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Intrinsically disordered Prosystemin discloses biologically active repeat motifs

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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

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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 (ProSys$_{1–178}$) 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/H_2O 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. Synthetized 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 MassHunterv.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-DDMQEEPKVKLHHEKGGDEKEKII 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-DDMQEEPKVKLHHEKG-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 (Rappsilber 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 acquirement. Each sample was diluted in ultrapure water in a final volume of 200 uL in a Hellma quartz cell with 1 mm light path (D'agostino et al., 2019). The final concentration reached for the experiments was 6.4 uM, 125 uM, and up to 367 uM 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) (deg cm^2 $dmol^{-1}$) 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:

$$\%helix = 100\frac{[\theta]_{obs\lambda}}{[\theta]_{H\lambda}}$$

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

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

where $[\theta]_{H\lambda\infty}$ is the maximum mean residue ellipticity for a helix of infinite length (−39500 for λ = 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 λ = 222 nm) and N is the number of residues. $f_H$is so defined:

$$f_H = i\overline{n}/N$$

where N is the total number of residues and $\overline{n}$ 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 ± 1°C and 60 ± 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 ± 1°C and 60 ± 5% RH, including 18:6 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. Délano-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-1α 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 ± 1 °C, 70 ± 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% ± 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 10^4 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^{-1}, 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. 1**B**). 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 synthetized, 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. 2**A,B** and Supplemental Fig. S3A). The propensity of the RMs to fold at increasing concentrations of TFE was also evaluated (Fig. 2**C, 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. 2**C**). 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. 2**D**), 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. 2**C**). 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. 3**A**). 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. 3**B** 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. 4**A**, RM-treated plants displayed a significant reduction of lesions

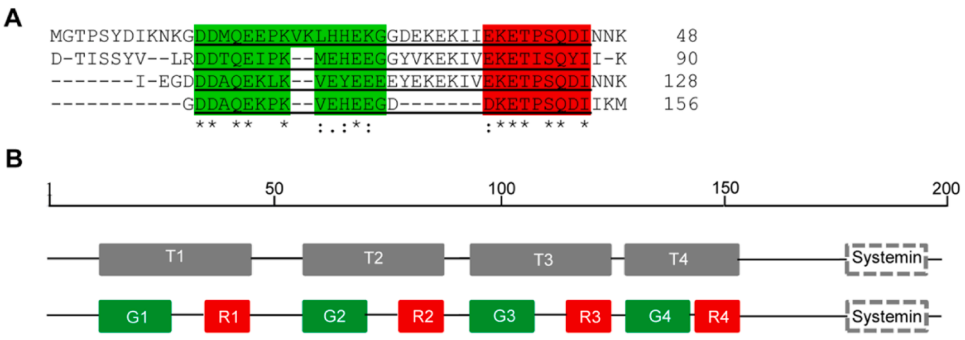

Fig. 1. Prosys contains repeat motifs. **(A)** Sequence alignment by T-Coffee. T1–4 sequences are underlined, G1–4 and R1–4 motifs are in green and red, respectively. **(B)** Schematic in-scale representation of repeat motifs disposition along ProSys sequence (200 AA long). Repeat motifs are shown as coloured rectangles. Systemin sequence is also reported and displayed as a dashed line rectangle.

Table 1
Primary sequences and physicochemical features of the repeat motifs (RMs) identified in ProSys.

RMs	Sequence	Hydroph.* (%)	polar (%)	charged (%)	pol+char* * (%)	Net charge	Length (AA)
T1	DDMQEEPKVKLHHEKGGDEKEKIIEKETPSQDI	21,21	27,27	51,52	78,79	-5	33
T2	DDTQEIPKMEHEEGGYVKEKIVEKETISQYI	22,58	35,48	41,94	77,42	-5	31
T3	DDAQEKLKVEYEEEEYEKEKIVEKETPSQDI	22,58	19,35	58,06	77,42	-8	31
T4	DDAQEKPKVEHEEGDDKETPSQDI	20,83	25,00	54,17	79,17	-7	24
G1	DDMQEEPKVKLHHEKG	18,75	31,25	50,00	81,25	-2	16
G2	DDTQEIPKMEHEEG	14,29	35,71	50,00	85,71	-5	14
G3	DDAQEKLKVEYEEE	21,43	14,29	64,29	78,57	-5	14
G4	DDAQEKPKVEHEEG	21,43	21,43	57,14	78,57	-4	14
R1	EKETPSQDI	22,22	33,33	44,44	77,78	-2	9
R2	EKETISQYI	22,22	44,44	33,33	77,78	-1	9
R3	EKETPSQDI	22,22	33,33	44,44	77,78	-2	9
R4	DKETPSQDI	22,22	33,33	44,44	77,78	-2	9

