FUNCTIONAL CHARACTERIZATION OF TOMATO PROSYSTEMIN AND PROSYSTEMIN REGIONS: NOVEL TOOLS FOR PLANT DEFENSE

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Alla mia splendida famiglia

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RIASSUNTO

Le piante sono costantemente esposte ad una vasta gamma di agenti sfavorevoli che ne possono influenzare e compromettere lo sviluppo e la sopravvivenza. Una considerevole parte della produzione agricola mondiale, infatti, è completamente distrutta o resa inutilizzabile dall'azione di parassiti, con una stima di una perdita complessiva delle principali *crops* che va dal 26 al 40% (Oerke, 2006).
La *crop protection* è una tematica da sempre considerata di grande interesse per l'agricoltura: la salvaguardia dei raccolti da agenti di danno è un nodo cruciale dello sviluppo della società per garantire una produzione che sia sufficiente sia in termini quantitativi sia qualitativi (Oerke e Dehne, 2004).
Negli anni numerosi studi si sono occupati di questa tematica proponendo vari strategie di controllo che hanno seguito nel tempo lo sviluppo dell'agricoltura (Ha, 2014). E' stato spesso utilizzato un approccio mono-disciplinare, proponendo soluzioni per lo sviluppo o l'adozione di sistemi a singolo componente come nuove varietà vegetali e, soprattutto, l'uso di specifici agrofarmaci (Schut *et al.,* 2014). Ciò ha comportato un ampio e a volte eccessivo uso di pesticidi e chimici.
L'uso massiccio di tali sostanze ha determinato molteplici effetti negativi quali l'insorgenza di resistenza nelle popolazioni target, effetti tossici negli organismi non-target (uccelli, pesci, insetti benefici e piante) oltre che problemi per la salute dell'uomo e per l'ambiente (Aktar, 2009). I problemi di salute nell'uomo connessi con i pesticidi posso essere di diversa natura (effetti indesiderati a livello dermatologico, gastrointestinale, neurologico, cancerogeno, respiratorio, riproduttivo e endocrino) e manifestarsi in maniera acuta o cronica (Nicolopoulou-Stamati, 2016); quelli per l'ambiente includono la diminuzione della biodiversità, la riduzione della fertilità del terreno ed accelerazione del fenomeno di erosione dei suoli.
Tutto questo ha portato ad una consapevolezza sempre maggiore della necessità di adottare strategie di controllo alternative all'uso esclusivo di agrochimici come protocolli di controllo biologico (o biocontrollo) e controllo integrato (*Integrated Pest Managagement*, IPM).
Il controllo biologico consiste nell'impiego di qualsiasi entità biologica vivente quali batteri, funghi, insetti predatori o parassiti, o di composti da essa derivati, allo scopo di contenere i danni causati alla pianta da parte di un parassita e/o patogeno (Pal e Gardener, 2006).
L'impiego esclusivo di tale strategia non può essere però considerato come unica alternativa al controllo chimico in quanto la sua efficienza è limitata da fattori temporali (monitoraggi continui) e necessita una conoscenza approfondita dei meccanismi che sono alla base del comportamento dei nemici naturali, conoscenza spesso non disponibile.
Più recentemente si sono sviluppate strategie di lotta integrata che prevedono l'utilizzo combinato di vari mezzi di controllo disponibili (chimici, biologici, genetici ecc.) per ottimizzare il contrasto degli agenti infestanti considerando a pieno le esigenze economiche, ambientali e sociali (Galea, 2010).
Il fine ultimo di questo metodo non è quello di debellare l'agente infestante ma di mantenere tali popolazioni al di sotto della soglia di danno economico.
L'utilizzo di molecole di origine naturale con attività biopesticida è uno strumento fondamentale per l'attuazione di buone pratiche agricole e per la salvaguardia della salute dell'uomo e dell'ambiente. A tale proposito l'identificazione di nuovi composti naturali utili per la protezione delle colture ed in grado di promuovere la riduzione

dell'uso di pesticidi chimici, è un obbiettivo di rilevante interesse per le biotecnologie vegetali applicate alla difesa delle piante agrarie.
Nelle *Solanaceae* le sistemine sono una famiglia di peptidi ad azione ormonale nota per essere coinvolta nell'attivazione dei geni di difesa in risposta alla ferita indotta da insetti masticatori o da danno meccanico (Ryan and Pearce, 2003).
In pomodoro la sistemina (Sys) è stata identificata come un segnale primario per l'attivazione dei geni di difesa (Pearce *et al.,* 1991). Si tratta di un ormone peptidico di 18 amminoacidi localizzato all'estremità della regione C-terminale di un precursore di 200 amminoacidi chiamato Prosistemina (ProSys).
Mediante meccanismi ancora poco noti, in seguito a ferita, il precursore è sottoposto ad una azione proteolitica mediata probabilmente da una fitaspasi, una proteasi aspartato-specifica della famiglia delle subtiliasi (Beloshistov *et al.,* 2018), che consente il rilascio della Sys. Questa viene rilasciata nell'apoplasto dove, attraverso l'interazione con recettori di membrana, attiva i segnali di difesa (Narvàez-Vàsquez e Orozco-Càrdenas, 2008).
Il genoma di pomodoro presenta una sola copia del gene della *ProSys*; esso è caratterizzato da una regione codificante di 4176 coppie di basi ripartita in 11 esoni, di cui l'ultimo codifica per la Sys.
In condizioni fisiologiche è noto che il gene della *ProSys* è espresso a livelli fentomolari nelle foglie, nei petali e nei fusti delle piante, ma non nelle radici (Pearce *et al.,* 1991; Narváez-Vásquez e Ryan, 2004). Viceversa, in caso di danno meccanico o ferita da parte di attacco di insetti masticatori, lo stesso risulta avere un'espressione incrementata.
Il ruolo del precursore/Sys nella difesa del pomodoro è stato ampiamente documentato attraverso lo studio di piante transgeniche sovraesprimenti il gene *ProSys* oppure silenziate per lo stesso gene. In particolare, la sovraespressione della ProSys ha dimostrato un aumento nella sintesi di proteine inibitrici di proteasi, sia di tipo I che II, e, quindi, una maggiore resistenza agli insetti (McGurl *et al.,* 1994); al contrario, la sottoespressione del gene determina la soppressione quasi completa della produzione degli inibitori di proteasi in seguito a ferita (McGurl *et al.,* 1992), che è stata associata ad una maggiore suscettibilità della pianta nei confronti di larve di *Manduca sexta* (Orozco-Cardenas *et al.,* 1993). Studi recenti hanno confermato che piante sovraesprimenti il gene della *ProSys* sono in grado di difendersi da numerosi stress biotici, attivando una vasta gamma di segnali difensivi (Coppola *et al.,* 2015): sono infatti capaci di rilasciare una maggiore quantità di composti volatili risultando più attrattive verso i parassitoidi (*Aphidius ervi*, Corrado *et al.,* 2007), maggiormente resistenti all'attacco di funghi necrotrofi (El Oirdi *et al.,* 2011; Coppola *et al.,* 2015) e di afidi (Coppola *et al.,* 2015), e allo stesso tempo tolleranti a condizioni di stress salino (Orsini *et al.,* 2010).
Tradizionalmente la funzione biologica di tale prodotto genico è sempre stata attribuita al peptide Sys, tuttavia, recenti dati di letteratura hanno evidenziato il ruolo del precursore privo della Sys nell'attivazione di geni di difesa in piante di tabacco. Corrado e collaboratori (2016) hanno infatti dimostrato che, trasformando piante di tabacco (endogenamente prive della ProSys) con il precursore privo della regione codificante per la Sys, la ProSys deleta viene sintetizzata dalla pianta e porta all'attivazione di una serie di geni associati alla difesa e maggiore tolleranza nei confronti del fungo *Botrytis cinerea*.
Ad oggi scarse e riduttive sono le conoscenze relative alla struttura di tale precursore. Pertanto, la comprensione delle caratteristiche biochimiche e strutturali della ProSys rappresentano un valido strumento per lo sviluppo di tecnologie a basso

impatto ambientale che prevedono l'utilizzo di molecole di origine naturali per la protezione delle colture.
Allo scopo di caratterizzare la ProSys e studiarne i meccanismi di attivazione e regolazione, il precursore è stato prodotto in forma di proteina ricombinante in *E. coli*.
Durante il lavoro di tesi sono state effettuate prove di clonaggio, di ottimizzazione dell'espressione e purificazione della proteina, al fine di ottenerne una quantità sufficiente da utilizzare, in forma nativa, per gli studi biochimici.
L'intera proteina è stata ottenuta in forma solubile e senza necessità di introdurre mutazioni all'interno della sua sequenza nucleotidica come precedentemente riportato (Délano *et al.,* 1999). Inoltre, l'approccio utilizzato per la purificazione, che ha previsto l'uso combinato di tre tecniche cromatografiche ad alta prestazione (cromatografia di affinità, cromatografia a scambio anionico e cromatografia a esclusione molecolare), ha consentito di ottenere una proteina caratterizzata da un elevato grado di purezza con una resa finale, maggiore di 4 mg/l. Queste rese hanno consentito un'ampia caratterizzazione biofisica della proteina ricombinante.
Le successive analisi bioinformatiche condotte sulla sequenza della proteina, insieme con i risultati spettroscopici ottenuti, hanno mostrato che la ProSys è una Proteina Intrinsecamente Disordinata (IDP).
Le IDPs sono proteine che, pur non avendo una definita struttura tridimensionale, presentano attività biologica. Una particolare caratteristica delle IDP è la loro capacità di interagire, con alta specificità e bassa affinità, con diversi target molecolari. Tali proteine si diversificano dalle proteine ordinate già a partire dalla loro sequenza primaria. Infatti, la loro peculiarità risiede nella predominanza di residui che promuovono il disordine, quali amminoacidi polari come Arg, Gly, Gln, Ser, Glu, e Lys e amminoacidi idrofobici come Pro e Ala, che ne definiscono l'abilità nel rimanere non strutturate.
Gli esperimenti volti a caratterizzare la proteina dal punto di vista spettroscopico hanno confermato la presenza di una struttura secondaria di tipo *random coil*, e hanno evidenziato la sua tendenza ad assumere una parziale struttura α-elicoidale in seguito all'aggiunta di trifluoroetanolo (TFE); un cosolvente che permette di evidenziare la propensione strutturale mascherata di peptidi/proteine mimando l'ambiente idrofobico che si realizza durante le interazioni proteina-proteina.
Le analisi spettroscopiche hanno poi evidenziato che incrementi di temperatura inducono nella proteina una trasformazione strutturale reversibile, che porta presumibilmente alla transizione da una conformazione altamente disordinata "*coil-like*" ad una conformazione parzialmente ripiegata "*pre molten globule-like*" (Uversky *et al.,* 1999). L'effetto peculiare è probabilmente dovuto alla maggiore forza delle interazioni idrofobiche che avvengono a temperature elevate e che fungono da forza motrice per il *folding* idrofobico.
A ulteriore supporto dei risultati ottenuti è stata effettuata un'analisi di *Light Scattering*. Le caratteristiche idrodinamiche emerse da tale analisi hanno confermato la natura disordinata della ProSys, evidenziando che la proteina ricombinante ottenuta è una specie monomerica altamente monodispersa con un raggio idrodinamico di 5.6 nm, indicativo di una proteina con una scarsa compattezza.
In considerazione del fatto che le interazioni delle proteine intrinsecamente disordinate con diversi interattori molecolari sono mediante dalle regioni disordinate (Dyson and Wright, 2005), i risultati ottenuti suggeriscono che ProSys possa modulare le risposte della pianta a molteplici agenti di stress in conseguenza del suo disordine intrinseco.

In seguito alla caratterizzazione biochimica e strutturale, l'attività biologica della proteina ricombinante è stata valutata attraverso l'analisi di espressione di geni precoci e tardivi coinvolti nella risposta a danno meccanico e/o *wounding* in seguito alla sua applicazione, mediante *spot* multipli, su foglie di pomodoro.
L'applicazione della proteina ricombinante su foglie ha evidenziato l'effetto positivo della stessa nell'attivazione dei geni coinvolti nella risposta di difesa attraverso i meccanismi molecolari già noti per la ProSys endogena.
Poichè le piante transgeniche esprimenti in maniera costitutiva la ProSys risultano inoltre fortemente resistenti contro insetti e contro funghi necrotrofi (McGurl *et al.,* 1994, Coppola *et al.,* 2015; El Oirdi *et al.,* 2011), si è voluto verificare che anche la proteina ricombinante applicata esogenamente riuscisse a conferire analoga protezione.
Un importante risultato è stato ottenuto dall'osservazione di piante trattate con la proteina ricombinante, le quali hanno mostrato una maggiore resistenza nei confronti del fungo *Botrytis cinerea* rispetto al controllo, sia in termini di dimensioni sia di sviluppo delle aree necrotiche. Inoltre, è stato registrato un ridotto peso e una precoce mortalità delle larve di *Spodoptera littoralis* alimentate con foglie trattate con ProSys e foglie distali a quelle trattate, rispetto a quelle controllo.
Ulteriori esperimenti sono stati eseguiti con lo scopo di investigare eventuali relazioni tra le molteplici regioni disordinate del precursore e la possibile presenza di attività biologica. A tal fine, quattro diverse regioni del precursore, i Frammenti I e III che ricoprono la regione N-terminale della proteina, e i Frammenti II e IV che ricoprono la porzione C-terminale (e quindi contenenti la regione della Sys), sono state clonate, espresse in *E. coli* e purificate mediante tecniche cromatografiche ad alta prestazione (cromatografia di affinità e anionico e cromatografia a esclusione molecolare) ottenendo buone rese (~1-2 mg/l).
Così come osservato per il precursore, tutti i frammenti hanno mostrato sia dall'analisi bioinformatica della sequenza amminoacidica che dai risultati delle diverse analisi spettroscopiche le tipiche caratteristiche biofisiche di disordine intrinseco.
Inoltre, le analisi di *Light Scattering* hanno dimostrato che i 4 frammenti ricombinanti sono monomerici ed hanno tutti una scarsa compattezza; è stato poi confermato che le proteine presentano una struttura prevalentemente di tipo *random coil* con un leggero incremento del contenuto di struttura secondaria in seguito all'incremento di temperatura.
Ulteriori esperimenti volti a caratterizzare le proteine dal punto di vista spettroscopico hanno inoltre evidenziato che i Frammenti II e III hanno la tendenza ad assumere una struttura ad α-elica in seguito all'aggiunta di TFE, mentre il Frammento I conserva in queste condizioni la sua struttura disordinata. Tali osservazioni rafforzano le predizioni di struttura relativa alla ProSys *full-lenght*, per la quale erano state predette due regioni che presentano elementi di struttura: la prima nella porzione centrale (dal residuo amminoacidico 75 al 110), e una seconda nella regione C-terminale (dal residuo amminoacidico 160 al 180).
È stata valutata la capacità dei singoli frammenti di indurre l'espressione dei geni coinvolti nella difesa mediante l'applicazione di *spot* multipli su foglie intatte di pomodoro. Tale analisi ha evidenziato che tutti i quattro frammenti ricombinati sono in grado di indurre l'espressione di geni coinvolti nella risposta di difesa. Ciascun frammento ha però mostrato un'attività differente sui geni studiati, e, in particolare, i Frammenti I e III hanno determinato una maggiore sovraespressione, e quindi una più efficace risposta da parte della pianta, rispetto agli altri.

Proprio tali frammenti sono stati selezionati per condurre saggi biologici volti alla valutazione della loro attività contro agenti di danno. Tali proteine ricombinanti sono state applicate sulle foglie intatte, ed è stata osservata una maggiore resistenza della pianta allo sviluppo del fungo *B. cinerea* e una riduzione dello sviluppo e della sopravvivenza di larve di *S. littoralis* alimentate con foglie trattate.
Questo nuovo risultato indica che la ProSys potrebbe non esaurire la sua attività biologica tramite il solo rilascio di Sys, ma contribuire all'attivazione delle difese endogene anche attraverso altre sue regioni.
I peptidi endogeni della pianta che stimolano le risposte di difesa contro gli invasori rappresentano un approccio molto sicuro alla protezione delle piante, a causa della bassa o nulla tossicità di queste molecole su esseri umani e organismi non bersaglio. Qui abbiamo identificato due nuovi peptidi, la cui somministrazione diretta alle foglie di pomodoro protegge efficacemente la pianta da due importanti agenti di stress biotico. In una prospettiva applicativa, sono molto promettenti rappresentando uno strumento biotecnologico sfruttabile per le strategie IPM. Come descritto nel Capitolo 1 in diversi lignaggi evolutivi, i peptidi si sono evoluti come segnali di difesa coinvolti nell'orchestrazione finemente sintonizzata dell'espressione genica alla base delle risposte immunitarie della pianta. Lo sviluppo di strategie di controllo degli stress biotici che implicano la loro applicazione diretta alle piante rappresenta uno strumento molto potente per l'agricoltura sostenibile, riducendo al minimo l'uso di input chimici fornendo al contempo qualità e sicurezza alimentare.

