uniformly cover the seed surface, left to air dry for 24 h and stored at 4°C until use. Seeds were germinated on Whatman[®] sterile filter paper (Sigma-Aldrich, Darmstadt, Germany), moistened with sterile distilled water, and placed in a growth chamber at 24 ± 1°C and 60 ± 5% RH in the dark. At the emergence of the cotyledons, germinated seedlings were individually transplanted into pots containing sterile commercial soil (Universal Potting Soil, Floragard, Oldenburg, Germany) and kept in a growth chamber at 26 ± 1°C and 60 ± 5% RH, 18L:6D photoperiod by lamps of 5000 lux. After 2 weeks, tomato seedlings were transplanted into 10-cm diameter plastic pots containing sterilized soil and grown for 2 weeks under the same environmental conditions. For *F. oxysporum* assay, seeds were planted on wet vermiculite in plastic trays and incubated in plant growth chambers for two weeks at 26 ± 1°C and 60 ± 5% RH.

2.5. In vivo *F. oxysporum* infection assays

Two weeks-old plants (Ctrl and T22 seed-treated), treated with Sys or PBS buffer 1X dissolved in 5 ml of distilled water (final Sys concentration 100 fM), were tested for resistance to *F. oxysporum*. Twenty-four hours after Sys application *Fol* infection was performed by dipping tomato roots in a microconidia suspension (5 × 10⁶ conidia/ml) from *Fol* wt or *fmk1Δ* strains (Di Pietro and Roncero 1998). Tomato seedlings were planted into minipots containing vermiculite and maintained in a growth chamber (28°C;

14L:10D photoperiod). Plant infection was recorded daily for up to 20 days and percentage of plant survival was calculated by the Kaplan–Meier method (López-Berges et al. 2012). Fifteen plants per treatment were used and each experiment was repeated on three independent occasions.

2.6. In vivo *B. cinerea* infection assays

The application of Sys and *T. afroharzianum* was tested either alone or in combination to evaluate the effects of each treatment on *B. cinerea* leaf colonization. Fully expanded leaves from four-week-old plants (Ctrl and T22) were treated with 15 spots of 2 µl of 100 fM Sys or PBS; spots were produced on the abaxial surface using a pipette. Leaves were tested for resistance to *B. cinerea* after 6 h by applying 10 µl drops of *B. cinerea* spores (1 × 10⁶ conidia/ml) between tomato leaf veins, using 8 different inoculation points per leaf from 5 different plants for each individual treatment. Detached leaves were placed on sponges soaked in sterile water and incubated in a growth chamber at 23°C, under 16L:8D photoperiod and 90% ± 5% RH. Disease severity was quantified by measuring the development of necrotic leaf areas caused by the fungal pathogen over time at different days post inoculum (DPI) using a digital caliper (Neiko 01407A; Neiko Tools, Taiwan, China).

2.7. In vivo *T. absoluta* assays

To evaluate the possible induction of repellency linked to the alteration of the profile of volatile organic compounds released by tomato plants, a choice test was performed using a random block experimental design. Three 4-weeks old plants (Ctrl and T22) were treated with 100 fM Sys or PBS by foliar spotting. After 6 h, plants were placed into a single mesh cage (60 × 60 × 180 cm; Vermandel, The Netherlands) at a distance of 20 cm from each other. Three to 5 days old mated females of *T. absoluta* were released into the cage keeping a 1:1 ratio between them and the plants. After 2 days (oviposition period), females were removed from the cage by an insect aspirator and the eggs laid were counted. The test was replicated four times totaling 12 females/plant tested. To evaluate antibiosis/antixenosis, a no-choice test was performed by feeding *T. absoluta* larvae with detached leaves from each treatment and relative control. Fully expanded leaves to be used in the test were cut from plants treated as described before and immediately placed through the petiole in a 5-mL tube capped by a cotton wick, sealed by parafilm tape, and filled with tap water. Larvae to be used in the test came from tomato plants exposed for 24 h to hundreds of *T. absoluta* adults in a mesh cage. Tomato leaves with eggs were transferred into aerated cages and a single hatched larva was gently transferred onto a detached leaf by a soft brush and kept in a plastic box (Duchefa, The Netherlands) constituting the experimental unit. Thirty larvae were tested for each treatment. Leaves were examined daily to check the larval longevity and survival and to record the time to pupation.

2.8. Gene expression analysis

Expression levels of defense-related genes in control and *T. afroharzianum* – and/or Sys-treated tomato plants were quantified by real-time PCR (RT-PCR). The selected genes were: *Allene Oxide Synthase* (AOS; Solyc11g069800),

Lipoxygenase D (LoxD; Solyc03g122340), *Wound-induced Proteinase Inhibitor I* (Pin I; Solyc09g084470), *Threonine deaminase* (TD; Solyc09g008670), and *Leucine aminopeptidase* (LapA; Solyc12g010030). Fully expanded leaves from 4 weeks old plants (Ctrl or T22) were collected 6 h after Sys treatment and immediately frozen in liquid nitrogen. Total RNA extraction, first strand cDNA synthesis and RT-PCR were performed according to standard procedures, as reported elsewhere (Corrado et al. 2012). RT-PCR was performed using Rotor Gene 6000 (Corbett Research; Sydney, Australia). For each sample, two technical replicates from each biological replicate were used for gene expression analysis. The housekeeping gene EF-1 α was used as endogenous reference gene for the normalization of the expression level of target genes (Müller et al. 2015). Relative quantification of gene expression was carried out using the $2^{-\Delta\Delta C_t}$ method and referred to the mock-treated control (relative quantification, RQ = 1). Primers and their main features are reported in Additional file 2: Table S1.

2.9. LC-HESI(-)HRMS of JA/SA and JA/SA-related metabolites

By using LC-HESI(-)HRMS analysis, the levels of JA, its glycosylated (tuberonic acid-glucoside) and methylated form (MeJA) as well as three of its intermediates (13(S)-HPOT, 12,13(S)-EOT and 12-OPDA), the levels of SA, its glycosylated form (Salicylic acid glucoside) together with five of its intermediates (DL-Phenylalanine, trans-Cinnamate, Benzoic acid, Chorismic acid, and Prephenic acid) were measured in control plants or in plants treated with Sys, T22 or both. Detection and quantification of JA/SA and JA/SA-related metabolites were performed as previously reported with slight modifications (Liu et al. 2010; Van Meulebroek et al. 2012; Grosso et al. 2018). Briefly, 100 mg frozen leaf samples of 4 weeks old plants (Ctrl or T22) were collected 6 h after Sys treatment and homogenized with 1 ml cold extraction buffer (methanol, ultrapure water, and formic acid (75:20:5, v/v/v) spiked with $10 \mu\text{g ml}^{-1}$ formononetin as internal standard), vortexed continuously at -20°C o.n. Following centrifugation at $15,000 \text{ g}$ for $20'$ at 4°C , 500 ml were transferred to 30 kDa Amicon Ultra centrifugal filter unit (Merck Millipore Corporation, MA, USA), and centrifuged for $15'$ at $15,000 \text{ g}$ and 4°C . Finally, extracts were evaporated by speedvac until a final volume of $50 \mu\text{l}$ was reached, and then transferred to the LC vials. LC-HESI(-)HRMS analyses