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

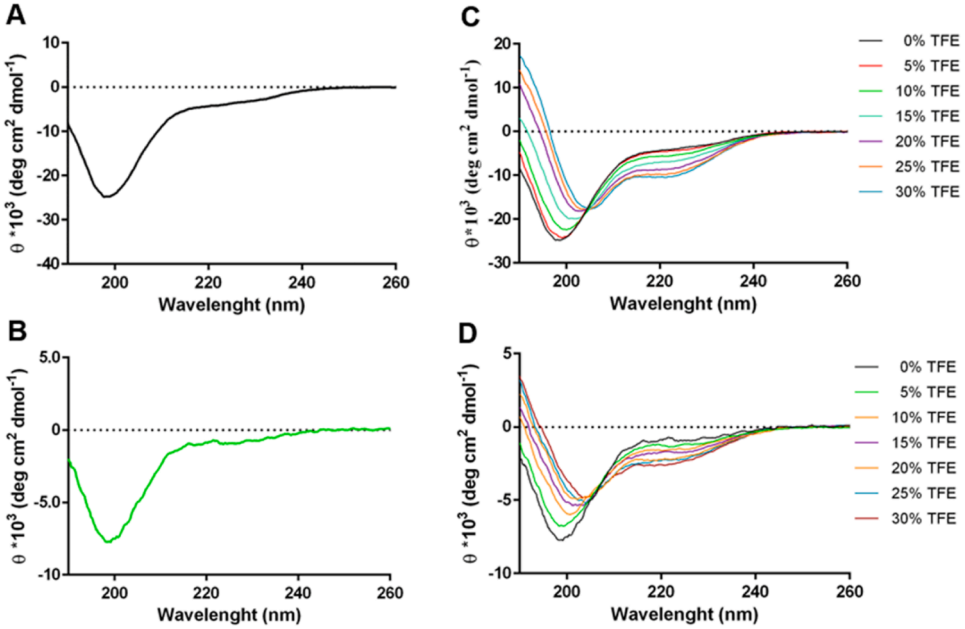

Fig. 2. Repeat motifs show a variable degree of plasticity. CD spectra of (**A**) T1 and (**B**) G1 synthetic peptides. Overlapping CD spectra of **(C)** T1 and **(D)** G1 peptides recorded at increasing concentrations of TFE (0%, 5%, 10%, 15%, 20%, 25%, and 30%). Spectra were acquired at 20 °C in water.

caused by the necrotrophic fungus (One Way ANOVA: $P < 0.0001$ and Supplemental Table S2). G1 peptide motif seems to be the most effective in the last two measurements, at 5- and 8-days post-inoculum (dpi). Furthermore, the bioassay using *A. alternata* (Fig. 4**B**) reveals that all RMs reduced fungal growth since 24 hpt lasting throughout the bioassay (One Way ANOVA: $P < 0.0001$ and Supplemental Table S3). Nevertheless, there are no appreciable differences among all assayed peptides.

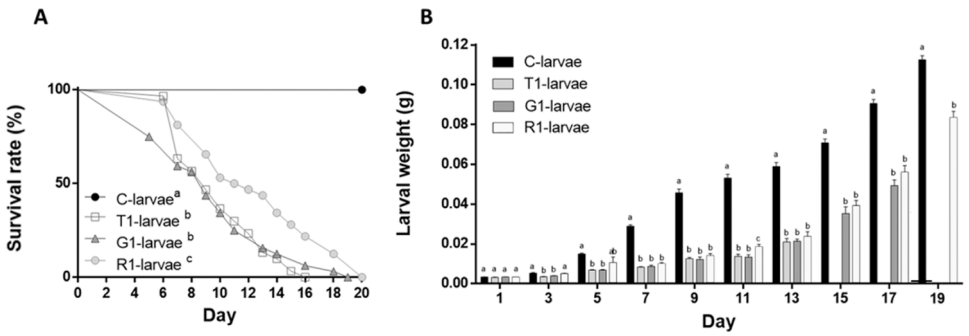

Fig. 3. Plants treated with repeat motifs disrupt insect growth and survival. Effects on survival and development of S. littoralis larvae fed on leaves treated with 100 fM of RMs or mock treatment. (**A**) Survival rate of larvae reared on tomato leaves obtained from plants treated with 100 fM T1, G1 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 ± SE. Punctual statistics is reported in the Supplemental Table S1.