Summary

Prosystemin (ProSys) is a pro-hormone of 200 aminoacidic residues which releases a bioactive peptide hormone of 18 amino acids called Systemin (Sys) involved in the activation of a complex signaling cascade that leads to the production of defense compounds. The tomato genome contains only one copy of *Prosys* gene; it is composed of 4176 bp and is structured into 11 exons, of which the last one codes for Sys. Sys peptide was traditionally considered as the principal actor that confers protection against both biotic and abiotic environmental challenges observed in tomato plant overexpressing the *ProSys*. Thus, a single peptide hormone is capable of eliciting multiple defense pathways to counteract a wide range of unfavourable conditions for the plant. So far, it was unknown whether ProSys had any biological function other than being an intermediate in the synthesis of Sys. However, recent evidences suggest that Prosys devoid of the Sys sequence contributes to defense responses. This observation prompted us to investigate the biochemical and structural features of the ProSys protein. To this purpose ProSys has been expressed in BL21 (DE3) *E. coli* cells and purified. A detailed characterization of this pro-hormone by means of multidisciplinary approach revealed for the first time that this precursor behaves like an intrinsically disordered protein (IDP) possessing intrinsically disordered regions (IDRs) within the sequence. However, to find out an alternative delivery strategy not relying on transgenic plants, we decided to investigate the effects of exogenous application of the recombinant pro-hormone on the defense responses and its potential use as a plant protection tool in tomato. In particular, plant assays revealed that ProSys direct treatment of leaves is biologically active being very effective in the induction, both locally and systemically, of tomato defense-related genes, conferring protection against different pests.
To our knowledge, this is the first biotic stress related IDP identified in plants, suggesting new interesting insights on the role of IDPs. into plant response against biotic stressors.
IDPs are functionally important proteins lacking a stable or ordered three-dimensional structure. Despite being highly flexible, it has been demonstrated that IDPs have crucial roles in signal transduction process, cell-cycle regulation, gene expression and molecular recognition. The role of IDPs in these processes has been systematically studied in the animal kingdom. In contrast, less reports of these proteins from the plant kingdom are available in the scientific literature. In plant biology, IDPs play crucial roles among plant stress responses, signaling, and molecular recognition pathways, that resemble the functional roles of ProSys in the tomato defense pathways activated upon several biotic and abiotic stresses. These evidences aimed our study focused on the establishment of a relationship between ProSys structure and its biological activity. To this purpose different regions of *ProSys* have been expressed in BL21 (DE3) *E. coli* cells, purified and then characterized by a biophysical and biochemical point of view. Results showed that the recombinant fragments are disordered in agreement with what previously shown for the whole precursor. It was subsequently investigated whether the recombinant ProSys Fragments had any biological activity in activating defense responses upon biotic or abiotic attacks. In particular, by using a combination of gene expression analysis and bioassays, we proved that the exogenous supply of the recombinant ProSys Fragments to tomato plants promotes early and late plant defense genes, but only two fragments (namely Fragment I and III, encompassing the N-terminal part of the protein) were found to be the most promising. In addition, it was observed that

the latter ones counteracted the development of *Spodoptera littoralis* larvae and the fungal leaf colonization. These results suggest that the direct application of these recombinant products, which are safe to humans and no-target organisms, may represent an exploitable tool for crop protection.

Chapter 1

General introduction

1.1 The main agents that cause crop yield losses

Plants, as sessile organisms, are constantly exposed to environmental changing which are often unfavorable or stressful for development, growth and productivity.
To limit the damage caused by these stress conditions, plants have evolved sophisticate mechanisms to detect changes and adapt to them through rapid, dynamic and complex mechanisms in their natural environment.
These adverse environmental conditions comprise abiotic stresses, such as water shortage (drought or dehydration), extremes temperature (cold, heat), excess of light, salt or toxic metals, and biotic stresses, such as herbivore attack or pathogens infections. Globally, the biotic and abiotic stress can decrease agriculture plant yields from 65% to 87% (Gursoy *et al.,* 2012).
Notably, water deprivation is one of the main prevalent environmental factors limiting plant growth, development and crop productivity (Bray, 1997), and climate change is increasing the frequency of extreme weather (Dai, 2013; Pineda *et al.,* 2016).
Other major abiotic stress factors like cold, heat, salinity and drought negatively influence the survival and yield of food crops up to 70% (Vorasoot *et al.,* 2003; Kaur *et al.,* 2008; Ahmad *et al.,* 2010; Thakur *et al.,* 2010; Mantri *et al.,* 2012; Ahmad *et al.,* 2012). Particularly, salinity and drought stress have a strong impact in many regions of the world causing salinization of more than 50% of available cultivable lands by the year 2050 (Wang *et al.,* 2003).
From an agricultural and physiological point of view, drought stress occurs when the available amount of water for plants in the soil decreases due to the low soil moisture at a specific time (Dai, 2011; Keyvan, 2010) and also when the transpiration rate from leaf cell surface is higher than the water uptake in the root zone (Lisar *et al.,* 2012), leading plant cells to swell or desiccation.
Moreover, high and low temperature stress also affects plant growth and productivity. In particular, heat stress may lead to membrane damage and protein denaturation. This latter phenomenon induces a change in the protein folding that may influence its biological activity. On the contrary, in cold stress conditions, water becomes ice that leads to a breach in the plant cells, causing significant yield losses.
When plants grow in soils with high salt content, the high amount of salt can reduce the ability of the plant to take up water and also enter in the transpiration steam of the plant and injury cells at the transpiring levels leading to reductions in the growth and productivity rate.
These abiotic stress conditions are also known to prompt the incident and the diffusion of weeds, insects and pathogens influencing plant physiology and defense responses (Coakley *et al.,* 1999; Seherm and Coakley, 2003; McDonald *et al.,* 2009; Ziska *et al.,* 2010; Peters *et al.,* 2014).
One of the complex changes in the plant induced by abiotic stressors is the accumulation of important molecular compounds such as sugars, amino acids and tertiary sulfonium and quaternary ammonium compounds that keep the main vital functions of the cell active (Slama *et al.,* 2015).
In addition to abiotic stress, plants have to defend themselves from several biotic stress agents such as insects, nematodes, viruses, bacteria and fungi that have a significant effect on plant growth being responsible for great losses of agriculture plant yields.
Plant fungal diseases represent the major threat to food security and safety worldwide as well as ecosystem health (Dean *et al.,* 2012). Based on their lifestyle,

phytopatogenic fungi can be classified into hemi-biotrophs, biotrophs and necrotrophs. The latter group represents the largest group of plant fungal pathogens and have an economic relevance on agriculture causing huge crop losses world-wide. For example, the necrotrophic fungus *Botrytis cinerea* infects over 200 plant species and causes worldwide losses of $10 to $100 billion per year (Wang *et al.,* 2014).

Necrotrophic pathogens are able to kill the living cells of the host tissues before or during colonization, feeding with nutrients released by damaged cells.

In contrast, biotrophic fungi must obtain nutrients from living cells and tissues during infiltration of their *hyphae* within the cell. Hemi-biotrophic pathogens, instead, start with a biotrophic phase during the first step of infection and display a necrotrophic phase only during the late stage of their life (Horbach *et al.,* 2011).

Phytopatogenic fungi include the powdery mildews, the rust fungi and species in the *Magnaporthe*, *Ustilago*, *Cladosporium* genera. For example, *Cladosprium fulvum* represents the most important pest on greenhouse growth tomatoes causing huge yield losses for Solanaceae species.

When a pathogen attempts to infect a plant host, the first line of defense set up by plant is the cell wall. The presence of mechanical barriers such as a thick cuticle and suberin and the deposition of callose on plants slow infectious processes, even though there are some fungi that have evolved mechanisms to evade these barriers. It was also shown that secondary metabolites produced by plants have important ecological functions to control phytopathogenic fungi (Bennett and Wallsgrove, 1994; Ribera and Zuniga, 2012).

Moreover, nematodes are recognized as one of the most widespread and economically important crop pests in the world (Webster, 1987). Plant-parasitic nematodes alone or in combination with other soil microorganisms have been found to attack all the parts of the plant including leaves, roots, seeds, stems and fruits and cause primarily soil-borne diseases related to nutrient deficiency, such as stunting and wilting.

Insects can cause severe physical damage to plants; land plants and insects have coexisted for as long as 350 million years and have developed a series of interactions which influence organisms from basic biochemical to population genetics levels (Gatehouse, 2002). Although some of these relationships are mutually beneficial, the most part of these interactions involve insect predation of plants and plant defense against insect pests.

Insect pests can damage vegetables in many ways depending on the type of mouth parts; they can cause a direct damage on plant tissues like chewing insect or indirect damage like piercing-sucking insects. The latter one can be extremely harmful to crops because they are able to produce a sugar rick substance which represents a nutrition source for other insects and saprophytic fungi.

Also, bacteria, generally bacilli, can cause severe physical damage to plants. Together with fungi, they can provoke vascular wilts, attack parenchyma tissues and develop rottenness or localized necrosis among other symptoms, and infect different part of the plant.

Finally, viruses represent a serious threat of all major cultures of agronomic importance in the world (Nicaise, 2014). These agents of disease need living cells to replicate other viral entities. They are generally transmitted to the plants by piercing-sucking insects producing local and systemic damage that causes curling, stunting, malformations at different part of the plants.

1.2 Several strategies for crop protection

The worldwide annual yield losses in crop production are caused by depicted biotic and abiotic stress and affect negatively the food request. It is well accepted that this situation will get worse considering that the agricultural production must be increased considerably for the next years to meet the estimating growth of human population and the consequent increase of food and feed demands. Indeed, the Food and Agricultural Organization of the United Nations (FAO) estimate that food production will have to increase by 70% over in the year 2050 (FAO, 2009).
However, crop production will become more difficult due to climate change, resource scarcity such as land, water, energy and nutrients, and environmental degradation, such as increased greenhouse gas emissions, declining soil quality and surface water eutrophication (Fan *et al.,* 2011).
Given these limitations, sustainable production at elevated levels is by far the best choice. Much of the increases in yield per unit of area can be attributed to more efficient control of biotic stress rather than an increase in yield potential.
Intensification of crop production over the last 50 years has come to be known as the 'Green Revolution' and has been achieved by the best combination of available technologies such as the use of genetically improved (high-yielding) varieties, enhanced soil fertility via chemical fertilization, pest control via synthetic pesticides and irrigation (Popp *et al.,* 2013).
For a long time, agronomic research was primarily mono-disciplinary and dose-effect oriented, and innovation often equaled the development, transfer and adoption of single component technologies such as new crop varieties or agrochemicals when combating pests and disease (Schut *et al.,* 2014). Although this practice offers a significantly increase in the agriculture production, the extensive use of agrochemicals, in particular, created new problems. Because of their intrinsic toxicity and limited species selectivity, pesticides exhibit undesirable harmful effects on food-safety, public health and environment. Therefore, the increase of social pressure to health and to the environmental safety, pointed the attention of the scientific community to replace chemically pesticides gradually with alternative control strategies which are safe for humans and non-target organisms.
Integrated Pest Management (IPM) is an effective and environmentally friendly sensitive approach to pest management that relies on a combination practices together overcomes the shortcomings of individual practices (Pineda *et al.,* 2010). The aim is not to eradicate pest populations but rather to manage them below levels that cause economic damage (Chandler *et al.,* 2011). One important aspect of this approach consists in the use of living organism, such as natural enemies, and their gene products with the aim to contrast pests and to favor positive interactions.
Some alternative control strategies of currently emerging plant diseases are based on the use of resistance inducers which offer the prospect of durable, broad-spectrum disease control using the direct activation of plant defenses, but also the priming of cells, resulting in stronger elicitation of those defenses, or other defenses, following pathogen attack (Goellner and Conrath, 2007). Such induced resistance rarely leads to complete control of pathogens but rather results in a reduction in lesion size and/or number (Kuć, 1982). Synthetic salicylic acid (SA), such as 2,6-dichloroisonicotinic acid and its methyl ester (both referred to as INA), and benzo (1,2,3) thiadiazole-7-carbothioic acid S methyl ester (BTH) analogs, were the first identified chemical substance capable of triggering induced resistance (Oostendorp *et al.,* 2001; Conrath *et al.,* 2002). A wide range of cellular responses has been

reported to be potentiated by these compounds, including alterations in ion transport across the plasma membrane, synthesis and secretion of antimicrobial secondary metabolites (phytoalexins), cell wall phenolics and lignin-like polymers, and activation of various defense genes (Aranega-Bou *et al.,* 2014).
Non-protein amino acid β-Aminobutyric acid (BABA) has received plenty of attention given its versatility; it has been shown to induce broad-spectrum resistance against many plant pathogens on a range of crop plants (Jakab *et al.,* 2001).
Cohen and colleagues (2011) demonstrated that roots or shoots application of BABA prevented oomycete pathogen *Bremia lactucae* development. Interestingly, BABA-induced protection of *Brassica napus* against the fungal pathogen *Leptosphaeria maculans* was also associated with the induced SA synthesis and expression of PR-1, but it was also found to have a direct fungitoxic effect against this pathogen (Walters et al., 2013).
Moreover, chitosan (poly-D-glucosamine) a polymeric deacetylated derivative of chitin (N-acetylchitosan) is an effective elicitor of proven efficiency in a wide range of experiments with different host plants and pathogens (Iriti *et al.,* 2010). It is able to perform several antimicrobial activities and contribute to reduce plant disease enhancing plant defenses which include oxygen-species scavenging and antioxidant activities, as well as octadecanoid pathway activation (El Hadrami *et al.,* 2010).
A novel approach is the introduction of beneficial organisms that could bio-stimulate plant performance and health, as *Trichoderma*-based products that are largely used in agriculture. It is well known that some *Trichoderma* spp. strains are able to interact directly with roots and prime colonized roots for an intense defense response to subsequent pest attack (Hanson and Howell, 2004; Segarra *et al.,* 2007; Pineda *et al.,* 2010; Tucci *et al.,* 2011; Regliński *et al.,* 2012).
Induced resistance is plagued by a low disease control compared with agrochemicals, reflecting the fact that various factors (such as genotype and environment) influence the expression of induced resistance under field conditions.
The introduction of environmentally sustainable strategies for plant protection represents a primary goal for crop production and requires a better understanding of genetic and molecular basis subtending plant responses against pests.
Only upgrading the knowledge about plants recognition, perception and responses against different stresses and the crosstalk between them, their conscious usage and fortification will be possible in order to enrich IPM programs.

1.3 Plant defense mechanisms against pests

In order to limit damages caused by diverse assemblage of pests, plants have developed during the evolution an impressive diversity of defense mechanisms.
Plant defenses are commonly distinguished based on the time of their activation, in constitutive and inducible.
Constitutive defenses include physical barriers on plant surface, as resin production and lignification, and chemical defensive traits, as deterrents of feeding or toxin, that plants have regardless the presence of pathogens (Wu and Baldwing, 2010).
Otherwise, inducible defenses are triggered only after the perception of the stress conditions and consist in the accumulation of defensive compounds.
Since the activation of defense mechanisms require a substantial input of energy, plants have developed complex regulatory systems to balance between growth, development and defense.

It is commonly known that induced defenses are favored by the natural selection since they lower the energy investment only in presence of herbivores, and/or limit damages made by auto-toxicity and/or allow for tailoring responses to different herbivores (Maffei *et al.,* 2012).
The first line of inducible defenses relies on the perception of general compounds, called elicitors, which are present either constitutively in the pests [such as microbe-associated molecular patterns (MAMPs) or pathogens-associated molecular patterns (PAMPs) or herbivore-associate molecular patterns (HAMPs)] or generated during their invasion [such as damage-associated molecular patterns (DAMPs)] by specific receptor proteins located on plant cell membrane (PRR) (Conrath *et al.,* 2015).
The PRRs specifically interact with these elicitors leading to activate intracellular signaling, transcriptional reprogramming, and biosynthesis of a complex output response that limits pests colonization (Olori-Great and Opara, 2017).
The system is known as PAMP-Triggered Immunity (PTI) and it is the first step of co-evolution of defense strategies described by the ZigZag model suggested by Jones and Dangl in 2006 (Figure 1).

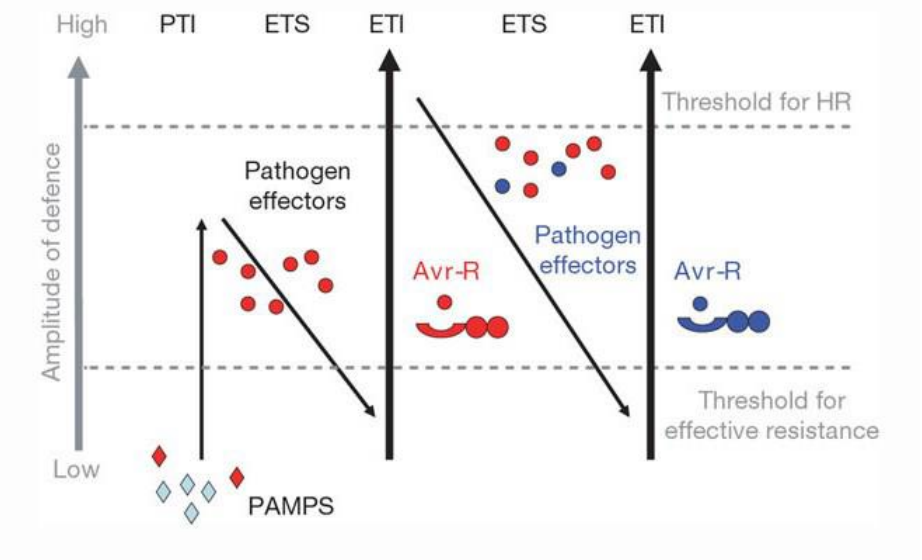

Figure 1: Figure 1: Zig-zag model illustrating the co-evolution between host and pathogen (Jones and Dangl, 2006).

Some pathogens have evolved effector factors that promote pathogens growth by suppressing the host's resistance baseline (PTI), which results in the effectors-triggered susceptibility (ETS) (Figure 1).
Plant can overcome suppression of PTI through the recognition of pathogen effectors by novel evolved plant receptors resulting in effector-triggered immunity (ETI). ETI is an amplified version of PTI response that often passed a threshold and induce a hypersensitive response (HR) and programmed cell death (PCD) at the infection site.
In a subsequent phase, some pathogens have been becoming able to counteract the ETI modifying the specifically recognized effectors and perhaps gaining a new effector. Subsequently, a new plant receptor allele is then evolved and selected that can recognize and bind the new developed effector, resulting again in ETI.
The rounds of ETS followed by ETI is continuous and reflect the evolutionary arms race between plants and their bioaggressors (Walling, 2009).