were carried out using a Dionex high performance liquid chromatography-diode-array detector (LC-DAD) coupled to high-resolution mass spectrometry equipped with a heated electrospray source operating in negative ion mode (ESI(-)HRSMS) (Thermo Fisher Scientific, Waltham, MA, USA). LC analysis was performed as reported before (Liu et al. 2010; Van Meulebroek et al. 2012; Grosso et al. 2018), whereas mass spectrometry analysis was performed using a quadrupole-Orbitrap Q-exactive system (Thermo Fisher Scientific, USA), using a single ion monitoring (SIM) method. More in detail, metabolite ionization was carried out using the following parameters: nitrogen was used as sheath and auxiliary gas (40 and 15 units, respectively); capillary and vaporizer temperatures were set at 300°C and 280°C , respectively; discharge current at 3.5 KV , probe heater temperature at 360°C , S-lens RF level at 50 V . The acquisition was carried out in the $110/1600 \text{ m/z}$ scan range, according to the following parameters: resolution 70,000, microscan 1, AGC target $1\text{e}6$, and maximum injection time 50. Data were analyzed using the Xcalibur 3.1 software (ThermoFisher Scientific, USA). Metabolites were identified based on their accurate monoisotopic and adduct masses (m/z) and MS fragmentation, using both in house database and public sources (i.e. KEGG, MetaCyc, ChemSpider, PubChem, Metlin, Phenol-Explorer), as well by comparing chromatographic and spectral properties with authentic standards, when available. Relative abundances of the metabolites studied were calculated using the Xcalibur 3.1 software (Thermo Fisher Scientific, USA). JA/SA, and JA/SA-related metabolites were quantified in a relative way by normalization on the internal standard amounts. Data are presented as means and standard deviations of three independent biological replicates.

2.10. Statistical analysis

Differences in the survival rate of tomato plants infected by *F. oxysporum* and tomato plants infested by *T. absoluta* larvae were analyzed using the Kaplan–Meier method and compared among groups using the log-rank test. One-Way ANOVA test, followed by Tukey's *post-hoc* multiple comparison test ($P < 0.05$), were used to evaluate: differences in relative transcripts abundance, in necrosis diameter development, the effect of Sys on T22 growth, and in *T. absoluta* oviposition rate. Welch's ANOVA test followed by Dunnett's T3 test was used to evaluate differences *T. absoluta* larval longevity. Student's *t*-test was used to identify metabolites whose abundance significantly changed with respect to the control condition.

3. Results

3.1. Sys peptide did not directly impact *T. afroharzianum* T22 growth

Sys treatment did not impact T22 proving that Sys does not have inhibitory effect on fungal growth (Figure 1).

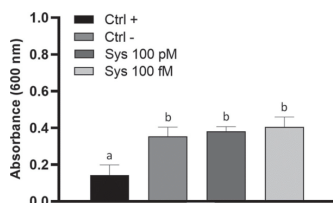


Figure 1. Effect of Sys on *T. afroharzianum* T22. Fungal growth in absence (Ctrl –) or presence of Sys (100 pM and 100 fM) was evaluated 24 h after fungal inoculation by assessing changes in optical density (OD_{600}). Switch (200 $\mu\text{g/ml}$) was used as a positive control (Ctrl +). Different letters indicate statistically significant differences (One-Way ANOVA, $P < 0.05$). Error bars indicate standard error ($n = 5$).

3.2. Sys and *T. afroharzianum* T22 reduce *F. oxysporum* infection

To evaluate if treatments with Sys and/or *T. afroharzianum* T22 were able to control *F. oxysporum* wilt disease on tomato

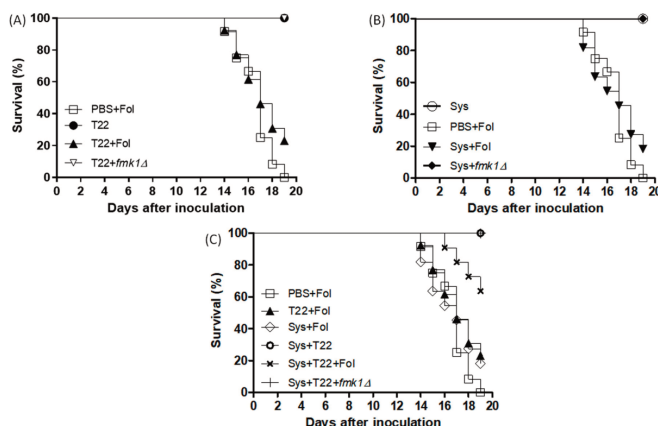


Figure 2. *T. afroharzianum* and *Sys* additively reduce *F. oxysporum* virulence on tomato plants. Kaplan-Meier plots showing survival of tomato plants treated with PBS (A, B, C), *T. afroharzianum* (A), *Sys* (B), or both (C), and not inoculated or inoculated by dipping roots into a suspension of 5×10^8 conidia/ml of the indicated *Fol* strains. Experiments were performed at least three times with similar results. Percentage survival of tomato plants was plotted for 20 days. Data shown are from one representative experiment.

plants, roots of two-week-old seedlings were left uninoculated, inoculated with the *Fol* wt strain or with the *Fol fmk1Δ* avirulent mutant strain and survival of treated versus untreated plants was recorded over time. As expected, in all experiments and regardless of the applied treatment (PBS control, *Sys*, or T22), uninoculated or *fmk1Δ* inoculated plants displayed neither wilting symptoms nor mortality throughout the whole evaluation process (Additional file 1: Figure S1).

On the contrary, most tomato plants germinated from Ctrl seeds and inoculated with the *Fol* wt strain showed progressive wilting symptoms and died after 19 days post inoculation (Additional file 1: Figure S1). Plants treatment with *Sys* or seed coated with *T. afroharzianum* T22 only partially, though not significantly, decreased *Fol*-mediated wilting and death of plants (Figure 2(A,B)) (T22 + *Fol*, Log-Rank test, $\chi^2 = 1.588$, $df = 1$, $P = 0.2077$; *Sys* + *Fol*, Log-Rank test, $\chi^2 = 0.771$, $df = 1$, $P = 0.3797$). Noteworthy, combined treatment of tomato plants with both *Sys* and *T. afroharzianum* T22 showed a stronger and significant reduction in plant mortality (*Sys* + T22 + *Fol*, Log-Rank test, $\chi^2 = 12.28$, $df = 1$, $P = 0.0005$) as only 40% of these plants had died after 19 DPI, contrarily to those derived from Ctrl seeds where 100% mortality was observed (Figure 2(C)).