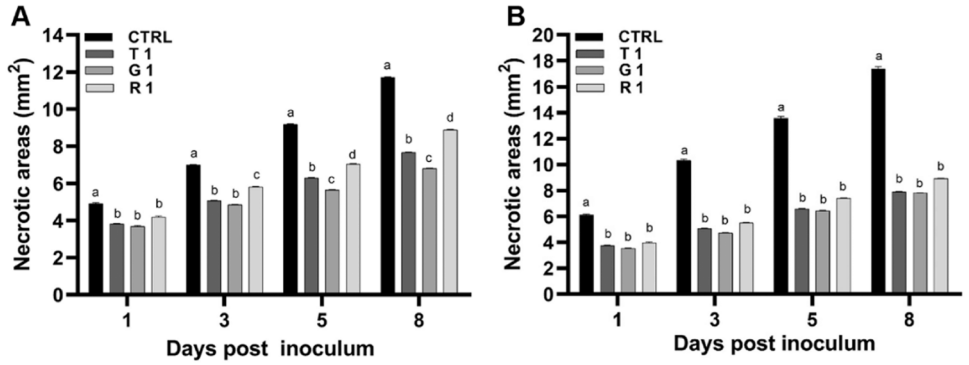

Fig. 4. Plants treated with repeat motifs show reduced fungal infection. B. cinerea (**A**) and A. alternata (**B**) infection progress after RMs or mock treatments. Bars represent means of necrotic areas measured 1, 3, 5, 8 days post-inoculum (dpi). Statistically significant differences are indicated 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).

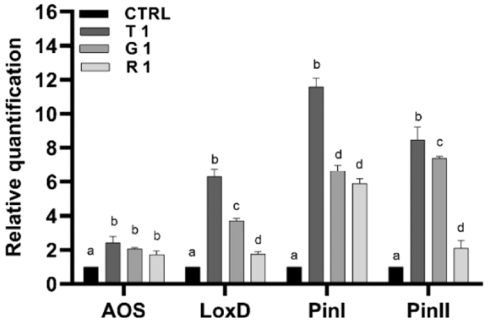

Fig. 5. Repeat motifs induce defence related genes. qPCR expression analysis of defense-related genes at 6 h after treatment with 100 fM RMs. Numbers are displayed in relation to the calibrator control condition, which is set to 1 (mock-treated plants). Different statistical groups are denoted by different 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 *m/z* → 827.4 *m/z*, 537.6 *m/z* → 600.2 *m/z*, and 537.6 *m/z* → 471.2 *m/z*) showed peaks at 16.0 min of retention time suggesting that the revealed peptide displayed the putative amino acid sequence, i.e. DDAQEKPKVEHEEG (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