Plant defenses can be also distinguished based on the mode of action in direct and indirect defense.
Direct defenses are triggered to interfere with pest metabolism, nutrition, growth and reproduction using physical or chemical barriers.
Physical barriers on plant surfaces, such as thorns, silica, cuticles and trichomes which may prevent colonization and limit movements are the first obstacles that herbivore has to overcome.
For instance, leaf trichome density negatively influences ovipositional preference, feeding and larval nutrition of insect pests (Handley *et al.,* 2005). Dense trichrome may also affect the herbivore mechanically and inhibit their movement, thereby reducing the access to leaf surface. Induction of trichome upgrade after pest attack has been observed in many plants only in developing leaves during or subsequent the attack (Agrawal, 1999; Agrawal *et al.,*2009).
Otherwise, trichomes are also able to complement plant chemical defenses producing substances that can be poisonous, olfactory or gustatory repellent, or trap herbivores, thus creating a combination of chemical and structural defense (Hanley *et al.,* 2007; Sharma *et al.,* 2009).
Epicular waxes, leaf toughness and cuticle thickness could affect the performance of the insect herbivores on its host plant (antibiosis), or their behaviour during host plant selection (antixenosis).
Direct defenses include also the production of primary and secondary metabolites and act as powerful chemical weapons interfering with herbivore metabolism and growth.
A typical example are plant lectins, sugar binding proteins ubiquitous in nature, that have a protective function against pests (Chakraborti *et al.,* 2009; Vandeborre *et al.,* 2011). The most important biological property of lectins is the ability to recognize and bind reversibly to specific carbohydrate structures of insect peritrophic membrane interfering with nutrient digestion and adsorption (Chakraborti *et al.,* 2009). Alteration of protein, lipid and carbohydrate metabolism provokes atrophy of tissues, thereby a disruption of the immunogical and hormonal status, threatening the development and growth of insects (Saha *et al.,* 2006; Chakraborti *et al.,* 2009; Vandenborre *et al.,* 2011).
Furthermore, tannins have a strong anti-nutritional activity on phytophagous insects because they are able to bind proteins causing a decrease of their absorption efficiency and midgut damages (Sharma *et al.,* 2009; Barbehenn and Constabel, 2011).
A rich font of chemicals involved in antibiosis is plant latex, a white sap exuded from leaf damage immediately after herbivores and pathogens attack. Most of the available literature suggest that plant latex defends plant against pests by trapping and immobilizing them due to its stickiness and also by its toxic activity thank to the presence of high concentration of various specialized metabolites and proteins such as proteinase, alkaloids, chitinases, terpenoids and glucosidases (Konno, 2011).
Moreover, the most popular anti-nutritive compounds are plant proteinase inhibitors (PIs) produced following herbivore injury and act as reducing the nutritional value of crops. In particular, PI suppress the activity of protein digestive enzymes in insect guts and thereby a deficiency of amino acids, resulting in a slow development, mortality and/or reduced fecundity (Azzouz *et al.,* 2005; Gatehouse, 2011).

Other defense-involved enzymes such as arginases, lipoxygenases, polyamine oxidases, peroxidases and ascorbate oxidases may have anti-nutritional properties (Mithöfer and Boland, 2012).
Indirect defenses, on the other hands, are plant traits that by themselves do not affect the susceptibility of host plants but can be attractive to the natural enemies of pests by releasing volatile organic compounds (VOCs) (Heil and Ton, 2008; Wu and Baldwin, 2010).
The production of VOCs, that mainly consist of terpenoids, fatty acids derivates and aromatic compounds, by infested plants mediates the attraction of parasitoids and predators which actively reduce the amount of feeding herbivores (Dudareva *et al.,* 2006; Maffei, 2010).
The plants normally emit an optimum quantity of volatile compounds, but in response to herbivores a different blend of volatiles is released into the atmosphere (Arimura *et al.,* 2009). Usually the volatile blend composition produced depends on plant and herbivores species, and also on their condition and stage of development (Arimura *et al.,* 2009; Maffei, 2010). Moreover, VOCs released by plants in response to herbivore attack is specific for a particular plant-herbivore interaction, including natural enemies (War *et al.,* 2012). Induced defenses are complex also because different types of herbivores could be recognized and activate different defense responses, depending upon their modes of feeding.
VOCs emitted from attacked plants can also act as signals in neighboring uninfested plants preparing their defensive machinery to respond more rapidly if they are subsequently attacked.
Some volatile compounds also play an important role in direct defenses by acting as a feeding and/or oviposition repellent to the attacking herbivores (Dudareva *et al.,* 2006; Arimura *et al.,* 2009). For instance, it was observed that nocturnal VOCs emitted by tobacco plants may have a direct repellent effect on *Heliothis virescens* behavior interfering with their oviposition on previously damaged plants (De Moraes *et al.,* 2001). Moreover, a previous study demonstrated that isoprene emitted by transgenic tobacco plants interferes with the feeding performance of tobacco hornworm, *Manduca sexta* (Laothawornkitkul *et al.,* 2008).

1.3.1 Plant responses against insects and pathogens

Insects are a class of the animal kingdom with more than one million described species, and nearly half of them feed on plants (Wu and Baldwin, 2010). Plants and insects have coexisted for more than 400 million years and have developed refined interactions that affect organisms at all levels, from basic biochemical to population genetics (Fürstenberg-Hägg *et al.,* 2013).
Based on their feeding strategies leading to different quantity and quality of damage on plant tissues, insects can be classified in two main group: chewers and phloem-feeders.
Chewing insects are able to destroy by grinding mandibles a big amount of leaf tissue causing a reduction of biosynthetic activity and an increase in respiration.
This feeding strategy promotes the release of cell-wall fragments and fatty acids, and favorites the interaction between substrates and enzymes physically separated at cellular level due to cell disruption, which alerts the plant of a possible biotic attack.
The fatty acids releasing from membranes induce the activation of the octadecanoid pathway in which jasmonic acid (JA) and its derivates are produced (Figure 2).

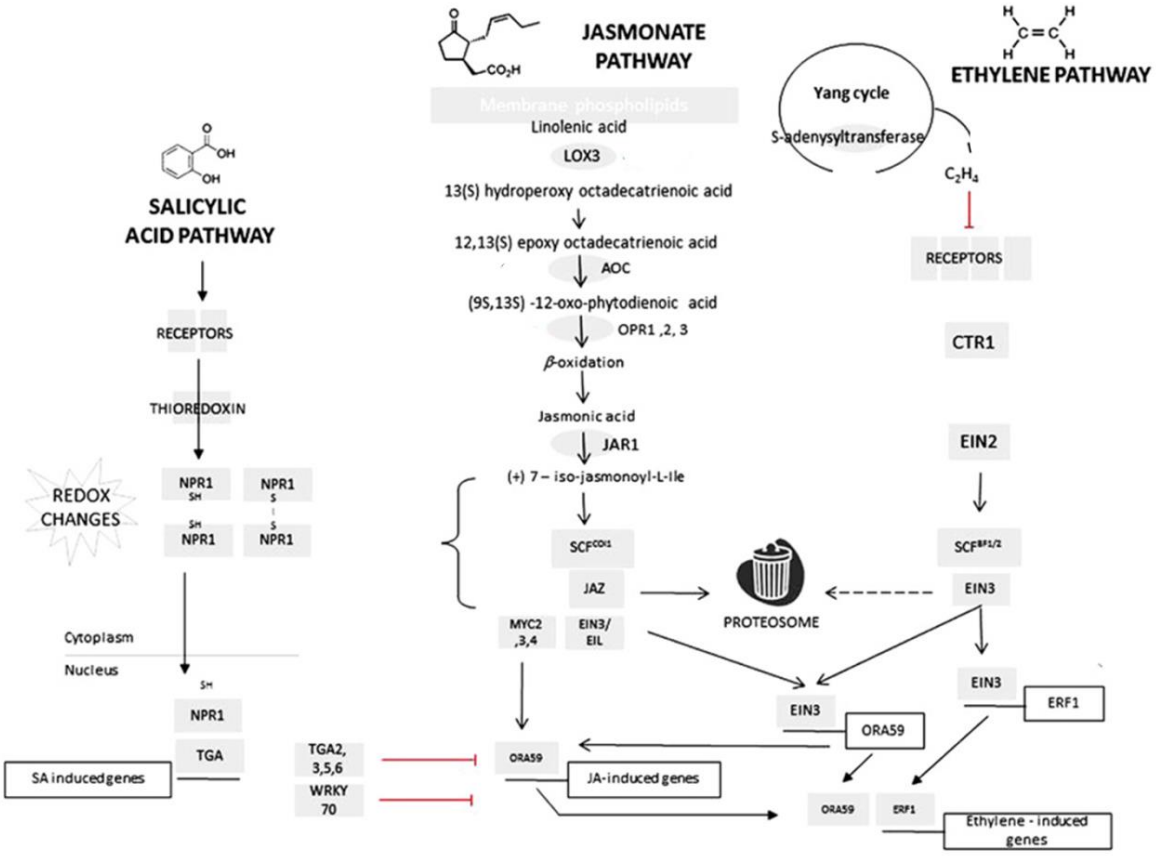

Figure 2: Plant herbivory-related pathways. Schematic versions of salicylic acid (SA), jasmonate (JA), and ethylene (ET) signaling cascade (Mur et al., 2013). Biosynthetic enzymes are represented as gray ovals and signaling components are gray rectangles. Abbreviations in the jasmonate biosynthetic pathway are as follows: LOX, lipoxygenase; AOC, allene oxide cyclase; OPR, oxo-phytodienoate reductase; for the ethylene biosynthetic pathway: ACS, 1-aminocyclopropane-1-carboxylic acid synthase; ACO, 1-aminocyclopropane-1-carboxylic acid oxidase. Genes and their regulatory promoters are represented as open boxes.

In particular, jasmonates regulate a complex defense network to herbivores by producing toxic secondary chemicals, enhancing emission of VOCs which attract predators or parasitic enemies and prime defense, stimulating wound signalling and perception, and increasing the levels of proteins that inhibit or deter insect feeding or growth (Frost *et al.,* 2008; Howe and Jander, 2008).

The importance of the octadecanoid pathway in the regulation of defense against a broad spectrum of insect herbivores has been deeply studied using mutants impaired in JA synthesis and signaling (Bostock, 2005; Sun *et al.,* 2011; Pangesti *et al.,* 2016). For example, *12-oxophytodienoate reductase 3* (*OPR3*) and *coronatine-insensitive 1* (*COI1*) mutants exhibit extremely sensitive to the feeding of *Pieris rapae* caterpillar (Reymond *et al.,* 2004).

COI1 encodes an F-box protein closely associated with the SCF-mediated protein degradation by the 26S proteasome and is required for most JA-mediated signaling (Xie *et al.,* 1998).

Moreover, Li and colleagues in 2005 showed that tomato mutants defective in acyl-CoA oxidase (ACX1A) that catalyzed the first step in the β-oxidation stage were weak in JA signaling processes resulting more susceptible to tobacco hornworm attack.

Phloem-feeders have an elongated, thin and flexible stylet containing two channels with which penetrate the phloematic cells obtaining plant nutrients and causing the

release of pre-digestive saliva. Among this group of insects, aphids are able to damage tomato causing relevant yield losses. During their feeding strategies aphids secrete two types of saliva: a gel saliva which creates a gelatinous wall isolating the full-length of the stylet within the apoplast facilitating penetration; and a water saliva which is also injected into the vascular tissues releasing a complex mix of enzymes and other components allowing nutrient digestion (Walling, 2008; Will and Vilcinskas, 2015).

Despite this kind of feeding produces a small damaged area compared to those caused by chewing insects, aphids activate in plants different and interconnected hormone dependent defense responses that are very similar to those activated by bacterial and fungal pathogens (Figure 2). Plant responses against piercing-sucking insects range from overlap with wounding to the promotion of SA-mediated defense pathways (De Ilarduya *et al.,* 2003; Kempema *et al.,* 2007).

Several researches based on study changes in gene expression induced by aphid revealed that many of these responses are involved in the regulation of genes involved in SA- and JA-mediated pathways, in Reactive Oxygen Species (ROS) production, cell wall organization, cell maintenance, protein metabolism, photosynthesis and secondary metabolites (Smith and Boyko, 2007; Kuśnierczyk *et al.*, 2008; Delp *et al.*, 2009; Coppola *et al.,* 2013).

Among plant pathogens, fungi represent the largest group and many of these adversely affect food security. A small fraction of the estimated 5 million fungal species are responsible for devastating diseases affecting agriculture and human health (Perez-Nadales *et al.,* 2014). In particular, there are more than 10000 fungal species known to cause diseases on plants, compared with roughly 50 species that cause disease in humans (Agrios, 2005). Plants have evolved complex defense strategies to limit cellular infection by microbial pathogens. The first line of defense during plant-pathogen interactions is an intact and impenetrable physical barrier composed of bark and waxy cuticle on the leaf surface and/or plant cell walls (Zeyen *et al.,* 2002; Micali *et al.,* 2011). Melotto and colleague in 2008 showed that natural plant openings, such as stomata and hydathodes, close upon detection of potential microbial pathogens to prevent its develop inside the leaf. Moreover, pathogenic bacteria have evolved strategies to suppress the closure of stomata through the production of phytotoxin which is able to force the pores back open (Olori-Great and Opara, 2017). Once the first line of defense is pervaded, plants must resort to a different set of defense mechanisms to fend off pathogens with different infection strategies and lifestyles. For instance, biotrophic pathogens, which are specialized to feed on living plant cells, are controlled by other basal defense mechanisms as compared to necrotrophic pathogens which often grow on plant tissues that are wounded or senescent and immediately kill host cells to metabolize their contents.

In particular, biotrophs mainly activate SA-dependent defense mechanisms, whereas necrotrophs induce JA- and ET-mediated responses. The SA- and JA/ET- regulated defense pathways crosstalk and can have antagonistically, additively, or even synergistically activity depending on the intensity and duration of the signals provided to the host plant (Mur *et al.*, 2006). These pathways are also able to interact with other defense signals such as ABA, auxin, GA, H_2O_2, and NO which are known to enhance or antagonize SA- and/or JA-defense signaling (Lopez *et al.,* 2008). This crosstalk can be influenced by herbivore and pathogens in order to avoid effective plant defenses.

1.4 Recognition of pest attack
1.4.1 HAMP elicitors

Compounds of biotic origin able to product and release molecules and patterns that elicit specific plant responses are defined “elicitors”.
They can be produced directly by the pest (exogenous elicitors, microbial elicitor) but also released from the plant by the action of the biotic agents (endogenous elicitors, host plant elicitors; D’Ovidio *et al.* 2004).
To distinguish the attack of the herbivore from other biotic stressors, plants have evolved the ability to recognize insect attack by their damage and by perceiving herbivore-derived chemical cues, such as herbivore-associated elicitors (HAE) or herbivore-associated molecular patterns (HAMPs, Bonaventure, 2012).
Several HAMPs have been isolated from herbivore oral secretions (OS), saliva and ovipositional fluid (Schmelz *et al.,* 2006; Alborn *et al.,* 2007; Wu *et al.,* 2007; Howe and Jander, 2008).
In particular, two major classes of elicitors have been isolated from the insect oral secretions which trigger plan defense responses against herbivores. The first one comprises lytic enzymes, such as β-glucosidase that was isolated from *Pieris brassicae* and induced the release of a volatile blend from wounded cabbage leaves that attract the parasitic wasp, *Cotesia glomerate* (Mattiacci *et al.,* 1995). Other lytic enzymes have been discovered from the oral secretion of other species, such as glucose oxidases in larval *Helicoverpa zea* saliva (Felton and Eichenseer, 1999); alkaline phosphatase in *Bemisia tabaci* saliva (Funk, 2001); and in a wide array of watery digestive enzymes from aphid saliva (Miles, 1999); but their roles as elicitors of defense responses have not yet been established (Kessler and Baldwin, 2002).
The second class of elicitors included fatty acid–amino acid conjugates (FACs).
The FAC volicitin or N-(17-hydroxylinolenoyl)-L-glutamine was the first discovered and fully characterized herbivore-derived elicitor isolated from the oral secretions of beet armyworm, *Spodoptera exigua* (Alborn *et al.,* 1997). As reported by Alborn and collegues (1997), the exogenous application of volicitin greatly enhances the release of a volatile blend in *Zea mays* seedlings, similar to that induced by *S. exigua*, which attracts natural enemies of the pest. In addition, several FACs have been identified in *Manduca sexta* regurgitate, the application of which to the wounded leaves of tobacco activated mitogen-activated protein kinase (MAPK), wound-induced protein kinase (WIPK), and JA, SA, ET accumulation (Wu *et al.,* 2007).
Since then, FAC has been isolated from the oral secretions of larval *Sphingidae*, *Noctuidae* and *Geometridae* (Pohnert *et al.,* 1999; Halitschke *et al.,* 2001; Spiteller and Boland, 2003) and also from fruit flies (*Drosophila melanogaster*) and in crickets (*Teleogryllus taiwanemma*) (Yoshinaga *et al.,* 2007).
In addition to FACs, other elicitors have been identified in the oral secretion of many insects, such as inceptins and caeliferins. In particular, inceptins from larval *Spodoptera frugiperda* saliva are disulfide-bonded peptides formed by proteolytic fragments of chloroplastic ATP synthase γ-subunit that are produced in the insect midgut and inducing defenses in cowpea and beans (Schmelz *et al.,* 2007). Even at very low concentration, inceptins introduced into wounded cowpea leaves increase the levels of JA, SA and ET (Schmelz *et al.,* 2007).
Caeliferins from the oral secretions of *Shistocerca americana* (American bird grasshopper) and other grasshopper species are disulfoxy fatty acids that enhance the emission of volatile terpenoids when applied to damages leaves of *Zea mays* seedling (Alborn *et al.,* 2007).

In contrast to these examples, a few number of elicitors from the insect oral secretion are known to act as suppressor of plant defense responses, as observed for salivary glucose oxidase (GOX) secreted by *Helicoverpa zea* and proteins identified in the salivary glands of *Myzus persicae* (Eichenseer *et al.,* 1999; Musser *et al.,* 2005; Bos *et al.,* 2010). Moreover, aphid watery saliva also contains elicitors, such as pectinesterases, lipases, peroxidases, amylases, cellulases, sucrases, proteases, and alkaline and acid phosphatases which are very important in response to phloem-feeding facilitating feeding from host plants.
Finally, insect oviposition fluids also contain substances that can elicit plant defense responses leading the attraction of egg parasitoids or strengthen its defense mechanisms in case of a future attack (Fürstenberg-Hägg *et al.,* 2013).
Two compounds in oviposition fluid are known to elicit plant responses, such as bruchins and benzyl cyanide. Bruchins, long chain diols that are mono- and diesterified with 3-hydroxypropanoic acid, was found in the ovipositional fluid of pea weevils (*Bruchus pisorum*) and only 0.5 pg applied to the pea leaf surface induces neoplasma growth on pods, which detaches egg from the oviposition site and elicits tumor-like growths that impedes larval entry into the pod (Kessler and Baldwin, 2002). While benzyl cyanide was identified in oviposition fluid of large cabbage white butterfly (*Pieris brassicae*) and only 1 ng applied on leaves of *Brassica oleracea* (Brussels sprouts plants) changes the leaf surface chemicals leading the arrest of parasitoid *Trichogramma brassicae (*Fatouros *et al.,* 2008*)*. More insight in the elicitation events could improve the knowledge about early signalling and the transduction of responses in plant.