3.3. *Sys* and *T. afroharzianum* reduce *B. cinerea* infection

Plants treated with *Sys* alone or in combination with T22 showed a significant reduction of foliar damage already one day after *B. cinerea* inoculation. Conversely, T22 alone treated plants exhibited significant differences in disease severity only after 5 days post inoculation when compared to control plants. A further decrease in necrotic leaf area development could be observed 7 DPI in plants concomitantly treated with both *Sys*

and T22, with about 63% reduction in disease severity compared to the control. Disease severity in these plants was significantly lower than the one observed in plants where each treatment was applied separately (–52% for *Sys* treatment and –40% for T22) (Figure 3).

3.4. *Sys* exogenous supply and *T. afroharzianum* colonization alters *T. absoluta* infestation

The number of eggs laid per plant was influenced by treatments (Figure 4(A), ANOVA, $df = 3$, $F = 10.03$, $P < 0.001$), resulting lowered by *Sys* and raised by T22 colonization. The combined application of T22 and *Sys* did not affect this parameter. Larvae survival curves revealed significant antibiotic effects of *T. afroharzianum* inoculation and *Sys*

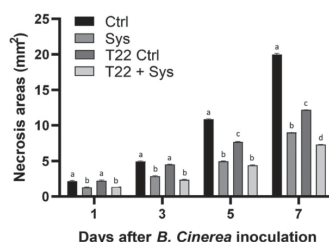


Figure 3. *T. afroharzianum* and *Sys* reduce *B. cinerea* virulence on tomato plants. Necrotic areas observed on *B. cinerea* inoculated leaves from tomato plants germinated from uncoated (Ctrl) or *T. afroharzianum*-coated seeds and treated or not with 100 mM *Sys*. The graph displays the average area of necrotic lesions formed 1, 3, 5, and 7 DPI. Letters indicate different statistical groups (One-Way ANOVA, $P < 0.05$). Error bars indicate standard error ($n = 90$).

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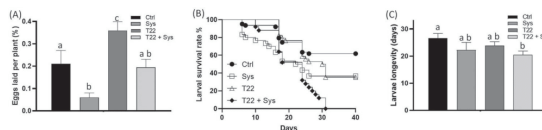


Figure 4. *T. absoluta* assays. (A) Eggs laid per plant by *T. absoluta* adults and (B) larvae survival curves on tomato plants treated with Sys and *T. afroharzianum* T22, their combinations and relative control. (C) Larvae longevity reared on the tomato plants treated as above described.

treatment on *T. absoluta* development when used alone or in combination (Figure 4(B), Log-Rank test, $\chi^2 = 21.6$, $df = 3$, $P < 0.001$). Survival rates, from eggs to pupation, were significantly lower for larvae fed on T22-treated leaves (Log-Rank test, $\chi^2 = 7$, $df = 1$, $P = 0.008$) and for larvae fed on Sys-treated leaves (Log-Rank test, $\chi^2 = 18$, $df = 1$, $P < 0.001$), in respect to control plants (Figure 4(B)). An additive effect was recorded for combined T22-Sys treatment (Pairwise log-Rank test T22 vs T22 + Sys $\chi^2 = 10.64$, $df = 3$, $P = 0.014$). Similarly, larval longevity was lowered by the combined application of T22 and Sys while no significant effect was recorded when the treatments were tested alone (Figure 4(C), Welch ANOVA, $F_{3,146} = 2.708$, $P = 0.047$).

3.5. Sys and *T. afroharzianum* enhance the expression of plant defense-related genes

A significant increase of AOS, Pin I, LapA, and TD, but not of LoxD, transcripts were recorded after Sys or T22 application). All gene transcripts, including those of the LoxD gene, were significantly more expressed in plants simultaneously treated with Sys and T22 compared to those treated with Sys or T22 alone (Figure 5).

3.6. Sys exogenous supply and *T. afroharzianum* colonization modify the accumulation of JA, SA, and related metabolites

JA over-accumulated in plants treated with Sys and T22, alone or in combination, with a maximum fold of 1.74 in T22 + Sys samples in addition, Sys treated plants also

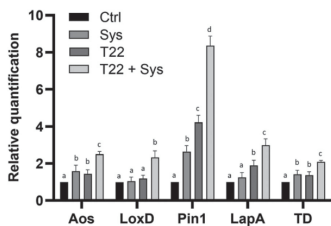


Figure 5. Level of defense-related genes expression in tomato plants grown from uncoated (Ctrl) or *T. afroharzianum*-coated seeds (T22) and treated or not with 100 fM Sys. Transcript levels of the following genes were analyzed: *Allene Oxide Synthase* (AOS), *LipoxygenaseD* (LoxD), *Wound-induced Proteinase Inhibitor I* (Pin I), *Threonine deaminase* (TD), and *Leucine aminopeptidase A* (LapA). Quantities of transcripts are relative to the calibrator Ctrl condition. Different letters indicate statistically significant differences (One-Way ANOVA, $P < 0.05$). Error bars indicate standard error ($n = 3$).

displayed higher levels in 12-OPDA in respect to controls, while another intermediate, 12,13(S)-EOT, increased in T22 + Sys plants. Accumulation of tuberonic acid-glucoside, involved in JA metabolism, was higher in single (Sys and T22) treatments, and unaltered in T22 + Sys plants. Finally, MeJA resulted over-accumulated in plants treated with both Sys and T22 (1.94 fold higher than Ctrl (Figure 6). Sys treatment significantly reduced the amounts of Chorismic acid, Prephenic acid, SA, and its glycosylated form while T22 increased Chorismic acid and decreased Prephenic acid and SA. The combined treatment increased chorismic acid (although to a lesser amount than the value reached by T22 treatment) and DL-Phenylalanine while decreasing Prephenic acid and SA (Figure 7).