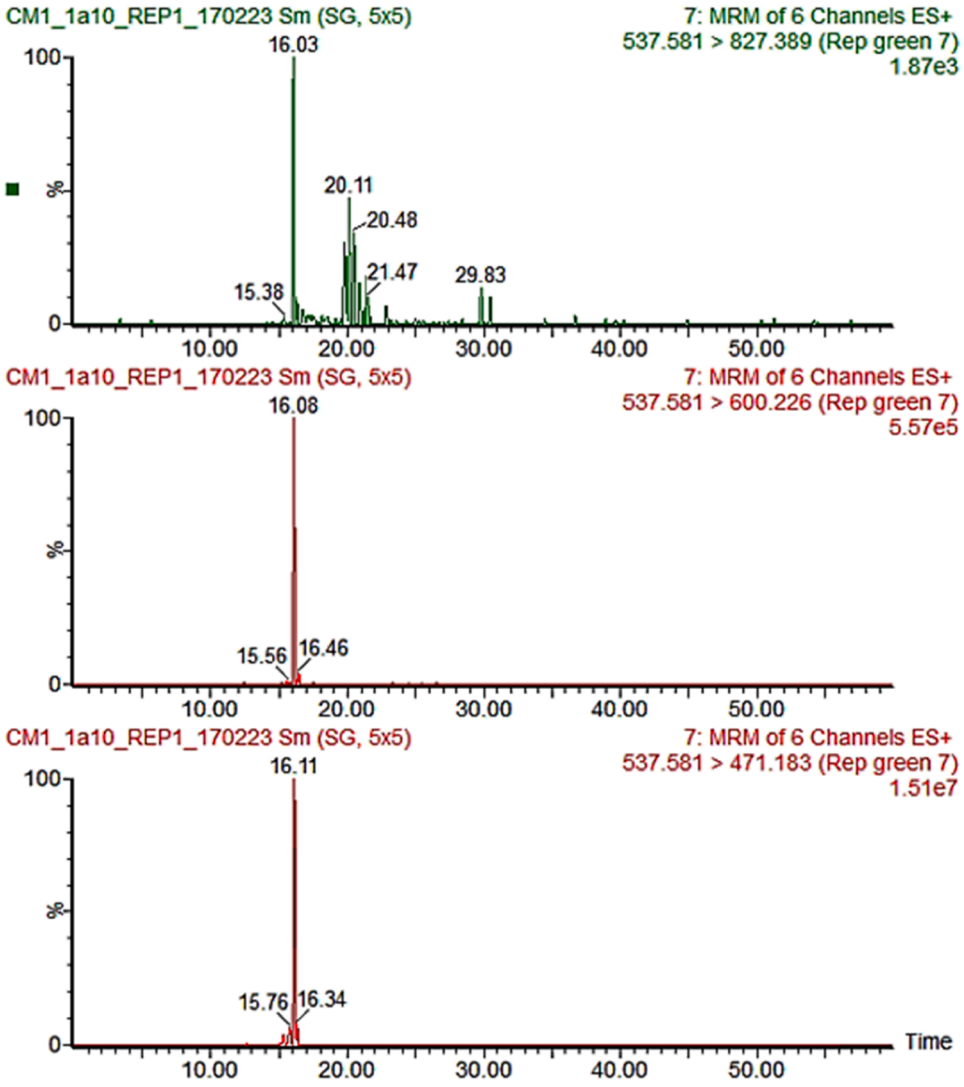

Fig. 6. In vivo detection of repeat motifs. Extracted ion chromatograms of three transitions (537.6 *m/z* → 827.4 *m/z*, 537.6 *m/z* → 600.2 *m/z*, and 537.6 *m/z* → 471.2 *m/z*) describing the fragmentation of DDAQEKPKVEHEEG peptide (G4).

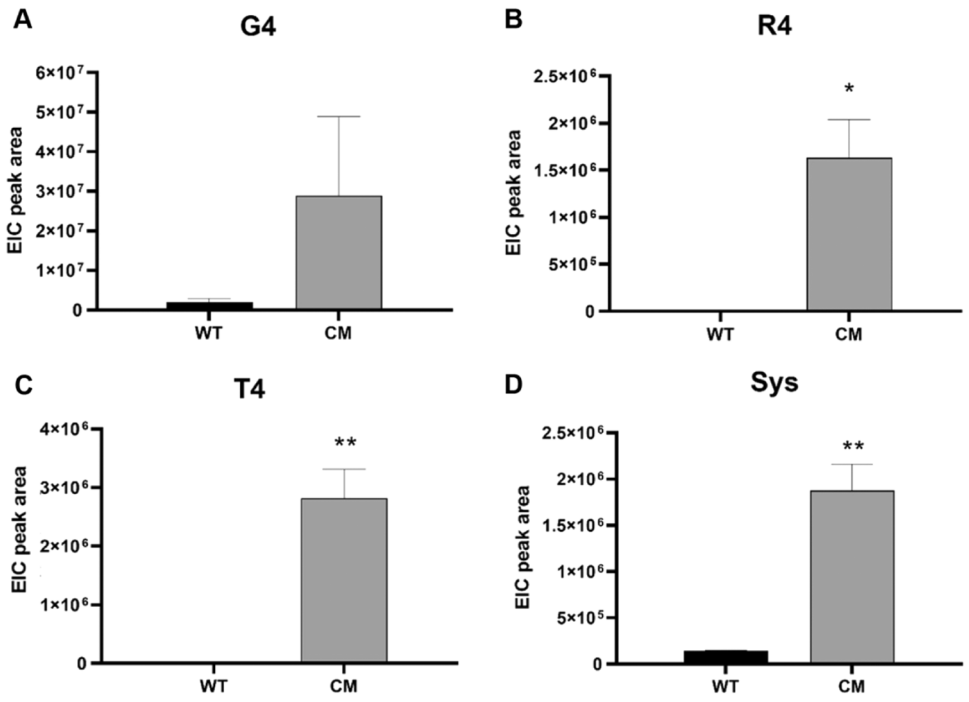

Fig. 7. Relative comparison of peak areas of repeat motifs. Relative comparison of peak areas of extracted ion chromatograms (EIC) detected for each detected peptide, i.e., G4 (panel A), R4 (panel B), T4 (panel C), Sys (panel D), between the wild-type (WT) and transgenic CM plants.

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. 1**B**). 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; Méndez-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), Cyclotides (Daly et al., 2009; Craik et al., 2010; Craik and Malik, 2013), and *Impatiens balsamina* antimicrobial peptides (Ib-AMPs) (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 prohormone 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 (Stührwohldt 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. **Criscuolo 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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