1.4.2 PAMP and DAMP elicitors

Plants are also capable of sensing and recognizing evolutionary conserved pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) that trigger a number of induced defenses in plants (He *et al.,* 2007).
Upon their perception by plant pattern recognition receptors (PRRs), the PTI response is activated which rapidly give rise to the activation of mitogen-activated protein kinase (MAPK) signaling cascades and rapid influx Ca^{2+}, production of reactive oxygen species (ROS) and transcriptional reprogramming that lead to a complex response of the plant that interfere with microbial growth (Wirthmueller et al., 2013).
Principally, the PRRs identified can be divided into two groups of plasma membrane-localized proteins: the receptor-like kinases (RLKs) and the receptor-like proteins (RLP). The first one consists of a ligand-binding extracellular region, a single-pass transmembrane domain and an intracellular serine/threonine kinase domain that activates the downstream signaling responses. RLPs differ from RLKs in the kinase domain because they only have a short cytosolic domain without an obvious signaling module. The extracellular region of PRRs showed a high diversity in amino acid sequence leading the plants to quickly adapt to the ever-changing structures of microbial elicitors (Shiu and Bleecker, 2001; Cock et al., 2002).
Example of PAMPs/MAMPs include conserved microbial products invariant among several groups of microorganisms such as lipopolysaccharides (LPS), peptidoglycans (PGN), bacterial flagellins, glucans, chitins, and lipids and other membrane components such as ergosterol, proteins and glycopeptides (Zipfel and Felix, 2005; Mishra *et al.,* 2012).

The first fully characterized PAMP is flagellin (flg22) which is perceived by the receptor-like kinase (RLK) flagellin-sensing 2 (FLS2) (Gomez-Gomez and Boller, 2000). Upon stimulation with flg22, FLS2 forms a complex with Brassinosteroid insensitive 1- Associate Kinase (BAK1) causing in turn transphosphorylates the BAK1-FLS2 protein complex and auto-activation of FLS2. The downstream signaling events of BAK1-FLS2 complex formation include a MAPK cascade and the activation of calcium-dependent protein kinases (Robatzek and Wirthmueller, 2013). This process has a feedback control mediated by the ubiquitination, internalization and degradation of the complex (Robatzek *et al.,* 2006; Lu *et al.,* 2011).
Moreover, many plant pathogens also release lytic enzymes to break the structural barrier of plant tissues in favor of their invasion. The products generated by these enzymes are released into plant apoplastic space and may function as endogenous elicitors or as Damage-Associated Molecular Patterns (DAMPs).
As already observed with PAMPs, also DAMPs are perceived by the PRRs and initiate and perpetuate innate immune response, PTI defense response.
Examples of DAMPs include cell wall fragments such as oligogalacturonides and cellulose fragments, cutin monomers, defensins, and phytosulfokines and peptides such as systemin (Ryan, 2000; Nuhse, 2012; Albert, 2013).
The classic examples of DAMPs are plant cell wall fragments released by the action of microbial enzymes during the infections or by the action of host enzymes that are released by the mechanical damage, the oligogalacturonides (OGAs) (Cervone *et al.,* 1989; Orozco-Cardenas and Ryan, 1999). They are able to elicit a wide range of defense responses, including an oxidative burst, accumulation of phytoalexins, an increase of glucanase, and chitinase activity, deposition of callose, increased hormone biosynthesis, and enhanced resistance to *B. cinerea* (Wang *et al.,* 2014).
Although their activity is well documented, little is known about the OGAs perception system. Interestingly, the extracellular domain of an *Arabidopsis* wall-associated RLK named wall-associated kinase 1 (WAK1) has the high ability to bind to OGAs in vitro (Decreux et al., 2006). It was observed that *Arabidopsis* plants which overexpress WAK1 showed an increased resistance to *B. cinerea* suggesting that WAK1 or its homologs might be part of the perception system for OGA.

1.5 Systemin and other defense peptides

Although a large number of DAMPs have been identified in the animal kingdom, until now only a few number of DAMPs have been found in plant kingdom. The best characterized class are polypeptides/peptides released from larger precursor protein (Choi and Klessing, 2016). These include a family of defense-related peptide hormones called systemins that are produced by plants of *Solanaceae* family in the activation of defense genes in response to injury, either locally or systemically (Ryan and Pearce, 2003). The 18-amino acid oligopeptide Systemin (Sys) was the first hormone signal bioactive peptide discovered in plant (Pearce *et al.,* 1991).
In tomato, Sys is generated by wound induced processing of a larger precursor protein of 200 amino acids called ProSystemin (ProSys) which is located in the cytosol of vascular phloem parenchyma cells.
Low levels of Prosys mRNA were found in unwounded tomato leaves, but increased levels were detected in both wounded and distal leaves in response to severe wounding (McGurl *et al.,* 1992), due to Prosys amplification upon persisting of insect attack (Lee and Howe, 2003; Li *et al.,* 2002).

Upon insect attack or mechanical wounding, the expression of Prosys precursor protein increases and the encoded protein is processed to released Sys peptide which appears to bind a receptor and then initiates a complex intracellular signaling pathways that leads to the accumulation of defense compounds (Ryan, 2000; Scheer and Ryan, 2002; Wang *et al.,* 2018).
In particular, at cellular level, the perception of Sys induces a depolarization of the plasma membrane, the alkalinization of the apoplast, the activation of MAPK and calmodulin, the activation of a phospholipase A2 (PLA2), and the release of linolenic acid from membrane phospholipids starting the octadecanoid pathway and the downstream biosynthesis of 12-oxophytodienoic acid (OPDA) and JA (Ryan 2000; Sun *et al.*, 2011).
Homologous sequences of *Prosys* gene are present in many other *Solanaceous* species including potato, bell pepper and nightshade (Constabel *et al.,* 1998) but not in tobacco in which there were identified functional homologues, *Hydroxyproline-rich systemin glycopeptides* (*HypSys*) (Pearce *et al.,* 2001).
In tobacco there were isolated three hydroxyproline-rich 18 aa polypeptides, called TobHypSys I, II and III, released from a single precursor of 165 amino acids that are active in the induction of defense responses (Pearce *et al.,* 2001).
Even in tomato three hydroxyproline-rich glycopeptides were discovered which involved in the regulation of defense genes in association with Sys, called SlHypSys I, II and III (long respectively 20, 18 and 15 amino acids), released from a larger precursor of 146 amino acids and synthesized in the endoplasmic reticulum and the Golgi apparatus (Pearce and Ryan, 2003).
Studies of overexpression and silencing both in tobacco and in tomato plants of this precursor showed that these peptides play an important role in the regulation of defense responses, indicating their cooperative action with the Sys in the activation of wound defense responses (Narvaez Vasquez *et al.,* 2007).
Other HypSys peptides, along with their precursor, have now been identified in other species such as petunia, black nightshade but also outside the *Solanaceae* family such as *Ipomoea batatas* (sweet potato, *Convolvulaceae*), *Populus trichocarpa* and *Coffea canephora* (Pearce, 2011).
Another family of peptide signals as Sys, the Plant Elicitor Peptides (Peps), was isolated and characterized in *Arabidopsis* (Huffaker *et al.,* 2006).
Peps are 23 amino acids peptides, derived by a larger precursor protein of 92 amino acids, ProAtPep1. The gene encoding this precursor is a part of a small family of at least seven members in *Arabidopsis*. In contrast to systemin, several candidate orthologues sharing sequence homology with ProAtPeps occur in diverse plant species including important crop plants (Schaller, 2008).
Peps are able to interact with PEPR receptors to stimulate accumulation of transcripts and metabolites associated with plant defense (Delano-Frier *et al.,* 2013).
As does Sys, the signal transduction processes activate by these peptides result in production and accumulation of JA, ET, reactive oxygen species, calcium flux and inhibition of a plasma membrane proton dependent ATP synthase (Howe *et al.*, 1996; Meindl *et al.,* 1998; Schaller and Oecking, 1999; Ryan, 2000; Orozco-Cárdenas *et al.,* 2001; Huffaker *et al.*, 2006; Qi *et al.,* 2010; Krol *et al.,* 2010; Huffaker *et al.,* 2011; Ma *et al.*, 2012).
The precursor protein of Sys, HypSys and Peps peptides do not share any sequence homology in their sequences but the presence of sequence proline, hydroxyproline and charged residues with common motives advances the hypothesis of a common origin. In fact, it is well known that proline residues have an important role in the

interactions of peptide ligands with their receptors conferring structural conformations in the backbone chains of these peptides (Rath *et al.,* 2005).

1.6 Research objectives

Even if the effectiveness role of Sys in promoting plant defenses has been well established, little is known about the biological activity of its precursor other than being an intermediate in the synthesis Sys. In particular, transgenic plants constitutively expressing ProSys showed the up-regulation of genes involved in several hormone-regulated defense pathways resulting resistant to insect and fungi infestations and tolerant to salt stress (Coppola *et al.,* 2015).
Recent evidences suggest an emerging role of ProSys N-terminal region in plant defense responses (Rocco *et al.,* 2008; Corrado *et al.,* 2016) and, possibly, of the whole precursor. To share more light on ProSys involvement in tomato responses and to evaluate of a possible use of this precursor as a defense instrument, a structural and biological characterization of the full-length pro-hormone was performed. The protein was expressed in native conditions and purified in a sufficient amount in order to perform biophysical, biochemical and structural analysis (Chapter 2), as well to evaluate its biological activity in *vitro* and in *vivo* (Chapter 4).
Surprisingly, our experimental results evidenced for the first time that ProSys is a member of the Intrinsically Disordered Proteins (IDPs) family possessing intrinsically disordered regions (IDRs) which are important for plant crucial roles such as stress responses, signaling, and molecular recognition pathways.
Finally, in order to shed light on the relationship between the several disordered regions of ProSys and their biological activity and thus possibly identify the minimal polypeptidic region(s) responsible of its activity, a structural (Chapter 3) and biological (Chapter 4) characterization of 4 ProSys fragments were performed.

Chapter 2

The analysis of the Prosystemin structure reveals that the protein is intrinsically disordered

Donata Molisso in collaboration with Emma Langella, Simona Maria Monti and Rosa Rao

Abstract

Prosystemin (ProSys) is a pro-hormone of 200 aminoacidic residues which releases a bioactive peptide hormone of 18 amino acids called Systemin (Sys) involved in the activation of defense genes in tomato in response to herbivore and pathogen attacks. It is presently unknown whether ProSys has any biological function other than being an intermediate in the synthesis of Sys. However, recent evidences suggest that Prosys N-terminal region could contribute to defense responses. This observation prompted us to investigate the biochemical and structural features of the ProSys protein. To this purpose ProSys has been expressed in BL21 (DE3) *E. coli* cells and purified. A detailed characterization of this pro-hormone by means a multidisciplinary approach reveals that this precursor is an intrinsically disordered protein (IDP) with some secondary structure elements within the sequence, suggesting new interesting insights on the role of ID proteins (IDPs) into plant response against biotic stressors.

2.1 Introduction

Plants are sessile organisms unable to escape adverse environmental changes. To contrast the environmental threats and all kind of pests, plants have developed during the evolution sophisticated defense responses. In particular, the molecular mechanisms underlying defense responses against insect attack are activated at the damage site and involve a complex regulatory network which allows a long-distance defense signaling, likely modulated by jasmonic acid (JA) and other phytormones (Schilmiller and Howe, 2005). In tomato, it was firstly shown that wound damage results both in local and systemic expression of defensive proteinase inhibitors (PIs) which interfere with the digestion activity of enzymes present in the herbivore midgut, reducing insect growth and vitality (Green and Ryan, 1972; Ryan, 1990). This mechanism is triggered by the 18-amino acid hormone peptide named Systemin (Sys) (Pearce *et al.,* 1991).
Upon wounding, Sys initiates a cascade of reactions associated with the induction of early signaling components of JA biosynthetic pathway that leads to the production of molecules active in defense responses against insect herbivores (Ryan, 2000; Schmiller and Howe, 2005; Corrado *et al.,* 2007; Degenhardt *et al.,* 2010; Coppola *et al.,* 2015). This small peptide is released from the C-terminal region of a larger cytosolic pro-hormone of 200 amino acids called Prosystemin (ProSys); it was recently suggested that processing of ProSys could be mediated by a proteolytic cleavage triggered by a phytaspase, an aspartate-specific protease of the subtilase family (Beloshistov *et al.,* 2018).
ProSys is encoded by a single gene (4176 nucleotides in length) that consists of 11 exons and 10 introns (Figure 2.1). The first ten exons are organized as pairs, and the last (single) exon encodes Sys. The nucleotide sequence of each of the five exon pairs in *ProSys* is homologous to that of every other pair, which suggests that they evolved by successive duplication or elongation events from a common ancestor (McGurl and Ryan, 1992). Notably, only Sys is coded by a single-copy exon.

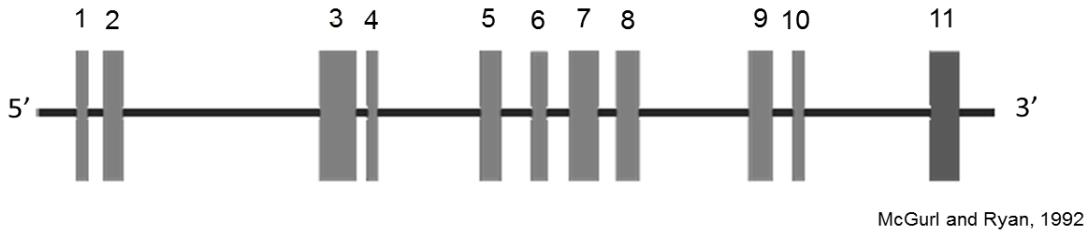

Figure 2.1: The organization of the *ProSys* gene. Exons are represented by vertical gray bars numbered 1 to 11. The five exons pairs are 1 plus 2; 3 plus 4; 5 plus 6; 7 plus 8 and 9 plus 10. Exon number 11, in dark gray, represents the sequence encoding Sys, it is located at the 3' end and it is unique when compared to the rest of the sequence of the larger precursor.

The function of the repeat regions and whether they have other signaling properties has not been investigated. Furthermore, sequence analysis of ProSys revealed that it contains no predicted membrane-spanning domain, prenylation sites or N-linked glycosylation sites and does not undergo other post-translational modification (Dombrowski *et al.,* 1999). A striking characteristic of this precursor is the absence of a signal peptide required for targeting to the secretory pathway (Von Heijne, 1990); it appears to be synthesized in the cytosol and transported outside the cell with an unknown mechanism (Pearce, 2011).

ProSys gene is transcribed at low level in physiological conditions while its expression increases after mechanical wounding or insect herbivore attack (McGurl *et al.,* 1994; Ryan, 2000, Coppola *et al.,* 2015).

The fundamental role of ProSys in plant defense was initially established by Ryan's group through the analyses of transgenic tomato plants expressing the *ProSys* gene in sense and anti-sense orientation. The overexpression of ProSys was related to the constitutive expression of PIs associated with a significant increase of resistance against insect herbivory (McGurl *et al.,* 1994; Dombrowski *et al*., 1999; Ryan, 2000). Conversely, plants expressing Prosys cDNA in anti-sense orientation showed almost a complete suppression of PIs expression after wounding (McGurl *et al.,* 1992) and were therefore compromised in their ability to counteract feeding damages imposed by *Manduca sexta* larvae (Orozco-Càrdenas *et al.,* 1993).

Recently, it was demonstrated that the constitutive production of ProSys in transgenic tomato plants resulted in an increased resistance also against some phytopathogenic fungi and aphids (Coppola *et al.,* 2015; Diaz *et al.,* 2002; El Oirdi *et al.,* 2011).

In addition, ProSys overexpressing plants showed a reinforced indirect defense, being more attractive towards parasitoids and predators (Corrado *et al.,* 2007; Degenhardt *et al.,* 2010) and present an increased tolerance to saline stress (Orsini *et al.,* 2010).

These observations suggest that ProSys plays a role in plant defense that is wider than previously thought. The biological mechanisms associated with its role are not fully understood likely due to the consequence that Sys peptide was traditionally considered as the principal actor of the described tomato plant defense responses. However, a very recent study showed that ProSys, devoid of the Sys aminoacidic sequence, exerts a consistent biological activity (Corrado *et al.,* 2016).

Due to the lack of structural information on the whole Sys precursor, a structural and biological characterization of the full-length pro-hormone was performed.

The protein was expressed in *E. coli* cells under native conditions in a sufficient amount in order to perform biophysical, biochemical and structural analysis, as well

as biological assays (described in Chapter 4). *E. coli* is one of the earliest and one of the most commonly used bacterial hosts for the production of recombinant proteins (Terpe, 2006). It can accumulate recombinant proteins up to 80% of its dry weight and survives in different environmental conditions (Demain and Vaishnav, 2009).
Surprisingly, experimental evidences in agreement with bioinformatics analyses proved that ProSys, is a member of the family of the Intrinsically Disordered Proteins (IDPs). Indeed, IDPs are known to be a class of proteins completely or only partially unstructured and nevertheless functional (Uversky, 2013). These results offer novel tools to understand how ProSys performs its biological functions since it was evidenced that many fundamental biological functions such as signaling and molecular recognition involve IDPs (Oldfield *et al.,* 2008).