4. Discussion

The discovery of novel strategies for pathogen or pest control able to reduce the use of chemical pesticides represents an important challenge in modern agriculture. Beneficial micro-organisms and natural-derived molecules with the potential to prime plant defenses are intriguing tools for the development of new generation biopesticides which could increase the sustainability of agricultural production. Some soil-dwelling fungi belonging to the genus *Trichoderma* have a profound impact on plant growth and protection limiting plant pathogen populations through mycoparasitism, competition, and antibiosis (Harman et al. 2004; Verma et al. 2007; Yuan et al. 2019). In addition to pathogen control, root colonization by *Trichoderma* spp. limits pest-induced damage on plants either through stimulation of plant defense or by attracting natural enemies of insect pests (Tucci et al. 2011; Battaglia et al. 2013; Coppola et al. 2019b). Despite the efficacy of these fungal species in promoting plant growth and resistance against biotic threats, their potential can be limited in certain ecological contexts where their fitness is low (Di Lelio et al. 2021). To overcome this limitation, a combination of *Trichoderma* and other natural-derived elicitors of plant's endogenous defense may represent an effective strategy to boost *Trichoderma* biocontrol activity both in fungal-friendly or harsh environments. A feasible strategy is represented by the combined use of these root symbionts with natural-derived compounds, including plant-derived signaling molecules (Isman 2006), semiochemicals (Witzgall et al. 2010), and interfering RNAs (Zhu et al. 2011; Koch et al. 2016) to attain satisfactory levels of plant protection. Natural molecules able to trigger plant immunity, such as Damage Associated Molecular Partners (DAMPs), are interesting players in pathogen and pest management. One of these is Sys, which acts as an elicitor of immune responses in tomato plants following insect or mechanical damage

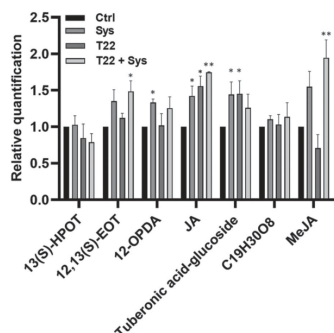


Figure 6. Targeted LC-HESI (-)HRMS analysis of semi-polar metabolites in the JA pathway. Metabolites were relatively quantified as fold internal standard level, normalized on the control (Ctrl; for more details, see materials and methods). Data represents the average of three independent biological replicates, and a student's *t*-test was used to identify metabolites whose abundance significantly changed with respect to the Ctrl condition. (** $P \leq 0.05$; *** $P \leq 0.01$).

(Ryan 2000; Ryan and Pearce 2003). Our group (Coppola et al. 2019c; Molisso et al. 2021) demonstrated that the exogenous supply of the peptide to intact plants enhanced their resistance against the noctuid moth *S. littoralis* and the fungal pathogen *B. cinerea*. These results are likely linked to the induction of plant's defense-related genes through the accumulation of active compounds.

An important prerequisite to carry out combined plant treatments with *Trichoderma* and natural-derived compounds is the absence of either direct or indirect antagonistic effects between the two treatments. Importantly, in our experiments, Sys addition to T22 fungal cultures did not show any growth inhibitory effect.

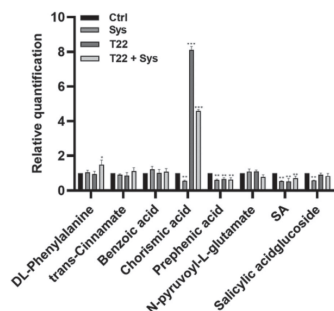


Figure 7. Targeted LC-HESI (-)HRMS analysis of semi-polar metabolites in the SA pathway. Metabolites were relatively quantified as fold internal standard level, normalized on the control (Ctrl; for more details, see materials and methods). Data represents the average of three independent biological replicates, and a student's *t*-test was used to identify metabolites whose abundance significantly changed with respect to the Ctrl condition (** $P \leq 0.05$; *** $P \leq 0.01$, *** $P \leq 0.001$).

Trichoderma and Sys ability to suppress different biotic threats resides in their propensity to prime plant defense responses through the modulation of different signaling pathways, including those of JA, ET, and SA (Shoresh et al. 2005; Martínez-Medina et al. 2017; Yuan et al. 2019). Priming is an intrinsic part of induced resistance during which the plant 'projects' a defense strategy against the potential invader while preparing its defensive approach for a faster and/or stronger reaction to future challenges (Mauch-Mani et al. 2017). The analysis of the expression of JA-associated genes in T22 and in Sys treated plants showed an up-regulation of *Aos*, an early defense-related gene, active in the octadecanoid signaling pathway that leads to JA biosynthesis, and of *Pin 1* and *TD*, two late defense-related genes responsive to JA. It is well described that JA activates a cascade of defense responses that are temporally (early and late) and spatially (local and systemic) regulated in tomatoes (Ryan 2000). The early and late wounding-response genes are regulated by distinct mechanisms. Local wounding leads to the processing of ProSys followed by Sys binding to SYR1, a membrane located receptor (Wang et al. 2018), and the subsequent activation of genes involved in JA biosynthesis. The increase of endogenous JA levels leads to the activation of late JA responsive genes that play a direct role in defense. Late defense genes include *Pls*, *TD*, and *LapA* (Howe 2004). Tomato *LapA* is important in deterring herbivory-induced damage and controlling insect growth (Fowler et al. 2009), while *Pin 1* prompts the formation of highly stable complexes with insect digestive proteases resulting in decreased digestion of dietary protein, depletion of essential amino acids, and, consequently, reduced rates of insect growth and development (Lorito et al. 1994). Furthermore, PIs in general and Pin I efficiently target microbial secreted proteases thus inhibiting a variety of plant pathogens including bacteria and fungi through suppression of cell growth (Hermosa et al. 2006; Turra and Lorito 2011; Turrà et al. 2020). *TD* is a gene involved in the resistance against chewing insects. Remarkably, TD activity in the insect's midgut correlates with reduced levels of free threonine, which is a dietary requirement for phytophagous insects (Chen et al. 2007). We observed that the combination of the two treatments (Sys and T22) results in higher levels of transcripts related to the activation of the aforementioned genes, and of *LoxD*, a gene that also contributes to JA biosynthesis consequently, enhancing resistance against insect herbivores (Yan et al. 2013; Thakur and Udayashankar 2019).

Metabolite data, obtained by LC-HRMS analyses, partly paralleled transcript profiles: single and double treatments were characterized by higher levels in JA, the end-product of the octadecanoic pathway involved in a series of physiological plant responses, in respect to control (Yang et al. 2019); the magnitude of alteration was stronger in T22 + Sys plants compared to single treatments. A significant increase of MeJA, the JA volatile methyl ester, was also registered in plants simultaneously treated with both Sys and T22. Tuberonic acid-glucoside, a metabolite produced in the frame of JA catabolism, so-called by virtue of its capacity to induce tuber formation in potato (*S. tuberosum*), also increased in Sys and T22 but not in the double-treated plants. Its synthesis looks to be part of a general mechanism aimed at turning on and switching off JA levels and functions (Miersch et al. 2008): thus, in this context, the higher JA content in T22 + Sys plants could reflect either the increased

upstream metabolic flux, as highlighted at the gene expression level, or the reduced catabolism in the double compared to the single treatments. Additional increases in JA intermediates were found in Sys and T22 + Sys samples, although this appears to be more related to stochastic events rather than to a solid tendency. As expected, metabolite data showed an impact of Sys and T22 plant treatment on SA pathway increasing phenylalanine and chorismic acid but decreasing SA. Since chorismate is a branch-point metabolite for SA and phenylalanine biosynthesis, it appears that the combined treatment 'pushed' the pathway's flow in the direction of phenylalanine production that should lead to phenylpropanoid biosynthesis, a class of compounds known to be produced by the plant under biotic stress. In addition, the reduction of SA should be connected to a faster and more intense T22 colonization since endophytic root expansion of the beneficial fungus is limited by SA pathway. Previous work has shown that endophytic colonization of *Arabidopsis thaliana* roots by *T. harzianum* is strictly dependent on the relative abundance of the two antagonistic plant hormones JA and SA. Indeed, exogenous application of SA reduces *Trichoderma* root colonization, while induction of the JA pathway or impairment of the SA one promotes it (Alonso-Ramírez et al. 2014; Martínez-Medina et al. 2017). In line with this, Sys treatment likely results in enhanced colonization of plant roots by *Trichoderma* which in turn contributes together with Sys to boost plant immunity and broad-spectrum resistance against both foliar and root fungal pathogens. The central role of JA in *Trichoderma* colonized plants in modulating defense responses has been demonstrated and confirmed through several mutant studies. For example, plants, of the JA-deficient defenseless1 (*def1*) mutant, were susceptible to disease development even after *Trichoderma* treatment, demonstrating that JA pathway is required for plant resistance in pathogen-challenged conditions (Martínez-Medina et al. 2013). It has also been shown that JA plays key role during plant defense against necrotrophic fungal pathogens, such as *B. cinerea* and *Alternaria brassicicola*. The mutation of COI1, a receptor involved in JA signaling, makes plants more susceptible to *B. cinerea* (An et al. 2019). Other results showed the protective role of JA against *Fusarium* wilt disease in tomato (Hernández-Aparicio et al. 2021). In addition, recent evidences shown that JA is involved in *Trichoderma virens*-mediated resistance against *F. oxysporum* in tomato plants. In this context, the activation of the JA signaling pathway in Sys + T22 treated plants nicely correlates with the reduction of *B. cinerea* disease severity and *F. oxysporum* induced mortality. JA is also a crucial player in plant defense response against insect pests (War et al. 2012). Exogenous supply of JA and MeJA through soil drenching or foliar sprays increases resistance against a broad spectrum of insects in numerous agricultural crops under greenhouse and field conditions (Haas et al. 2018; Nouri-Ganbalani et al. 2018; Stella de Freitas et al. 2019). Strapasson (2014) found that the treatment of tomato seeds with MeJA significantly decreased the pupal weight of *T. absoluta*. Our findings showed for the first time that Sys strongly reduces the survival rate of larvae of *T. absoluta*, and corroborate previous findings showing that *Trichoderma* endophytic colonization negatively affects *T. absoluta* fitness through significant reduction of larval survival (Agbessenou et al. 2020). Furthermore, our study highlighted the usefulness of combined treatments to enhance plant resistance to

T. absoluta. Previous works had shown a synergistic effect of different treatments on its fitness. For example, the combination of *Beauveria bassiana* and *Bacillus thuringiensis* strongly reduced *T. absoluta* larvae survival (Tsoulara and Port 2016). Yet, the usefulness of co-expression of different PIs to enhance plant resistance to *T. absoluta* has been highlighted (Hamza et al. 2018). Nonetheless, this is the first study to evaluate the impact of combined peptide treatment and endophytic colonization.

Apparently, the combined application of Sys and T22 did not alter the oviposition behavior of *T. absoluta* with respect to the single powerful treatment with Sys. This result could have many explanations. For example, *T. absoluta* develops many overlapping generations/year, therefore mated females frequently oviposit on highly infested plants that are highly activated as Sys treatment. The combined treatment showed an additive effect on larval longevity/survival of *T. absoluta* in respect to the single treatments and this could correlate to the larger accumulation of JA-derived metabolites. It will be now interesting to test the effect of the combined treatments on the natural antagonists of *T. absoluta*, known to exploit plant VOCs to locate their victim (Lins et al. 2014; Gontijo et al. 2019).

In conclusion, the combined application of *Trichoderma* and Sys on tomato plants increases the level of JA while decreasing the amount of SA, thus promoting a defense priming state and likely enhancing T22 plant colonization. Altogether, these events result in a boost of plant immunity. Since JA and its derivatives trigger VOCs emission (Tamogami et al. 2012), the increased levels of JA produced in treated plants may amplify the attractiveness of natural enemies of pests, thus promoting indirect defenses of treated plants that could be protected also against other agents or biotic stresses.

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Author contributions

AMA performed bioassays, gene expression studies; MC; supervised and participate to the experimental work; DT, SV designed and carried out experiments with *F. oxysporum*; PC, EG designed and carried out *T. absoluta* bioassays; AF, GD, performed metabolomic analysis; CA, AR synthesized and purified Sys; VC contributed to gene expression analyses; SW supervised experimental work and revised the manuscript; RR conceived and supervised the work and wrote the manuscript. All the authors contributed to manuscript writing.

ORCID

Pasquale Cascone  <http://orcid.org/0000-0002-4097-1974>
Emilio Guerrieri  <http://orcid.org/0000-0002-0583-4667>

Notes on contributors

Anna Maria Aprile is a PhD student in Biotechnology at the Department of Agricultural Sciences, University of Naples "Federico II", Italy.

Mariangela Coppola is a Postdoctoral Researcher at the Department of Agricultural Sciences, University of Naples "Federico II", Italy.

David Turri is an Associate Professor in Plant Pathology at the Department of Agricultural Sciences, University of Naples "Federico II", Italy.

Stefania Vitale is a Postdoctoral Researcher at the Institute for Sustainable Plant Protection (IPSP), Italian National Research Council (CNR), Italy.

Pasquale Cascone is a Researcher at the Institute for Sustainable Plant Protection (IPSP), Italian National Research Council (CNR), Italy.

Gianfranco Diretto is a Research Scientist at the Italian Agency for New Technologies, Energy and Sustainable Development (ENEA), Italy.

Alessia Fiore is a Research Scientist at the Italian Agency for New Technologies, Energy and Sustainable Development (ENEA), Italy.

Valeria Castaldi is a PhD student in Biotechnology at the Department of Agricultural Sciences, University of Naples "Federico II", Italy.

Alessandra Romanelli is an Associate Professor in General and Inorganic Chemistry at the University of Milan, Italy.

Concetta Avitabile is a Researcher at the Institute of Crystallography (CNR) Bari, Italy.

Emilio Guerrieri is a Research Director at the Institute for Sustainable Plant Protection (IPSP), Italian National Research Council (CNR), Italy.

Sheridan Lois Woo is an Associate Professor in Plant Pathology at the University of Naples "Federico II", Italy.