2.2 Results

2.2.1 Recombinant ProSys shows hydrodynamic features typical of intrinsically disordered proteins

2.2.1.1 Expression of ProSys in bacteria and purification of the recombinant protein

The cDNA encoding ProSys protein was cloned in pETM11 expression vector (a kind gift from EMBL, Heidelberg) which is suitable for *E. coli* expression. It contains a strong bacteriophage T7 polymerase promoter for the chemical induction by Isopropyl-β-D-1-thiogalactopyranoside (IPTG). This vector allows the expression of the recombinant protein with a six histidine N-terminal tag [$(His)_6$-tag] and a fusion Tobacco Etch Virus (TEV) protease recognition site. Thanks to the presence of the $(His)_6$-tag, the recombinant protein was firstly purified by an Ion Metal Affinity Chromatography (IMAC). The protein eluted at 125 mM imidazole (Figure 2.2A) and fractions containing $(His)_6$-ProSys were analysed by 15% SDS-PAGE (Figure 2.2B).

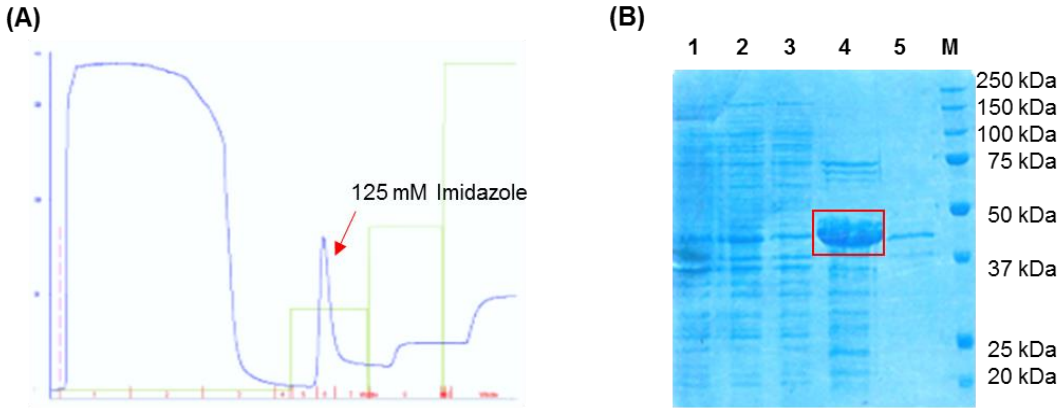

Figure 2.2: IMAC and 15% SDS-PAGE of $(His)_6$-ProSys. (A) $(His)_6$-ProSys eluted in 125 mM imidazole (red arrow). (B) 15% SDS-PAGE of the collected fractions; 1: total fraction, 2: soluble fraction, 3: unbound fraction, 4-5: $(His)_6$-ProSys, M: molecular weight marker.

After dialysis, the pooled fractions were further purified through an Ion Exchange Chromatography (IEC) at pH 8.0. The fractions, containing the recombinant protein eluted at 300 mM NaCl (Figure 2.3A), were analyzed by 15% SDS-PAGE gel (Figure 2.3B).

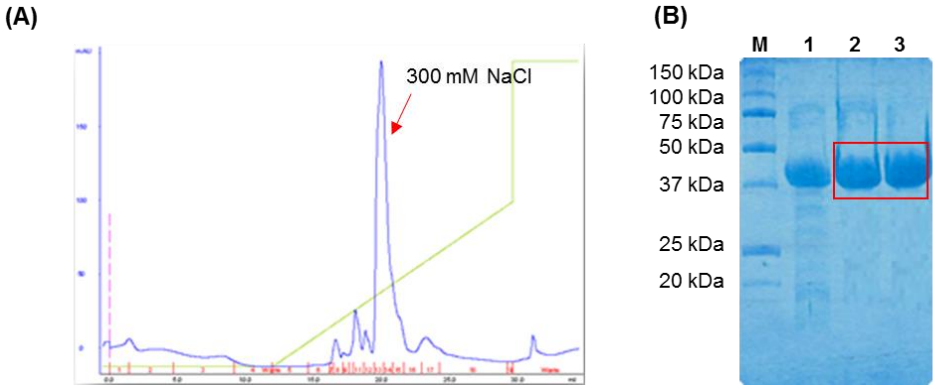

Figure 2.3: IEC and 15% SDS-PAGE of ProSys. (A) ProSys eluted at 300 mM NaCl (red arrow). (B) 15% SDS-PAGE of the collected fractions; M: marker of molecular weight, 1: input sample, 2-3: ProSys protein.

Finally, a Size Exclusion Chromatography (SEC) was performed in order to obtain a highly purified protein (>98% purity) suitable for subsequent analysis (Figure 2.4). A final yield of 4 mg/l of growth medium was achieved.

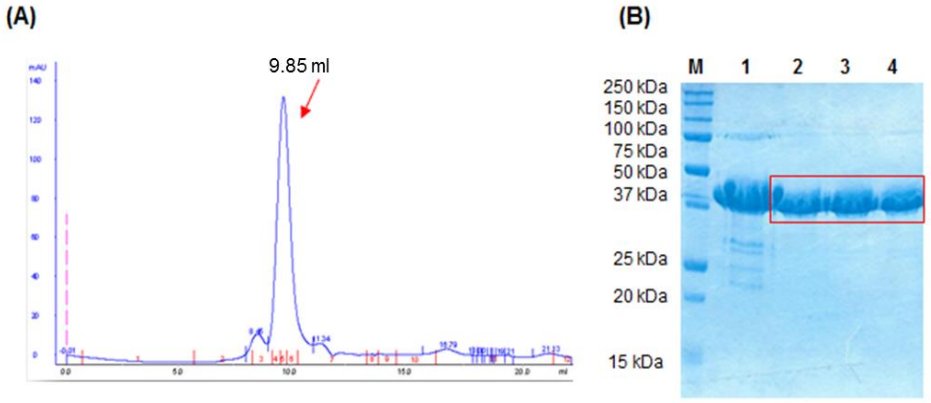

Figure 2.4: SEC and 15% SDS-PAGE of ProSys. (A) ProSys eluted at 9.85 ml (red arrow). (B) 15% SDS-PAGE of collected fractions; M: marker of molecular weight, 1: input, 2-3-4: eluted ProSys.

2.2.1.2 ProSys identification

Since the first step of purification, ProSys showed peculiar features which caused difficulties in its identification. Indeed, the recombinant protein showed an aberrant migration by SDS-PAGE, migrating as a protein with a molecular mass of 40 kDa greater than the expected molecular mass of 26 kDa (comprehensive of the $(His)_6$-tag) (Figure 2.5). A preliminary positive identification of the recombinant protein was carried out by western blot analysis with anti-$(His)_6$-tag antibodies (Figure 2.5B), but only by mass spectrometry analysis it was definitively confirmed that the purified protein was ProSys. In fact, mass analysis showed an experimentally evaluated

molecular mass of 26126.3 Da in agreement with the theoretical one (26125.9 Da) (Figure 2.6).
Indeed, the SDS-PAGE aberrant migration is compatible with the prediction of the SDS PAGE-displayed molecular weight of ProSys recombinant protein that has been estimated 9 kDa greater than the theoretically predicted one, using the equation recently described by Guan and his colleagues (2015).

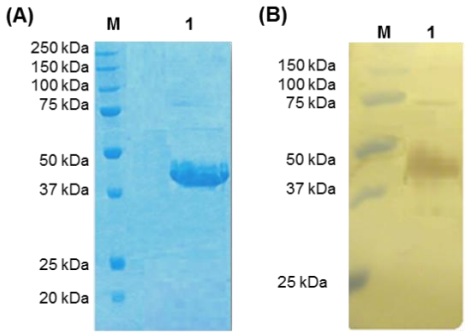

Figure 2.5: 15% SDS-PAGE (A) and western blot analysis (B) of ProSys pooled fractions. M: marker of molecular weight, 1: $(His)_6$-ProSys.

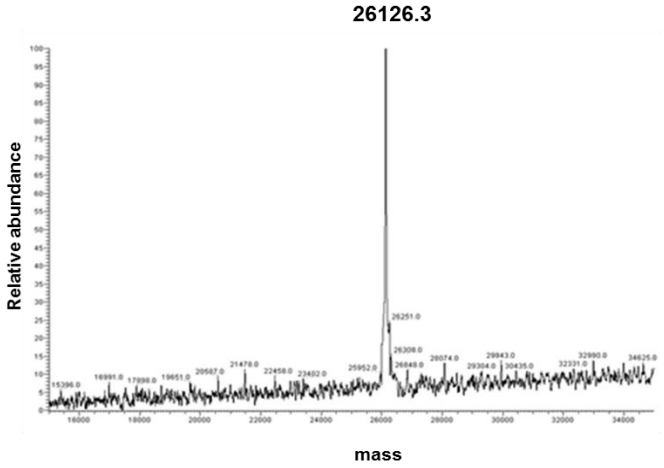

Figure 2.6:LC-ESI-MS analysis of recombinant ProSys: deconvoluted mass spectrum.

2.2.1.3 Light-scattering analysis

Furthermore, SEC analysis showed that the recombinant protein exhibited a retention volume of 9.85 ml indicative of a protein with an apparent molecular mass (MM_{app}) of about 71 kDa, as estimated by the calibration curve (Figure 2.7).

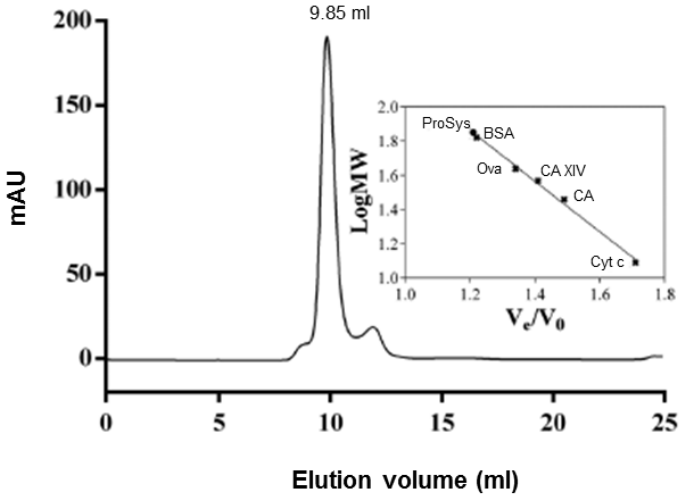

Figure 2.7: Elution profile of ProSys on a Superdex 75 10/300 SEC column. Inset, molecular mass deduced from the calibration curve.

Since this value was higher than the expected (MMtheo), it was suggested that ProSys was present in solution either as a folded trimeric protein or as a protein with a low compactness.
SEC equipped with Multi Angle Light Scattering and Quasi-Elastic Light Scattering (SEC-MALS-QELS) was used to investigate the oligomeric state of the recombinant protein. This analysis suggested that in solution ProSys occurs as a monodisperse monomeric protein with a molecular weight of 23.7 ± 0.1 kDa (Figure 2.8). Dynamic Light Scattering (DLS) showed an apparent hydrodynamic radius of 5.6 nm (data not shown), which is indicative of a protein with a scarce compactness. The same DLS measurements were carried out in presence of urea. In these analysis, ProSys showed an increase of the hydrodynamic radius to 8.6 nm (data not shown), suggesting that the protein in native conditions contains some residual structural content which is lost in a denaturing solution (Habchi *et al.,* 2010).

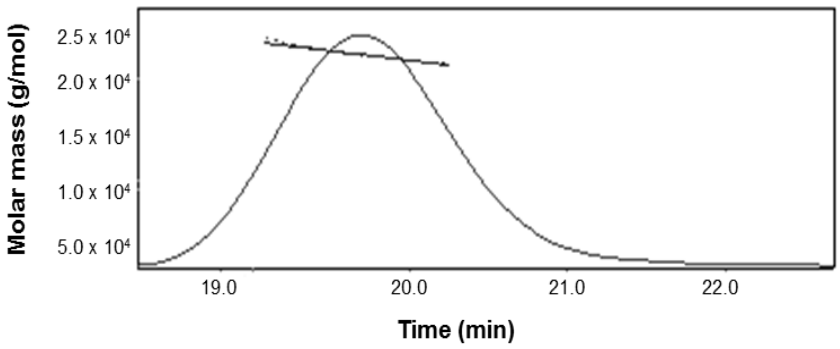

Figure 2.8: Molecular mass value of ProSys determined by SEC-MALS-QELS.

2.2.2 Spectroscopic features of ProSys and effects of temperature

The secondary structure content of ProSys was analyzed by means of circular dichroism (CD). The obtained Far-UV CD spectrum showed at neutral pH a low ellipticity value at 190 nm and a large negative ellipticity at 198 nm (Figure 2.9).

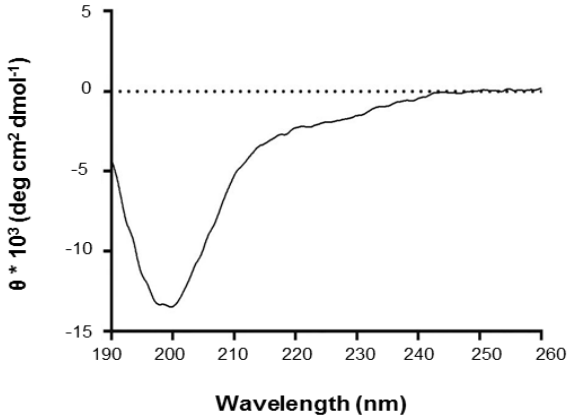

Figure 2.9: Far-UV CD spectrum of ProSys. CD spectrum was recorded in 10 mM phosphate buffer, pH 7.4 at a protein concentration of 4 µM.

These features are indicative of proteins in a largely disordered conformation. Furthermore, presence of residual secondary structure was consistent with the ellipticity values observed at 200 and 222 nm (Habchi *et al.,* 2010; Sun *et al.,* 2010; Marín *et al.,* 2012). According to Uversky (2002), extended disordered proteins can be divided into two different groups, premolten globule-like (PMG-like) group and random coil-like (RC-like) group based on the ratio of the ellipticity values at 200 and 222 nm. ProSys protein falls in the twilight zone between PMG-like and RC-like group, suggesting that the protein exhibits some residual secondary structure (Figure 2.10).

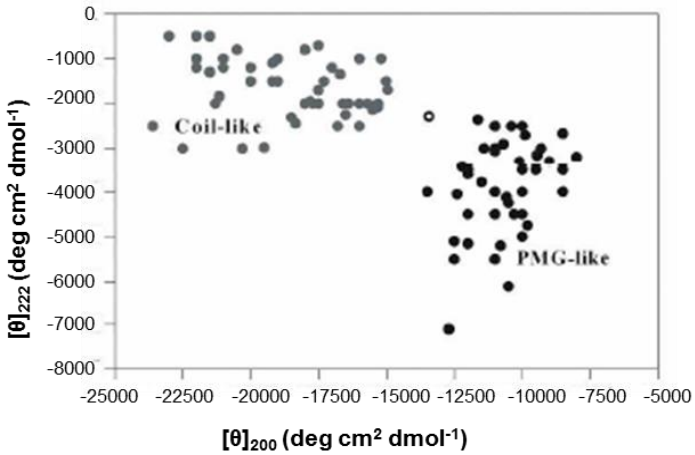

Figure 2.10: $[\theta]_{222}$ vs $[\theta]_{200}$ ellipticity plot modified from Uversky (Uversky, 2002); $[\theta]_{222}$ of a set of well-characterized coil-like (gray circles) and premolten globule-like subclasses (black circles) has been plotted against $[\theta]_{200}$. The position of ProSys is indicated with an empty circle.

This result is in agreement with data obtained by DLS analysis that revealed the presence of intramolecular interactions in native conditions, a typical behavior of proteins belonging to PMG-like group (Habchi *et al.,* 2010).
It was observed that ProSys undergoes also temperature-induced changes; as the temperature increases, modest but discernible far-UV CD spectral changes were evident, due to the formation of secondary structure (Figure 2.11).

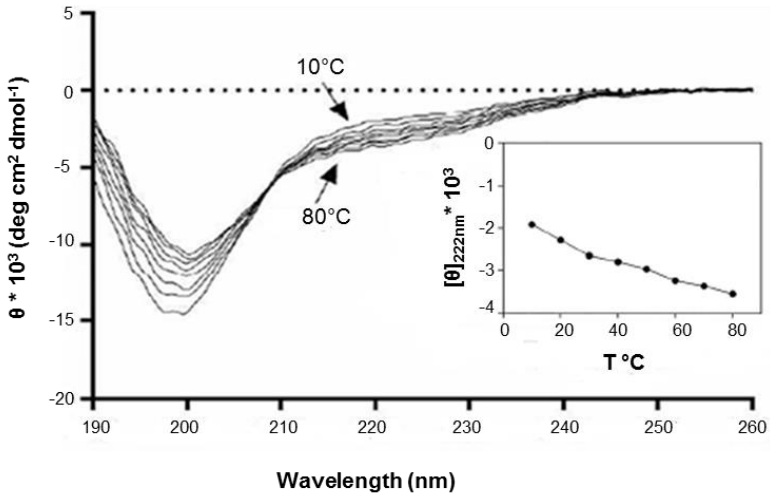

Figure 2.11: Temperature effect on ProSys (at 10°C, 20°C, 30°C, 40°C, 50°C, 60°C, 70°C and 80°C). CD spectra were recorded in 10 mM phosphate buffer, pH 7.4 at a protein concentration of 4 µM. The ellipticity $[\theta]_{222nm}$ versus temperature is shown in the inset.

The structural changes induced by heating were completely reversible and not driven by a cooperative behavior (inset Figure 2.11). Heat-induced structuring is a typical feature of IDPs, in contrast to globular proteins, which undergo unfolding upon heating (Uversky, 2002). The peculiar effect is likely due to the increased strength of the hydrophobic interactions occurring at high temperature, which act as a driving force for hydrophobic folding (Sun *et al.,* 2010; Uversky, 2002).

2.2.3 TFE induces folding of ProSys

It was further tested the propensity of ProSys to fold in water/trifluoroethanol (TFE) mixtures. TFE is often used to investigate propensities of proteins and peptides to fold because it can mimic a hydrophobic environment (Buck, 1998) typical of protein-protein interactions (Tantos *et al.,* 2013). To this aim, CD spectra were recorded at increasing concentration of TFE. When TFE was added, ProSys showed an increased alpha helical content as indicated by the characteristic maximum at 190 nm and the double minima at 208 and 222 nm (Figure 2.12).