Rosa Rao is Full Professor in Plant Genetics at the University of Naples "Federico II", Italy.

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Conference abstracts



Plant peptides as new eco-chemicals: an innovative approach to boost a healthy microbiome

Valeria Castaldi, Martina Chiara Criscuolo, Wisnu Adi Wicaksono, Gabriele Berg, Rosa Rao

Keeping up with new, sustainable approaches that might ensure the health of crop plants becomes challenging in the current context of intense environmental changes. Therefore, it is intriguing to investigate the natural stress response mechanisms found in plants while drawing inspiration to improve them. For instance, since the employment of plant-derived peptides as plant resistance inducers is becoming an established reality, it is necessary to look at their impact at multiple levels, microbiome included. In this study, a promising short peptide derived from tomato Prosystemin defense protein was further examined for its impact on tomato phyllosphere microbiome, aiming to determine whether the peptide effect on host defences involves also a shift in the microbial community. The trial considered common agricultural treatment practices, in which resistance inducer molecules are applied to plants through spray delivery every couple of weeks from the submergence of the plantlets until two months of growth. Through a shotgun metagenomics approach, here we show that the peptide treatment cause a shift in the bacterial community, reflected by a decreasing bacterial diversity as well as a specific selection of taxa. Indeed, the performed differential abundance analysis on peptide-treated plants revealed an increase in microbial genera such as *Acinetobacter*, *Sphingobium*, *Sphingomonas*, *Brevundimonas*, *Massilia*. These genera are known to help them in terms of stress responses and/or growth performance. Interestingly, such shift is further supported by bacterial networks visualization, where the control is characterized by a dense single network and the peptide has instead two ordered networks, one of which is mainly characterized by above mentioned peptide-enriched taxa. Current functional profiling focusing on nutrient uptake and microbial metabolism may provide additional insights. Overall, beyond the indirect activity against tomato insects and necrotrophic fungi, Prosystemin peptide shows additional positive properties also on a microbiome scale.

Phyllosphere microbiome, small peptides, plant defense, shotgun sequencing, crop protection

valeria.castaldi@unina.it

Department of Agricultural Sciences, University of Naples Federico II, Via Università 100,
80055, Portici, Naples, Italy

Graz University of Technology, Institute of Environmental Biotechnology, Petersgasse 12, 8010,
Graz, Austria

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P33: Phyllosphere microbiota composition of tomato plants treated with Prosystemin-derived peptide

Valeria Castaldi (1,2), Martina Chiara Criscuolo (1), Giuseppina Sequino (1), Anna Maria Aprile (1), Francesca De Filippis (1), Rosa Rao (1)

(1)Department of Agricultural Sciences, University of Naples Federico II, Via Università 100, 80055, Portici, Naples, Italy; (2)Graz University of Technology, Institute of Environmental Biotechnology, Petersgasse 12, 8010, Graz, Austria

The employment of growth or defense inducers, commonly used in agriculture to preserve crop health, may have an impact on both structure and behaviour of plant microbial population. In this context, plant phyllosphere is a flexible and challenging habitat involved in a dynamic microbial selection. Recent findings suggest that the application of protein hydrolysates or plant hormones can shape plant immunity responses, positively impacting the leaf microbiota. It is still unclear what molecular processes are responsible for the selection or the recruitment of beneficial microbes, but many efforts have to be made. In this direction, we explored the impact of an elicitor peptide derived from Prosystemin protein, notably involved in tomato defense responses against biotic stress, on the epiphytic community of tomato leaves. The phyllospheres of peptide- and mock-treated tomato plants (cv. San Marzano Nano) were analysed for their 16S rRNA communities. According to our findings, the microbiota of leaves changes after peptide treatment compared to control plants, with the main variations occurring in bacterial abundance. For instance, peptide-treated samples showed an increase in the genera *Photobacterium* and *Psychrobacter*, which are linked to features that promote plant growth or that are antagonistic to plant pathogens. The present exploratory study emphasizes the importance of using tools derived by nature, such as plant-derived peptides, to safeguard plant health on a variety of levels, including the promotion of beneficial plant-microbe interactions.



P17

Eco-friendly strategies for crop protection: exploring Prosystemin bioactive peptides

Martina Chiara CRISCUOLO^{1,3}, Valeria CASTALDI¹, Emma LANGELLA², Martina BUONANNO², Simona Maria MONTI², Rosa RAO¹

¹Department of Agricultural Sciences, University of Naples Federico II, Via Università 100, 80055, Portici, Naples, Italy.

²Institute of Biostructures and Bioimaging, National Research Council (IBB-CNR), via Pietro Castellino 111, I-80114, Naples, Italy.

³MATERIAS S.r.l., Corso Nicolangelo Protopisani, 70, 80146, Naples (Italy)

martinachiara.criscuolo@unina.it

TOPIC: Biotechnology

ABSTRACT

To counteract environmental stresses on plants, chemical pesticides are still widely used. However, they represent a serious risk for non-target organisms, human health and environment. Therefore, it is necessary to find effective eco-friendly tools that provide protection against stress agents^[1]. Plant immunity system is based on the recognition of molecular patterns (called elicitors), released during plant-pathogen interaction, that triggers defense responses. Among elicitors, tomato Systemin (Sys) activates a wide array of defense responses amplifying jasmonic acid pathway. Sys is proteolytically released, after injury, from C-terminal region of a 200 amino acids precursor, ProSystemin [ProSys]^[2]. Interestingly, ProSys devoid of Sys peptide, confers protection against biotic stressors, when delivered on tomato leaves^[3]. In order to identify the shortest regions, which confer the bioactivity, the amino acid sequence of ProSys was analysed through bioinformatic tools. This investigation showed the presence of amino acid regions with putative biological activity and regions that are likely miss it. Here we show that the precursor contains several amino acid regions harbouring biological activity able to counteract the plant colonization by the fungus *Botrytis cinerea* and also to reduce growth and vitality of *Spodoptera littoralis* larvae when delivered on tomato plant at hormonal concentration. In addition, the same regions induce the expression of defence-related genes in treated plants. Interestingly, we also identified other amino acid stretches unable to protect the plant and activate defence related genes. Thus, our data suggest ProSys may be a promising source of natural sequences useful in crop protection in an eco-sustainable perspective.

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57 - Systemin is not alone: its precursor provides new peptides with still unexplored functions

Valeria Castaldi ⁽¹⁾ - Emma Langella ⁽²⁾ - Martina Buonanno ⁽²⁾ - Ilaria Di Lelio ⁽¹⁾ - Anna Aprile ⁽¹⁾ - Martina Chiara Criscuolo ⁽¹⁾ - Simona Maria Monti ⁽²⁾ - Angela Amoresano ⁽³⁾ - Gabriella Pinto ⁽³⁾ - Anna Illiano ⁽³⁾ - Francesco Pennacchio ⁽¹⁾ - Rosa Rao ⁽¹⁾

Department of Agricultural Sciences, University of Naples "Federico II", Portici, Italy ⁽¹⁾ - National Research Council, Institute of Biostructures and Bioimaging, Naples, Italy ⁽²⁾ - Department of Chemical Sciences, University of Naples "Federico II", Naples, Italy ⁽³⁾

In the history of tomato defense responses, the focus has often been the systemin peptide (Sys) as a key component of plant immunity, activating multiple responses to a wide range of stressors. However, the mechanisms linked to such a broad protection capacity are also possibly linked to the intrinsic disorder of its precursor protein, known as Prosystemin (ProSys). Since recent findings suggest that ProSys has its own biological functions besides Sys sequence, we assumed that this precursor provides other peptide motifs with a role in defense-related pathways. Candidate peptides were identified *in silico* and synthetically produced to investigate their performance in protecting tomato plants from different biotic stressors. Our results reveal that ProSys harbours several repeats motifs that, when exogenously supplied in small doses, are able to trigger plant immune responses against pathogens and pest insects. An intriguing result comes from mass spectrometry analysis performed on plants overexpressing ProSys, validating the existence *in vivo* of three peptide motifs. These experimental data shed new light on ProSys unreported functions and provide a new chapter to be explored in the study of plant peptidome during stress events.



60 - Beyond Systemin: An in-deep screening of molecular mechanisms underpinning tomato Prosystemin

Martina Chiara Criscuolo ⁽¹⁾ - Valeria Castaldi ⁽¹⁾ - Anna Maria Aprile ⁽²⁾ - Emma Langella ⁽³⁾ - Martina Buonanno ⁽⁴⁾ - Simona Maria Monti ⁽⁵⁾ - Rosa Rao ⁽¹⁾

Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy ⁽¹⁾ - Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy ⁽²⁾ - Institute of Biostructures and Bioimaging, National Research Council, Napoli, Italy ⁽³⁾ - Institute of Biostructures and Bioimaging, National Research Council, Napoli, Italy ⁽⁴⁾ - Institute of Biostructures and Bioimaging, National Research Council, Napoli, Italy ⁽⁵⁾

As known plants constantly counteract several stresses through a very sophisticated immunity system, based on the recognition of the enemy's molecular patterns, called in general elicitors, that act as danger signals that trigger plant defenses. Among them tomato Systemin (Sys) promotes long-distance defense responses by amplifying the jasmonic acid pathway, leading to the production of defense compounds. Sys is proteolytically released from the C-terminal region of its precursor, Prosystemin (ProSys). Interestingly, ProSys is an intrinsically disordered protein and this suggest that it may interact with different molecular partners belonging to diverse biological pathways. In addition, we demonstrated that the exogenous application of ProSys devoid of Sys peptide (here referred like ProSys(1-178)) on tomato leaves, is able to confer protection against different stress agents, highlighting different functions of ProSys(1-178). To deeply understand the role of ProSys(1-178) in tomato defenses we designed ad hoc experiments in which we compared the impact on tomato plants of ProSys(1-178) and Sys peptide, when exogenously delivered. Preliminary studies, conducted through the expression analysis of genes related to different biosynthetic pathway, suggested that ProSys(1-178) may be involved in different phytohormone pathways. Our previous works and this preliminary findings highlight the multifaceted roles of ProSys(1-178) and open up new opportunities of research on its functional mechanism.



SESSION XI

GENETICS, GENOMICS AND BIOTECHNOLOGY

PS1-70 and PS1-120 peptides reduce the damages produced by *Phthorimaea absoluta*, (Lepidoptera, Gelechiidae) infestation in tomato plants

Anna Maria Aprile¹, Pasquale Cascone², Liberata Gualtieri², Valeria Castaldi¹, Martina Criscuolo¹, Michelina Ruocco², Emilio Guerrieri², Rosa Rao¹

¹ University of Naples "Federico II", Italy; ² Institute for Sustainable Plant Protection, National Research Council of Italy

The leafminer, *Phthorimaea absoluta* Meyrick (Lepidoptera: Gelechiidae) is currently considered as one of the most devastating invasive lepidopteran pest of tomato crops, in both greenhouse and outdoor production. Native to South America, this Microlepidoptera has invaded more than 113 countries outside of South America becoming a serious threat to tomato production worldwide. *P. absoluta* larvae destroy all aerial parts of plants (leaves, stems, fruits, buds, and flowers), resulting in severe yield losses. In the Mediterranean basin, the most widespread control strategy against *P. absoluta* relies on chemical insecticides. Continuous overuse of pesticides may lead to adverse effects on non-target organisms, environmental contamination and to the development of resistance in populations, which is relatively common in this species. In the present context of growing demand of sustainable agriculture practices and healthy food, plant resistance inducers acting by stimulating plant defense mechanisms, are seen as an eco-friendly and promising option to conventional pesticides. Two protein fragments biologically active against different pests, namely PS1-70 and PS1-120 have been recently identified in the N-terminal region of Prosystemin (ProSys), the protein precursor of tomato Systemin, a very well-studied defense-signaling peptide. Here, we report the ability of these fragments to confer protection against *P. absoluta*. We demonstrated that plants treated with foliar spray of PS1-70 and PS1-120, were less damaged by *P. absoluta* attack. Larvae fed with treated leaves were severely impaired in their survival rate. This phenotype was associated with the upregulation of genes associated with the octadecanoid signaling pathway that is known to play a central role in plant defense against a wide range of biotic stressors. Moreover *P. absoluta* oviposition preference was significantly reduced by PS1-70 treatment. Such behavioral observation appeared to be associated with VOC profiles of PS1-70 treated plants. Our results showed that PS1-70 and PS1-120 are effective in the stimulation of tomato immunity and the consequent protection of the plants against *P. absoluta*.

KEY WORDS: Gene expression analysis, crop protection, peptide, *Solanum lycopersicum*, biotic stress, resistance inducers, leafminer, *Phthorimaea absoluta*.