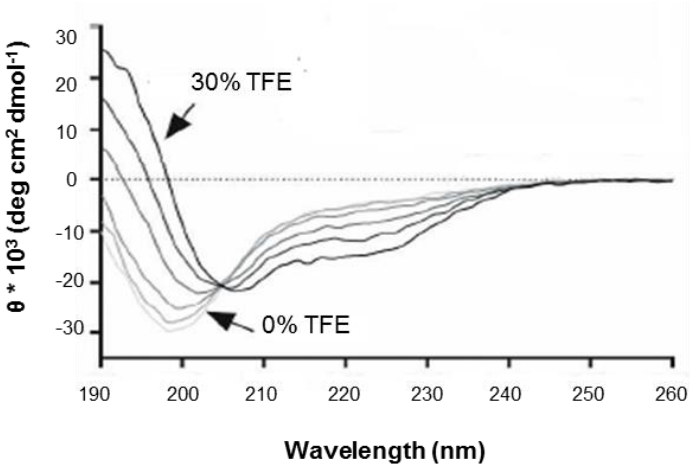

Figure 2.12: TFE induced folding of ProSys (at 0%, 5%, 10%, 15%, 20%, 30%). CD spectra were recorded in 10 mM phosphate buffer, pH 7.4 at a protein concentration of 4 µM.

In particular, a slight increase of alpha helical content was observed between 5 to 15% TFE, whereas most of the unstructured-to-structured transitions were observed between 20-30% TFE.

2.2.4 *In silico* analyses of the 'disorder tendency' of ProSys

Since previously discussed experimental data provide evidence that ProSys is an IDP not completely unfolded, but with a certain degree of compactness and a residual secondary structure, an extensive *in silico* analysis was performed in order to achieve disorder predictions. It is well known that ProSys has a unique amino acid sequence, rich of charged residues with some repetitive sequence elements (McGurl and Ryan, 1992). However, an in-depth sequence analysis aiming at studying the sequence-structure relationship of ProSys is lacking. For this reason, different tools available *in silico* were used for an in-depth analysis of ProSys structure. Firstly, ProSys primary sequence was compared to that of proteins within the Swiss-Prot database (Habchi *et al*., 2010) by Composition Profiler, which allows to identify statistically significant patterns of amino acid enrichment or depletion along the sequence (Vacic *et al.*, 2007). As can be seen from Figure 2.13, ProSys showed a peculiar amino acid composition, depleted in the so-called "order promoting" residues, that include Cys, Asn, Leu, Val, Trp, Phe, and Tyr which are regularly represented in the hydrophobic core of folded globular proteins (Uversky, 2013). On the contrary, it is enriched in most "disorder promoting" residues such as Gln, Asp, Glu, Lys and Pro.

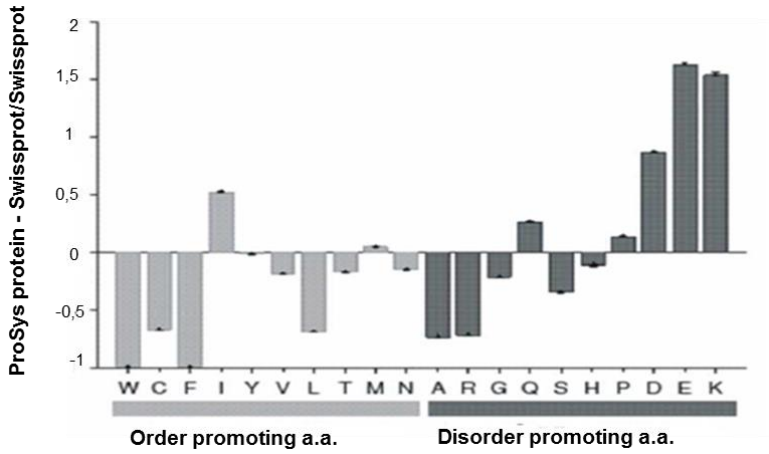

Figure 2.13: Sequence properties of ProSys. Amino acid composition analysis performed by means of Composition Profiler tool. ProSys sequence is compared to the reference value of the average amino acid frequencies of the Swiss-Prot database (Vacic *et al.,* 2007). Bar heights indicate enrichment or depletion of indicated residue.

All these features suggested that ProSys behaves as an intrinsically disordered protein. Moreover, the high number of charged residues (Asp, Glu, and Lys) and the lack of hydrophobic residues (Trp, Phe), or their scarceness (Tyr), are indicative of a high net charge and a low mean hydrophobicity, respectively. When correlating these two parameters (Uversky *et al*., 2000) ProSys is predicted to be a disordered protein (Figure 2.14).

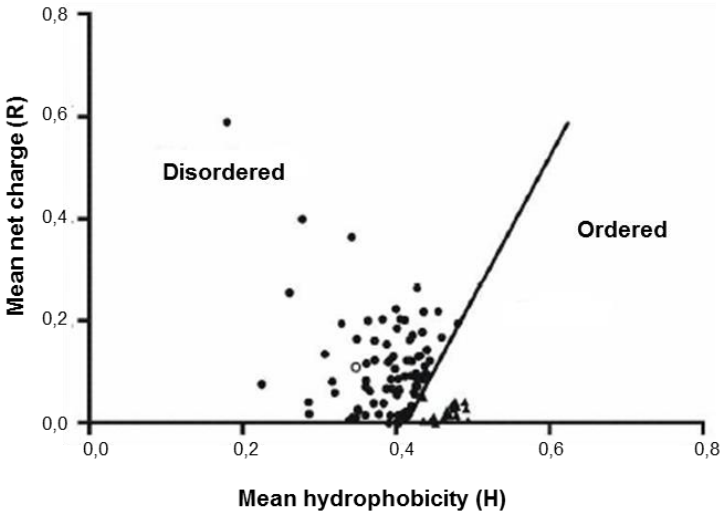

Figure 2.14: Sequence properties of ProSys. Charge-Hydrophobicity plot generated as described by Uversky, (2002). Black dots, intrinsically disordered protein reported in literature (data partially taken from Uversky, 2002), black triangles, natively folded proteins randomly taken from PDB. Solid black line, border between the ID and the natively folded proteins, described by the equation H=(R+1.151)/2.785, where H and R are the mean hydrophobicity and the mean net charge, respectively. Empty black circle, ProSys.

Furthermore, other *in silico* tools such as PONDR-FIT (Xue *et al*., 2010) and DisMeta (Huang *et al*., 2014) were used to predict the degree of disorder in the sequence.

The obtained results suggest that ProSys is mainly disordered with local propensities to order, as indicated by the disorder score close or below 0.5 in regions 75-105 and 160-180 which belong to the Central and the C-terminal part of ProSys, respectively (Figure 2.15). These results are in agreement with previously described evidences that suggested that ProSys is a PMG-like protein, thus not completely unfolded but containing some residual secondary structure and some degree of compactness.

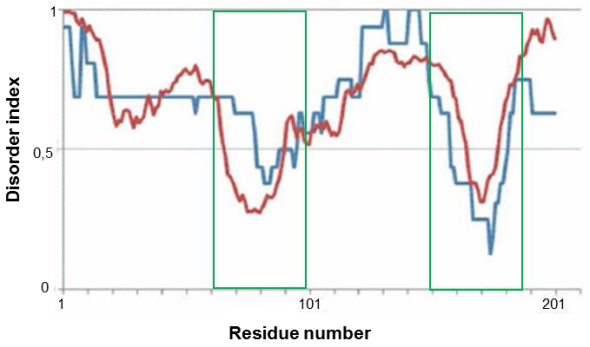

Figure 2.15: Sequence properties of ProSys. Predictions of intrinsic disorder by PONDR-fit (red line) and DisMETA (blue line) predictors. Values higher than 0.5 indicate a propensity for disorder, and lower than 0.5 indicate a propensity for order. Regions with a disorder score close or below 0.5 are indicated with green boxes.

The intrinsically disordered features of Prosys which came out upon biochemical and bioinformatics investigations, made it impossible to perform X-Ray crystallographic studies. Thus, further structural characterization was carried out by means of *in silico* tools.

Secondary structure calculations were performed using different predictors: PSIPRED (Buchan *et al*., 2013), SSPro (Pollastri *et al*., 2002) and QUARK (Xu and Zhang, 2012). Results shown in Figure 2.16 indicate that most of the sequence is random coil with local tendencies to assume a regular secondary structure (α-helix or β-strand), mainly in the Central and C-terminal region of the protein, consistently with the above discussed disorder predictions.

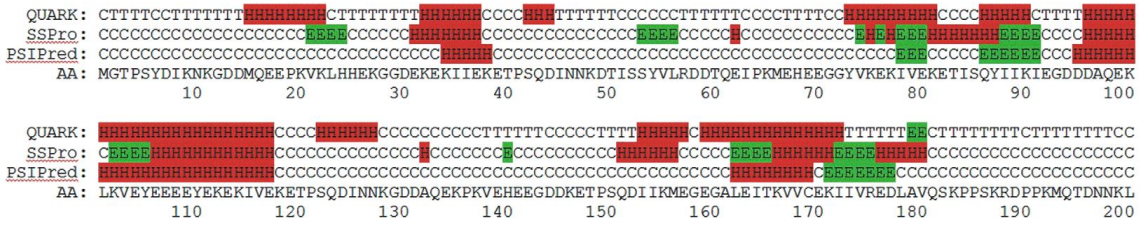

Figure 2.16: Structure predictions of ProSys. Secondary structure predictions by QUARK, SSPro and PSIPred. The amino acid (AA) sequence is also shown. H: helix, E: strand, C: coil, T: turn.

Finally, predictions of the three-dimensional structure of ProSys were carried out using QUARK *ab initio* server (Figure 2.17) (Xu and Zhang, 2012).

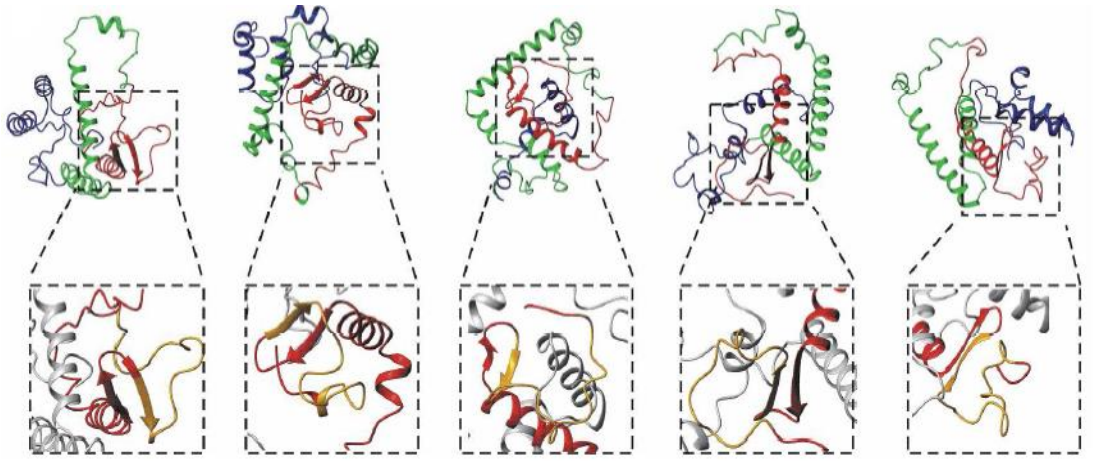

Figure 2.17: Structure predictions of ProSys. Ribbon representation of five out of the ten 3D-structure models predicted by QUARK *ab initio* server for ProSys full-length. N-terminal region (1-70) is in blue; Central region (71-140) is in green; C-terminal region (140-200) is in red. Details of beta-hairpin (172-180) which precedes the Sys polypeptide in the C-terminal region (140-200) of ProSys in each model have been highlighted in dashed boxes. The region corresponding to Sys polypeptide is in orange.

The models generated by QUARK have a quite low TM-score value (TM-score = 3.1), which indicates no single-high probability model for a specific fold but an ensemble of energetically similar conformations. This finding further suggested that ProSys is an IDP, devoid of a unique well defined global fold, possessing long disordered regions. Moreover, the analysis of the structural models provided insights into the local propensity of protein regions to assume a regular secondary structure. In details, in all the models we found that: *i*) the N-terminal region (blue ribbon in Figure 2.17) is highly disordered, having long random coil segments; *ii*) the central part of the protein (green ribbon in Figure 2.17) consists of two long V-shaped α-helices with a varying inter-helices angle; *iii*) the C-terminal region (red ribbon in Figure 2.17) displays a long random coil segment and an α-helix/β-hairpin motif which precedes the disordered Sys region. According to the QUARK models (Figure 2.17), these three regions can be reciprocally oriented in many different ways, leading to an ensemble of possible conformations.
Notably, there is a good agreement between the different *in silico* predictors used, indicating that ProSys is mainly unstructured and possesses some residual secondary structure elements in the Central and C-terminal region of the protein.

2.3 Discussion

In this study different biochemical and bioinformatic approaches were used for a detailed characterization of the recombinant ProSys. The pro-hormone, which does not require any post-translational modifications, was produced as bacterial recombinant protein, overcoming the expression problems encountered in earlier studies (Delano et al., 1999). Indeed, previous data on bacterial expression of the full-length pro-hormone showed many problems due to the presence of a classic bacterial start site (Shine-Delgardo sequence) just upstream of an internal ATG codon (Délano *et al.,* 1999). This sequence was probably responsible of a directed translation of a truncated form of ProSys (ProSys 185) missing of first 14 initial amino

acids. Moreover, a high degree of nucleotide complementary in the 5' coding region of ProSys with the ribosome binding site of the pET 11d expression vector contributed to the low production of the expressed protein.
These problems were partially solved by producing a mutated protein (Met15Ala) and by introducing six conservative mutations in the 5' coding region of the ProSys sequence (Délano *et al.,* 1999). The present study shows that the use of pETM11 vector allowed the expression of the full-length protein in *E. coli;* highly purified ProSys was obtained at high yields (4 mg/l culture). The advantages of fast growth at the high density in an inexpensive medium enable *E. coli* to offer a mean of a rapid, high yield, and economical production of a recombinant protein that does not require any post-translational modification.
Since the beginning, the purified recombinant protein showed an unusual experimental behavior. A difference between the theoretically predicted and the SDS PAGE- displayed molecular weight was observed likely due to the high content of the acidic residues within the aminoacidic sequence which might be responsible of its scarce binding to SDS (Tompa, 2002; Alves *et al.,* 2004; Habchi *et al.,* 2010). Obtained results fitted the prediction of SDS-PAGE displayed molecular weight as calculated by the equation y=276.5x-31.33 where x represents the percentage of acidic residues and y the average of the variation of the molecular weight per residues (Guan et al., 201). SEC-MALS-QELS and native DLS analysis revealed the lack of a globular structure within the protein. These results were in agreement with CD and non-native DLS investigations which confirmed the presence of a largely disordered conformation with few secondary structure elements. All these results show, for the first time, that ProSys is an Intrinsically Disordered Protein (IDP) having within its sequence some residual secondary structure which is typical of PMG-like proteins. To gain further insights into the structural features of the pro-hormone, a computational analysis was performed. The different predictors used to assess the degree of disorder along the sequence, as well as the secondary and tertiary structure agreed that ProSys is a highly disordered protein with few regular structural elements in the central (sequence 75-110) and C-terminal region (sequence 160-180).
IDPs are biologically active proteins although lacking an ordered structure under physiological conditions. Indeed, IDPs do not possess a unique three-dimensional structure, but are entirely or in part disordered (Dyson and Wright., 2005; Uversky and Dunker, 2010). However, their unstructured state is crucial for their biological function (Dyson and Wright., 2005; Diella *et al.,* 2008; Uversky and Dunker., 2010). IDPs/IDRs show a wide diversity in their degree of disorder; indeed they can exhibit collapsed disordered conformations with some regions of fluctuating secondary structure (thus, resembling a molten globule) or can attain an extended-disordered state (random coil-like) (Daughdrill *et al.,* 2005; Cortese et al., 2008; Uversky and Longhi, 2011). The latter group can be also expanded into two subgroups. The first one comprises proteins devoid of any ordered secondary structure, while the second group consists of so-called premolten globules, containing some hydrophobic cluster and some residual secondary structure (Cortese et al., 2008; Uversky, 2014; Habchi *et al.,* 2014). Proteins harboring long stretches of intrinsic disorder are key components of signal transduction cascades. Notably, IDPs may display binding plasticity by accommodating diverse binding sites of different partners during protein interactions and may undergo binding-induced folding. As a result of the disorder-to-order transition, IDPs adopt a preferred ordered conformation upon binding to their biological partners (Dyson and Wright., 2002; Fuxreiter *et al.,* 2004; Uversky *et al.,*

2005). Accordingly, CD experimental results show that ProSys adopts an ordered three-dimensional structure upon TFE addition, exhibiting alpha-helices formation. This result suggests that a large part of Prosys consists of intrinsically disordered regions (IDRs) which may undergo a disorder-to-order transition upon binding an interaction partner (Dyson and Wright, 2002; Uversky, 2002). Therefore, it appears that ProSys, as other IDPs, is able to interact with different molecular partners produced in the plant under stress conditions providing a fast mechanism to obtain complex, interconnected and versatile molecular networks underpinning defense responses (Chavali *et al.,* 2017).
Moreover, computational analysis predicted the presence of a β-harpin motif, beside the Sys sequence at the C-terminal region of ProSys (Figure 2.17). It is tempting to speculate that this structural motif could represent a recognition site for hormone-releasing enzymes responsible for the cleavage and release of Sys. But until now little is known about the molecular mechanism of Sys release from the precursor.