POSTER

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SESSION XI

GENETICS, GENOMICS AND BIOTECHNOLOGY

Plant immunity inducers: new peptide motifs from Prosystemin to face plant stressors

Valeria Castaldi¹, Ilaria Di Lelio¹, Emma Langella², Martina Buonanno², Andrea Becchimanzi¹, Simona Maria Monti², Francesco Pennacchio¹, Rosa Rao¹

¹ University of Naples "Federico II", Italy; ² Institute of Biostructures and Bioimaging, National Research Council, Italy

Chemical pesticides represent a pivotal tool in agriculture to control pest agents and secure both quality and yield in plant production. Nevertheless, their extensive use in crop protection led to a widespread toxicity that involved also non-target organisms as well as the surrounding environment, endangering ecosystems sustainability. Therefore, the increasing demand for food products low or free in pesticide residues, spurred the scientific community to develop more effective, sustainable, and eco-friendly solutions for pest control. Promising biotechnological strategies include molecules that act as plant immunity inducers, known as elicitors. Among them, tomato Systemin (Sys) has often been the focus of attention as a key component of plant resistance phenotype towards a wide range of stressors. This 18-amino acids peptide is released upon wounding from the C-terminal end of its 200 amino acids precursor called Prosystemin (ProSys), activating an array of local and systemic defenses. However, ProSys is not only a mere scaffold for the Sys peptide, but itself contains other biologically active regions. In addition, the defence mechanism underpinned by the protein is likely linked to the intrinsic disorder of its sequence that may have its own biological function beyond the release of Sys. Indeed, our recent findings suggests that ProSys hides other functional peptide motifs able to activate multiple stress-related pathways. To contribute to this knowledge, we identified and produced different synthetic peptides derived from ProSys precursor that when exogenously supplied to tomato plant, induce defense-related genes protecting plants against necrotrophic fungal pathogens such as *Botrytis cinerea* and chewing insects like *Spodoptera littoralis* larvae. Larvae fed on treated leaves are unable to reach the required weight to moult, dying either during moulting or shortly afterwards. It is intriguing to notice that dying larvae exhibit a strange "albino" phenotype, remaining contracted and trapped in the old cuticle and lacking sclerotization of the new cuticle. Since peptides act only on plant defenses rather than directly on larvae, the observed unusual phenotype is possibly linked to the induction of genes that promote the production of substances with insect hormone-like activity that impact on larvae development. Overall, our data open new perspectives on ProSys functionality, since it contains multiple biologically active sequences that may be novel exploitable tools in crop protection programs.

KEY WORDS: small peptides, sustainable agriculture, biotic stress, plant elicitors, *Spodoptera littoralis*.

POSTER

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Poster Communication Abstract – 3.06

PROSYSTEMIN PEPTIDES AS NOVEL TOOLS FOR SUSTAINABLE TOMATO CROP PROTECTION

CASTALDI V.*, MOLISSO D.*, LANGELLA E.***, BUONANNO M.***, DI LELIO I.*, APRILE A. M.*, CRISCUOLO M.***, MONTI S. M.**, PENNACCHIO F.*, RAO R.*

*) Department of Agricultural Sciences, University of Naples "Federico II", Portici (Italy)

**) Institute of Biostructures and Bioimaging, National Research Council, Naples (Italy)

***) MATERIAS S.r.l., Naples (Italy)

small peptides, resistance inducers, pest agents, crop protection, eco-friendly tools

Chemical pesticides represent a pivotal tool in agriculture to control pest agents and secure both quality and yield in plant production. Nevertheless, their extensive use in crop protection led to a widespread toxicity that involved also non-target organisms as well as the surrounding environment, endangering ecosystems sustainability. Therefore, the increasing demand for food products low or free in pesticide residues, spurred the scientific community to develop more effective, sustainable, and eco-friendly solutions for pest control.

Promising biotechnological strategies include molecules that act as plant immunity inducers, known as elicitors. Among them, tomato Systemin (Sys) was traditionally considered as the principal actor of plant resistance phenotype, triggering multiple defense pathways in response to a wide range of biotic/abiotic stressors. This 18-amino acids peptide is released upon wounding from the C-terminal end of its 200 amino acids precursor called Prosystemin (ProSys), activating an array of local and systemic defenses. The mechanisms that underpin such a broad protection capacity are possibly linked to the intrinsic disorder of ProSys sequence that promotes the binding to different molecular partners. However, since our recent findings suggests that ProSys is likely more than a simple precursor of Sys peptide, we supposed that it contains other peptide sequences able to activate

multiple stress-related pathways. To contribute to this knowledge, both bioinformatic and gene-expression analysis were carried out. We identified and produced different synthetic peptides which are derived from ProSys precursor. We demonstrated that these peptides, among which none of them included Sys, when exogenously supplied can induce defense-related genes protecting tomato plants against *Spodoptera littoralis* larvae and towards necrotrophic fungal pathogens such as *Botrytis cinerea* and *Alternaria alternata*. Our data give a significant contribution to the understanding of the functional mechanism of ProSys, demonstrating that is not only a simple scaffold of Sys, but it contains biologically active sequences that may be novel exploitable tools for crop protection.

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Poster Communication Abstract – 5.10

NOVEL PROTEIN FRAGMENTS FOR TOMATO RESILIENCE TO SALT STRESS

MAGLIULO R.*, CIRILLO V.*, MOLISSO D.*, MONTI S. M.***, LANGELLA E.**,
BUONANNO M.***, APRILE A. M.*, CRISCUOLO M. C.***, CASTALDI V.*, MAGGIO A.*,
RAO R.*

*) Department of Agricultural Sciences, University of Naples Federico II,
Portici (Italy)

**) Institute of Biostructures and Bioimaging, National Research Council,
Naples (Italy)

***) MATERIAS S.r.l., Naples (Italy)

*protein fragments, plant resistance, salt stress, biostimulants, eco-
friendly tools*

Environmental stresses are critical constraints to crop production. Novel tools, that may improve plant resilience to biotic and abiotic stressors, need to be developed to cope with a growing food demand due to an increasing world population. Understanding and exploiting intrinsic mechanisms of tolerance to multiple stresses in plants is the new frontier, since these often occur simultaneously in natural and agricultural systems. We recently identified two fragments, named PS1 and PS3, in the scaffold of Prosystemin (Prosys), the protein precursor of tomato systemin, a very well-known defense-signaling peptide that efficiently protect tomato plants against *Botrytis cinerea* and *Spodoptera littoralis* larvae by inducing defense-related genes. Both fragments belong to the N-terminal region of the scaffold, lacking of systemin sequence. Since it was previously demonstrated that Prosys protects tomato plants also against soil salinity, we analyzed the ability of PS1 and PS3 to confer salt tolerance. We demonstrated that plants treated with exogenous foliar spray of PS1 and PS3, followed by salt stress, were less affected in terms of plant biomass and root area compared to control plants. Treated plants showed a comparable stomatal density to plants non-irrigated with saltwater. Noteworthy, we observed an unexpected growth improvement effect on PS1/PS3-treated tomato plants in the absence of salt stress, making us hypothesize a further activity of these protein fragments as biostimulants and not only

as stress protectant. Finally, a gene expression analysis revealed the upregulation of salt stress-related genes such as CAT2 and APX2 in all treated plants, showing the activation of antioxidant and scavenging responses in stressed plant cells. Overall, these results lay the foundations for further investigations on the role of PS1 and PS3 in salt stress tolerance and growth enhancement. On a practical aspect, the two protein fragments may be exploited in plant protection strategies against multiple stresses.