2.4 Materials and Methods

2.4.1 Materials

Expression host strain *E. coli* BL21(DE3) and plasmid pETM11 were a kind gift from EMBL, Heidelberg. *E. coli* strain TOP10F' was obtained from Invitrogen (San Diego, CA, USA). QIAprep spin miniprep kit was from Qiagen (Germantown, MD, USA). PCR Clean-Up DNA Purification System for elution of DNA fragments from agarose gel was purchased from Qiagen (Germantown, MD, USA). Enzymes and other reagents for DNA manipulation were from New England Biolabs (Ipswich, MA, USA), from Promega (Madison, WI, USA) and from Qiagen (Germantown, MD, USA). All other chemicals were from Sigma-Aldrich (Milano, Italy).

2.4.2 Production of the recombinant ProSys protein

2.4.2.1 Cloning of ProSys protein in pETM11 vector

The full-length ProSys cDNA (Genebank: AAA34184.1) was amplified by PCR using forward primer:
5'-CGCGCG<u>CCATGG</u>GAACTCCTTCATATGATATCA-3'
and reverse primer:
5'-CGCGCG<u>CTCGAG</u>TTACTAGAGTTTATTATTGTCTGTTTGCATTTTGG-3',
that incorporated desired restriction sites at the 5'- ends, respectively Nco I and Xho I (underlined). The PCR product was digested with Nco I and Xho I and gel purified using QIAquick PCR Purification Kit (Qiagen). The purified product was then directionally ligated to Nco I/Xho I double digested expression vector pETM11.
The generated plasmid was transferred into TOP10F' electrocompetent *E. coli* cells and the nucleotide sequence of ProSys cDNA was checked by DNA sequencing.
The recombinant plasmid was isolated from the overnight grown positive clone using QIAprep Spin Miniprep Kit (Qiagen).

2.4.2.2 Large-scale expression

The expression of the recombinant protein was performed using the BL21(DE3) *E. coli* host cells in Luria Bertani (LB) medium. Single clones of *E. coli* strain, chemically transformed with the recombinant expression vector and grown at 37°C on LB agar

containing the appropriate antibiotic, were inoculated into 2 ml of LB medium and after 3 hours transferred in 10 ml of pre-warmed growth medium.
Once the optical density (OD) had reached the 0.5/0.6 at 600 nm, this was in turn inoculated into 800 ml of pre-warmed LB medium.
The cell culture was grown at 37°C under shaking until it reached 0.5/0.6 OD at 600 nm and the expression was induced with 2.0 mM IPTG. The temperature was then decreased to 22°C and the growth continued for 16 hours.
After centrifugation (20 minutes at 6000 g at 4°C) the induced bacterial pellet was dissolved in the lysis buffer [20 mM Tris-HCl pH 8.0, 20 mM imidazole, 50 mM NaCl, 1 mM Dithiothreitol (DTT), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 5 mg/ml DNase I , 0.1 mg/ml lysozyme and 1 µg/ml Aprotinin, 1 µg/ml Leupeptin, 1 µg/ml Pepstatin protease inhibitors (Sigma-Aldrich)], incubated with shaking at room temperature for 30 minutes then stored on ice and sonicated at 9 W amplitude for 10 minutes with a pulsing cycle of 10 second on/10 second off, using a Misonix Sonicator 3000 apparatus with a macro tip probe. The sonicated suspension was then centrifuged for 30 minutes at 30000 g at 4°C and the supernatant (soluble fraction) collected and analyzed by SDS-PAGE to verify the presence of the recombinant protein of interest. The difference between the predicted and SDS PAGE-displayed molecular weights of Prosys was calculated from the equation y = 276.5x − 31.33, where x is the percentage of acidic amino acids ranging from 11.4% to 51.1% while y is the average ΔMW per amino acids, as recently reported (Guan *et al.,* 2015). In the case of Prosystemin x is 27,5% and the number of residues is 226.

2.4.2.3 Purification of the recombinant protein

The soluble fraction was loaded onto a pre-equilibrated nickel-immobilized affinity chromatography column (1 ml His Trap FF column, GE Healthcare) and purified by FPLC, using an ÄKTA system. The elution was performed using an imidazole gradient combining buffer [A] (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole, 1 mM DTT and 100 µM PMSF) and buffer [B] (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 500 mM imidazole, 1 mM DTT and 100 µM PMSF) according to the manufacturer's instruction (GE Healthcare). The purity level of the eluted protein was assessed by 15% SDS-PAGE, using Biorad Precision Plus Protein All Blue Standards (10-250 kDa) as molecular mass marker. The purified proteins were dialyzed in 20 mM Tris-HCl, 50 mM NaCl, 0.1 mM PMSF, 1 mM DTT pH 8.0 using a membrane with a molecular weight cut-off (MWCO) of 3500 Da for 16 hours at 4°C.
The protein was further purified by an anion exchange chromatography, associated with an ÄKTA system, using a MonoQ 5/5 HR column (GE Healthcare) and two different buffers: [A], 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM DTT, 100 µM PMSF and [B] 20 mM Tris-HCl pH 8.0, 1 M NaCl, 1 mM DTT, 100 µM PMSF. The purity level of the eluted protein was assessed by 15% SDS-PAGE, using Biorad Precision Plus Protein All Blue Standards (10-250 kDa) as molecular mass marker.
The last step of purification was performed using a Size Exclusion Chromatography (SEC) utilizing a Superdex 75 10/300 HP column (GE Healthcare) connected to an ÄKTA FPLC. SEC was performed using 20 mM Tris-HCl, 150 mM NaCl, 100 µM PMSF, 1 mM DTT buffer, pH 8.0. Calibration was carried out using the following standard (Sigma Aldrich, St. Louis, MO, USA): horse cytochrome c (12400 Da), chicken ovalbumin (45000 Da), bovine serum albumin (66400 Da), carbonic anhydrase from bovine erythrocytes (29000 Da) and recombinant carbonic anhydrase XIV (37000 Da, homemade). The purity level of the eluted protein was

assessed on 15% SDS-PAGE, using Biorad Precision Plus Protein All Blue Standards (10-250 kDa) as molecular mass marker.
The purified protein was finally extensively dialyzed in PBS 1X (Phosphate buffer saline, 10 mM phosphates, 140 mM NaCl, 2.7 mM KCl, pH 7.4, Sigma-Aldrich) at 4°C.

2.4.3 Biochemical and biophysical characterization of ProSys

2.4.3.1 Western Blot analysis

Pooled purified protein separated by 15% SDS-PAGE, was transferred to a Immun-Blot® PVDF membrane (BIO-RAD) in Blotting Buffer (19 mM Tris-HCL pH 8.0, 140 mM glycine and 20% methanol). Before transfer, SDS-gel and PVDF membrane were equilibrated in Blotting Buffer for 10 minutes. After the electroblotting, the membrane was blocked with 5% skim milk (BioRad) in TTBS 1X buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.3% Tween 20) for 1 hour at room temperature. After the incubation, the membrane was washed for three times with TTBS 1X buffer and then incubated with the 1:1000 diluted α-His-mouse antibody HRP (Horse Radish Peroxidase) conjugated for 1 hour at room temperature. Finally, the membrane was washed for three times with TTBS 1X buffer. The detection was performed using Opti-4CN™ Colorimetric Kit (Biorad) following the manufacturer's instructions.

2.4.3.2 LC-ESI MS experiments

ProSys purity and molecular weight were checked on LC-ESI-MS system comprising a LCQ DECA XP ion trap mass spectrometer (Thermo Electron Corporation), equipped with an OPTON electrospray source and a complete Surveyor HPLC system.
Analyses were performed loading sample on a 300 Å narrow bore 250x 2 mm Jupiter C4 column (Phenomenex, Torrance) and applying a linear gradient of [B] buffer (0.05% TFA in CH_3CN) in [A] buffer (0.08% TFA in H_2O) from 30% to 70% in 40 minutes. Mass spectra were recorded continuously in the mass range 400 to 2000 amu, in 27 positive mode (LC-MS, condition 1). Multicharge spectra were deconvoluted using the Biomass program implemented in the Bioworks 3.1 package provided by the manufacturer's instruction. Mass calibration was performed automatically by means of selected multiple charge ions, in the presence of a calibrant agent (UltraMark; Thermo Electron Corporation). All masses were reported as average value.

2.4.3.3 Light-scattering

Multi-angle light scattering analysis of the protein was performed by combining SEC with MALS-QELS equipment.
In particular, the experiments were run at 0.5 ml/min in 20 mM Tris-HCl, 50 mM NaCl, 100 µM PMSF, 1 mM DTT pH 8.0 using a Superdex 75 10/300 GL (GE Healthcare) column linked to an FPLC ÄKTA coupled to a light scattering detector (mini-DAWN TREO, Wyatt Technology) and to a refractive index detector (Shodex RI-101). All data collected were processed using the ASTRA 5.3.4.14 software (Wyatt Technologies Corporation).
Dynamic light scattering (DLS) measurements were obtain using a Malvern nano zetasizer (Malvern, UK). The sample with a concentration of 0.4 mg/ml was placed in

a disposable cuvette and held at 25°C during analysis. Each sample was recorded six times with 11 sub-runs using the multimodal mode. The Z average diameter was calculated from the correlation function using the Malvern technology software.
The same experiment was carried out in presence of urea 7.4 M (Sigma-Aldrich).

2.4.3.4 Circular dichroism spectroscopy

All circular dichroism (CD) spectra were recorded using a Jasco J-715 (Easton, MD) spectropolarimeter equipped with a Peltier temperature control system (Model PTC-423-S). Molar ellipticity per mean residue, [θ] in deg cm^2 x $dmol^{-1}$, was calculated from the equation: $[\theta]= [\theta]_{obs}$ x mrw/10 x l x C, where $[\theta]_{obs}$ is the ellipticity measured in degrees, mrw is the mean residue molecular mass, C is the protein concentration in mg X ml^{-1} and l is the optical path length of the cell in cm. Far-UV measurements (from 190 to 260 nm) were carried out at time constant 4 seconds, 2 nm band width, scan rate of 20 nm min^{-1}, using an Hellman quartz cell of 0.1-cm-path length and a protein concentration of 4 µM in 10 mM sodium phosphate buffer pH 7.4. CD spectra were signal averaged over at least three scans, and the baseline was corrected by subtracting a buffer spectrum.
The same parameters were applied to perform measurements in the temperature range of 10-80°C and titration with increasing concentration of trifluoroethanol (TFE) from 5% to 30%. DICHROWEB was used to analyze data. CDSSTR was used as a deconvolution method to evaluate the percentage of α-helical content of the protein.

2.4.3.5 Bioinformatic sequence analysis and ab initio modeling

Composition Profiler tool (http://www.cprofiler.org) was used to perform the amino acid compositional analysis by comparing the ProSys sequence with the reference value of the average amino acid frequencies of the Swiss-Prot database (http://us.expasy.org.sprot) (Vacic *et al.,* 2007).
Composition analysis was carried out using the relation (CPX –CSX)/CSX where CPX means the content of an amino acid X within the protein of interest whereas CSX is the typical composition of X in SWISS-PROT proteins.
The Charge-Hydrophobicity (CH) plot was generated using data reported in literature as described by Uversky (Uversky, 2002) (data taken from Uversky *et al.,* 2000 for the intrinsically disordered protein, and randomly taken from PDB for natively folded proteins). The mean net charge <R> and the mean hydrophobicity (H=(R+1.151)/2.785) were determinated using the protParam program at the EXPASY server (http://us.expasy.ch/tools) (Habchi *et al*., 2010). In particular, R was calculated as ratio of the absolute value of the difference between the number of positive and negative charged residue at neutral pH to the total number of the amino acid residues. Whereas, H was defined as the ratio between the sum of the normalized hydrophobicities of each residue and the total number of amino acid residues minus 4 residues (to take into account fringe effects in the calculation of hydrophobicity). Individual hydrophobicities values were estimated using Protscale program at the Expasy Server (http://www.expasy.ch/tools), with the options “Hphob/Kyte&Doolittle”, window size of % and normalizing the scale from 0 to 1 (Habchi *et al.,* 2010). Results obtained were compared with those of proteins already studied by the CNR group (i.e. CDCA1-R3 (Alterio *et al.,* 2012), PhEST (Alterio *et al.,* 2010), blLAP (Cappiello *et al.,* 2006), EST2 (De Simone *et al.,* 2000), AFEST (De Simone *et al.,* 2001), hCA IX-domain (Alterio *et al.,* 2009),hCA II (Di Fiore *et al.,*

2015), hCA VII (Di Fiore *et al.,* 2010), hCA XIV (Alterio *et al.,* 2014), ANK (Di Lelio *et al.,* 2014) and with two intrinsically disordered proteins already reported in literature [α-Prothymosin (Uversky *et al.,* 1999) and Chromogenin A (Uversky *et al.,* 2000)].
The disorder predictions were carried out using two meta-predictors PONDR-FIT (Xue *et al.,* 2010) and DisMeta (Huang *et al*., 2014), which perform a combined consensus prediction. Secondary structure calculations were performed using different predictors: PSIPRED (Buchan *et al*., 2013), SSpro (Cheng *et al*., 2005) and QUARK (Xu and Zhang, 2012; Buonanno, 2017). Three-dimensional structure of ProSys full-length was predicted by QUARK *ab initio* server (Xu and Zhang, 2012). QUARK program builds 3Dstructure models by replica-exchange Monte Carlo simulation under the guide of an atomic-level knowledge-based force field.

Chapter 3

Identification and characterization of ProSys regions with a putative biological activity

Donata Molisso in collaboration with Emma Langella, Simona Maria Monti and Rosa Rao

Abstract

Intrinsically Disordered Proteins (IDPs) are functionally important proteins lacking a stable or ordered three-dimensional structure. Despite being highly flexible, it has been demonstrated that IDPs have crucial roles in signal transduction process, cell-cycle regulation, gene expression and molecular recognition. The role of IDPs in these processes has been systematically studied in the animal kingdom. In contrast, less reports of these proteins from the plant kingdom are available in the scientific literature. Our results (Chapter 2) evidenced for the first time that ProSys behaves like an IDP possessing ID regions (IDRs). These results, together with the recent finding that ProSys devoid of Sys still retains biological activity (Corrado *et al.,* 2016), prompted us to continue our study of ProSys. Indeed, we decided to study (isolate) different regions of the protein (ProSys Fragments) in order to check for their biological activity. These fragments were fully characterized also from a structural point of view, aiming to establish a structure-activity relationship. To this purpose four different regions of ProSys have been expressed in BL21 (DE3) *E. coli* cells, purified and then characterized by means of biophysical and biochemical tools. Results showed that the recombinant fragments are disordered in agreement with what previously shown for the whole precursor. These fragments have been subsequently tested for their biological activity (Chapter 4).

3.1 Introduction

Most proteins need to assume a unique and stable three-dimensional structure to carry out their specific function. However, in the last twenty years, several experimental and computational data showed a large fraction of proteins which does not adopt a defined three-dimensional structure in physiological conditions but are nevertheless functional. These proteins are known as Intrinsically Disordered Proteins (IDPs), either being completely unfolded or containing ordered domains and ID Regions (IDRs) (Dyson and Wright, 2002; Tompa *et al.,* 2009). IDPs are generally characterized by a reduced level of the so-called order-promoting amino acids such as Ile, Leu, Val, Trp, Tyr, and Phe, which usually form the hydrophobic core of folded globular proteins, and an increased level of the so-called disorder-promoting amino acids, such as Arg, Gly, Ser, Glu, Lys, Pro, and Ala (Uversky, 2013).
The lack of structure does not affect their biological function since IDPs/IDRs may undergo a binding-induced folding upon interaction with their partners (Uversky, 2013). Such binding-induced folding is further characterized by the presence of short protein segments, so-called short linear motifs (SLiMs) located within extended disordered regions, so-called molecular recognition features (MoRFs) which are able to recognize the interacting partners thus playing a key role in initiating a disorder-to-order transition (Cheng *et al.,* 2007; O'Shea *et al.,* 2017). This transition has been proposed to allow a great binding flexibility and promiscuity conferring the ability to adapt IDP/IDR biding site to structurally different and/or multiple partners with relatively high specificity and low affinity (Dyson and Wright, 2005). These structural and biochemical properties are typical of proteins that play essential roles in cellular functions such as signaling transduction, transcriptional and translation activation and chromatin remodeling (Dyson and Wright, 2005; Radivojac *et al.,* 2007; Cortese *et al.,* 2008).

The role of IDPs/IDRs in these processes has been systematically studied in mammalian systems. However, only few experimental confirmation of disorder predictions from the plant kingdom are available (Kragelund *et al.,* 2012; Sun *et al.,*2013). For instance, plant-specific GRAS proteins are transcriptional regulators in the early stages of plant defense signaling (Day *et al.,* 2003; Czikkel and Maxwell, 2007; Sun *et al.,* 2012); another example is given by a vast majority of LEA family members which are involved in the sequestration of small molecules (such as sugars, ions and reactive oxygen species) and protection of membrane enzymes conferring tolerance to dehydration, cold and salinity stresses (Tolleter *et al.,* 2007; Haaning *et al.,* 2008; Boucher *et al.,* 2010; Rahman *et al.,* 2010; Tolleter *et al.,* 2010). In our case, the detailed characterization of ProSys by means of a multidisciplinary approach (Chapter 2) revealed for the first time that this pro-hormone is a member of the IDP family, giving new insights on the multiple roles of ProSys in tomato defense mechanisms (Orsini *et al.,* 2010; Coppola *et al.,* 2015). In order to investigate a putative role of the different regions of ProSys and to investigate the structure/function relationship of the different regions of the protein, four Fragments of ProSys (hereafter referred as Fragment I, II, III, IV) were designed and characterized from a biochemical (this chapter) and functional (Chapter 4) point of view. The design of the fragments was made in accordance with the results of modeling analysis made on the whole pro-hormone (Chapter 2) as well as with the experimental laboratory feasibility.

3.2 Results

3.2.1 Design, cloning, expression and purification of ProSys Fragments

According to the models previously obtained by Quark *ab initio* server (paragraph 2.2.4), four different fragments of ProSys, namely Fragment I, III (ProSys regions that not contain Sys sequence) and Fragment II and IV (ProSys regions that contain Sys sequence) were designed (Figure 3.1), cloned and produced by means of heterologous expression in *E. coli*. cDNA encoding each ProSys fragment was cloned in pETM11 expression vector, expressed in bacterial strain BL21(DE3) cells and purified by IMAC.

The pooled fractions were dialysed and further purified by SEC at pH 7.4 (Figure 3.3).

3.2.2 Biochemical and structural characterization of ProSys Fragments

3.2.2.1 Light-scattering analysis

3.2.2.2 Spectroscopic features of Fragments I-IV and effects of temperature

The secondary structure content of Fragments I-IV was analyzed by means of circular dichroism (CD).

The propensity of ProSys fragments to fold in water/trifluoroethanol (TFE) mixtures was tested collecting spectra at increased concentration of TFE.

3.2.2.3 *In silico* analysis of ProSys fragments

As expected, the recombinant products showed a disordered behavior in solution.

3.3 Discussion

Due to the “hub role” of ProSys in plant defense mechanisms, it was hypotized that ProSys could have a biological function other than being a simple intermediate in the synthesis of Sys. To this aim we designed, produced and characterized four recombinant fragments which encompassed the whole precursor sequence to be used for further biological activity assays (Chapter 4). Indeed, the knowledge of the biochemical and structural features of the biomolecules to be used for biological assays is a fundamental step for the rational comprehension of any obtained results.
The fragments were cloned in pETM11 vector, expressed heterologously in BL21(DE3) *E. coli* cells and highly purified with a final yield ranging from 0.7 to 2 mg/l.

3.4 Materials and Methods
3.4.1 Materials

The same materials as those described in the paragraph 2.4.1 were used for these experiments.

3.4.2 Production of recombinant ProSys fragments
3.4.2.1 Cloning and expression and purification

According to data obtained by models generated by QUARK (see paragraph 2.2.4), ProSys cDNA was amplified using different synthetic primer sets (Table 3.1) in order to obtain 4 different ProSys fragments.

Table 3.1 List of primer sequences, expected amplicon size and number of PCR cycles used

*Nco I restriction site is underlined; Xho I restriction site is italicized and underlined**.**

These fragments were cloned into pETM11 vector using Nco I and XhoI restriction sites. The generated plasmids were transferred into TOP10F' electrocompetent *E. coli* cells and the nucleotide sequences were checked by DNA sequencing. The recombinant plasmids were isolated from the overnight grown positive clone using QIAprep Spin Miniprep Kit (Qiagen). The recombinant constructs were expressed in *E. coli* BL21(DE3) cells in different selective media. In particular it was observed that ProSys fragments showed high expression levels in different media, in particular LB was optimal for Fragment I and IV, SOB for Fragment II and 2-YT for Fragment III. Induction was performed with a final concentration of 2 mM IPTG for Fragment I-IV. The expression was carried out at 22°C for 16 hours. Cells were harvested by centrifugation (20 minutes at 6000 g at 4°C) and the final cell pellets were stored at -80°C until use. Purification of the recombinant fragments was carried out as described in Par 2.4.2.3

3.4.3 Biochemical and biophysical characterization of ProSys fragments

3.4.3.1 Western Blot and LC-ESI MS analysis

The identification of ProSys fragments was carried out using the same procedures as described in paragraph 2.4.3.1 and 2.4.3.2.

3.4.3.2 Light-scattering analysis

Multi-angle light scattering analysis of the proteins were performed by combining SEC with light scattering equipment.
In particular, the experiments were run at 0.5 ml/min in PBS 1X, 100 µM PMSF, 1 mM DTT pH 8.0 using a SEC 2000 column (Phenomenex, Torrance, CA) linked to an FPLC ÄKTA coupled to a light scattering detector (mni-DAWN TREO, Wyatt Technology) and to a refractive index detector (Shodex RI-101). All data collected were processed using the ASTRA 5.3.4.14 software (Wyatt Technologies Corporation).

3.4.3.3 Circular dichroism (CD) spectroscopy

CD measurements were performed using the same methods as described in paragraph 2.4.3.4. CD at increasing of temperature were collected for Fragment I-IV with a final concentration 4.4 µM, 4.4 µM, 3.5 µM and 12.23 µM respectively.
CD at increasing concentration of TFE were recorded for Fragment I-IV with a final concentration of 5.15 µM, 2.76 µM, 3.5 µM and 14.76 µM, respectively.

3.4.3.4 Bioinformatic sequence analysis

Bioinformatic analysis of the primary sequence of ProSys fragments was carried out following the same procedures as described in paragraph 2.4.3.5.

Chapter 4

The recombinant products are biologically active in tomato

Donata Molisso in collaboration with Francesco Pennacchio and Rosa Rao

Abstract

Pesticides are substances or mixtures of substances extensively used in agricultural and other settings in order to protect crops against pest agents. Because of their widespread use, selective toxicity and bioaccumulation, synthetic pesticides represent the most toxic substances which contaminate the environment and cause a range of human health problems. Therefore, there is an increased social pressure to substitute them with an ecofriendly alternative, such as biopesticides. Biopesticides include living microbes and those biochemicals derived from microorganisms and naturally occurring substances from plants. In this contest, an investigated system is Systemin (Sys) peptidic hormone which is released by its precursor Prosystemin (ProSys) in response to herbivore attacks and plays an important role in the activation of the defense genes in *solanaceous* plants. However, it was recently shown that ProSys itself possesses a biological activity wider than expected, even when devoid of the Sys sequence; these results fitted with the finding that we have shown how that the precursor is an intrinsically disordered protein. In this study we investigated whether fragments of ProSys might exert any biological activity in activating defense responses upon biotic or abiotic attacks. In particular, by using a combination of gene expression analysis and bioassays, we proved that the exogenous supply of the recombinant ProSys and ProSys fragments to tomato plants promotes early and late plant defense genes. In addition, it counteracts the development of *Spodoptera littoralis* larvae and the fungal leaf colonization. These results suggest that the direct application of these recombinant products, which are safe to humans and no-target organisms, may represent an exploitable tool for crop protection.

4.1 Introduction

Pesticides (farm chemicals or agrochemicals) are substances or mixtures of substances mainly used in agriculture in order to protect crops from pests, weeds or diseases and to guarantee high plant yields. Herbicides are the widely used class, followed by other pesticides, fungicides, insecticides and plant growth regulators (US-EPA, 2011; Stoytcheva, 2011). A wide range pesticide has been shown to be associated with a variety of adverse health and environmental effects (Hayes *et al.,* 2006; Sanborn *et al.,* 2007; Mnif *et al.,* 2011; Goulson, 2014; Zheng *et al.,* 2016) and the use in agriculture of some of these products has been abolished (Stoytcheva, 2011). In particular, since their limited species selectivity and high intrinsic toxicity, pesticides can negatively affect non-target organisms such as humans, birds, fish, domestic animals and wildlife populations as well as contaminate soil and water media (Hernández *et al.,* 2011a).

In order to perform a toxicological risk assessment of the exposures to these chemical products, the US Environmental Protection Agency (USEPA) and other regulatory committees require a premarket testing on animals (Ekström and Åkerblom, 1990; Pitot and Dragan, 1996; Fenske *et al.,* 2000).

However, the adequacy of this assessment can be under discussion because toxicologic tests are usually performed on a single active compound for a short time to inbred strains of animals, whereas humans are frequently exposed to a mixture of chemical substances during the lifetime which may have additive and or synergistic effect (Ekström and Åkerblom, 1990; Pitot and Dragan, 1996; Dai *et al.,* 2001; Tang

et al., 2002; Kortenkamp, 2007). Moreover, the problem lies in the massive use of substances exceeding legal limits and, in the meantime, the frequent abuse of old and banned compounds, often in developing countries.
Human can be exposed to pesticides by drinking water, dust, air, food and by different routes of exposure such as ingestion, inhalation, or skin contact (Naik and Prasad, 2006). The individual chemical category of pesticide, the dose, the duration and route of exposure are determining features in the type and severity of the health outcome. In particular, long-term exposure may cause numerous health effects including dermatological, respiratory, reproductivity, carcinogenic and endocrine effects (Semchuk *et al.,* 1992; Sanborn *et al.,* 2007; Bassil *et al.,* 2007; Mnif *et al.,* 2011; Kanavouras *et al.,* 2011; Hernández *et al.,* 2011b; Thakur *et al.,* 2014). Furthermore, high accidental or intentional exposure to pesticides can lead to the hospitalization till the death (Gunnell *et al.,* 2007).
There is an increasing demand of consumers for food products that are pesticide-residue low or residue free. This is particularly important for vegetables and fruits which are usually consumed fresh without any processing. To meet these requirements, the scientific community is pointing the attention on the development of alternative control strategies such as biological control with the aim to reduce the use of pesticides in agricultural practices. Biological control strategies are often included in Integrated Pest Management (IPM), a system that combines different techniques and methods in a compatible manner with accurate monitoring of pests and their natural enemies (Bajwa and Kogan, 2002; Flint and Van den Bosch, 2012). The final aim of this approach is not to destroy pest populations but rather to manage them to tolerable levels.
Biopesticides represent an important tool of IPM strategy. They include living organisms (i.e. natural enemies) or their genes and molecules as well as naturally occurring substances that can be used to control pests. The most commonly used biopesticides are microorganisms (bacteria, fungi, oomycetes, viruses) specific for a pest of interest, no dangerous for the environment and human health.
An interesting approach to find new biopesticides is the screening of naturally occurring substances in plants such as plant extracts, pheromones or fatty acids (Isman, 2006; 2008). Since plants are sessile organisms unable to escape attackers, they produce and exude a wide variety of secondary metabolites which have an important role in defense mechanisms. The mixture of these metabolites can be deterrent to insect pests for longer period a single compound and different physical properties may allow more persistence of defense responses. Most of the studies were carried out on plant extracts and essential oils, evaluating the lethal doses, the time required to achieve lethal effects, and the mode of actions (Lewis *et al.,* 1993; Roeder, 1994; Broeck *et al.,* 1995; Zafra-Polo *et al.,* 1996; Enan, 1998, 2001; Kostyukovsky *et al.,* 2002; Priestley *et al.,* 2003; Rattan, 2010).
The introduction of natural biopesticides and naturally occurring substances for crop protection is highly desirable and there is a continuous search for natural substances useful for the protection of agricultural crops.
In *Solanaceae*, a family of defense-related peptide hormone called systemins are involved in the activation of defense genes in response to injury (Bergey *et al.,* 1996; Schilmiller and Howe, 2005). Systemin (Sys) is an 18-amino acid oligopeptide located at the C-terminus of a cytosolic precursor protein of 200 amino acids called prosystemin (ProSys). In tomato plants, upon insect attack, Sys is released from its cytosolic precursor and recognized by an unidentified receptor to lead the activation of a complex signaling cascade that induce the production of defense compounds

(Ryan, 2000; Scheer and Ryan, 2002). In addition, in tomato plants were also identified three functional homologues, hydroxyproline-rich glycopeptides (called TomHypSys I, II and III) of 15-20 amino acids in length that are synthetized from a single precursor protein of 145 amino acids (Pearce and Ryan, 2003). Sys and HypSys are involved cooperatively in the activation of systemic wound signaling in tomato (Narváez-Vásquez *et al.,* 2007).
The peptide-mediated resistance has also been observed in other crop species (Huffaker *et al.,* 2006, 2011; Pearce *et al.,* 2010; Yamaguchi *et al.,* 2011) suggesting that plants have a peptide-based signaling system involved in the activation and amplification of plant defenses against pathogen an herbivore attacks (Schaller, 2008). These evidences suggest that plant defense-related peptides could represent an intriguing tool for the development of sustainable crop protection strategies. This hypothesis is corroborated by the observation that transgenic plants overexpressing the precursors of these peptides are resistant to the attacks of different biotic stressors (Coppola *et al.,* 2015; Huffaker *et al.,* 2006).
One of such precursors is ProSys. It was previously demonstrated that the constitutive expression of Prosys in tomato, promotes the up-regulation of an array of defense genes, controlled by different signaling pathways, that confer protection against chewing and sap-sucking insects and necrotrophic fungi. In addition, transgenic plants tolerate a salt and heat stress (McGurl *et al.,* 1994; Orsini *et al.,* 2010; Coppola *et al.,* 2015, Rao, unpublished). Furthermore, we observed that the overexpression in tobacco of the deleted ProSys of lacking the final exon encoding Sys triggers the expression of defense-related genes and confers resistance against a phytopathogenic fungus (Corrado *et al.,* 2016). Very recently it was demonstrated that the exogenous supply of Sys to tomato plants is able to modify the metabolome of the treated plants and modulate plant to plant communication (Coppola *et al.,* 2017).
Based on these observations here we investigated the effect of the exogenous application of the purified recombinant ProSys and ProSys fragments to tomato plants to evaluate if: (i) the treated plants are able to perceive the exogenous supply of the recombinant products and react to the treatments; (ii) the recombinant products are biologically active against biotic stresses, (iii) Prosys includes regions, diverse form the Sys one, able to trigger defense-related genes and protect tomato plants.
We demonstrate that the recombinant products are biologically active being able to induce early and late defense genes and to protect plants against *B. cinerea* and *S. littoralis* larvae.

4.2 Results

4.2.1 Induction of the expression of plant defense-related genes

The biological activity of the purified recombinant ProSys protein and ProSys fragments was analyzed by the evaluation of the expression of some defense-related genes in response to their exogenous application on tomato leaves. In particular, four genes were selected: two early genes active in the octadecanoid pathway, that lead to the synthesis of JA, *Lipoxygenase* (*LoxC*) and *Allene oxide synthase* (*AOS*), and two late JA related defense genes, encoding for proteinase inhibitors I and II (*Pin I* and *Pin II,* respectively*)*.

4.2.1.2 RNA and cDNA quality

In order to analyze the expression profile of defense genes in response of the foliar application of the ProSys protein and its fragments, analysis at RNA and protein levels were performed on treated and control samples. Total RNA was isolated from treated and control leaves and quantified by measuring absorbance using a spectrophotometer.
All samples showed ratios $A_{260/280nm}$ and $A_{260/230nm}$ very close to 2. These values allowed us to assess RNA purity detecting the presence of contaminants such as proteins, phenols, carbohydrates and aromatic compounds.
Moreover, RNA quality was checked by looking at the integrity of the extracted RNA on 1.2% (w/v) agarose gel. An example of agarose gel electrophoresis of total RNA is shown in figure 4.1.

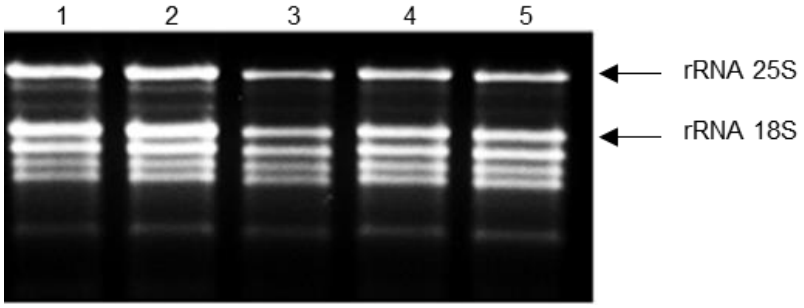

Figure 4.1: Agarose gel electrophoresis on 1.2% (w/v) of 2 µg of some RNA samples prepared from leaves of treated and control tomato plants.

RNA integrity was checked evaluating the presence of defined bands while its good quality was assessed when the fluorescence intensity of the first band from the top to down (ribosomal RNA 25S) resulted twice than the third band (ribosomal RNA 18S).
Isolated RNA was used for the synthesis of the first-strand cDNA. The produced cDNA was checked by PCR primers StbEF Fw and LeEF Rv (Table 4.1) that are able to amplify a region between two consecutive exons of the gene *EF-1α,* constitutively expressed in all tomato plant tissues (Pokalsky *et al.,* 1989). The use of these primers allows to verify cDNA synthesis and to check the presence of contaminant genomic DNA. This is the consequence of the fact that the used primers anneal on two contiguous exons. Therefore, the amplicon size depends on the kind of template: 765 bp from genomic DNA template or 687 bp from cDNA template. PCR products were separated on 1.5% agarose gel (Figure 4.2).

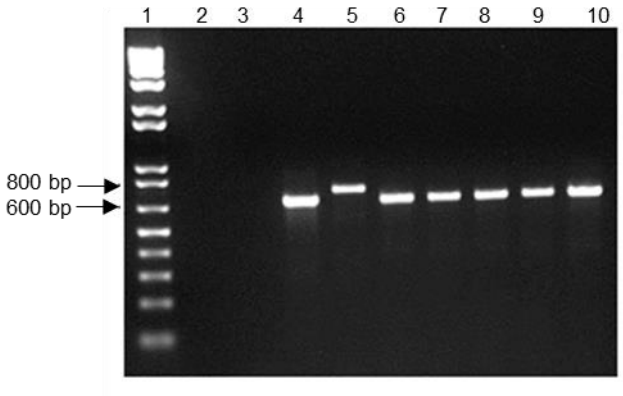

Figure 4.2: Agarose gel electrophoresis 1.5% (w/v) of *EF-1α* gene amplification products obtained from some cDNA preparations. 1: marker. 2: empty lane; 3: negative control; 4: positive control; 5: amplicon of genomic DNA; lane; 6-10: amplicons of cDNA samples.

No DNA contaminations were found in the cDNA samples. RT-PCR was carried out to monitor the expression levels of defense-related genes. The relative quantification of gene expression was performed using as calibrator the cDNA synthesized from 'Red Setter' control plants treated with PBS 1X. All the fluorescence data were standardized with those obtained from the amplification of the constitutive endogenous gene *EF-1α.* The relative quantification (RQ) of transcripts were calculated using the method of $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

4.2.1.3 Transcripts quantification

The evaluation of the perception and of the effect of the exogenous supply of ProSys and ProSys fragments on defense gene expression was performed quantifying the transcripts of the selected genes by means of qRT-PCR.
The biological activity of ProSys was carried out monitoring the expression of defense-related genes in response to the application of purified recombinant pro-hormone on wounded leaves.
In order to circumvent the effect of endogenous ProSys on the activation of defense genes following wounding, a time point analysis in a wounding experiment was carried out. To this aim leaves of healthy tomato plants were wounded and the endogenous ProSys transcripts quantified after 3, 6 and 24 hours after wounding. As shown in Figure. 4.3, the expression of the endogenous ProSys largely decreased 24 hours after wounding.

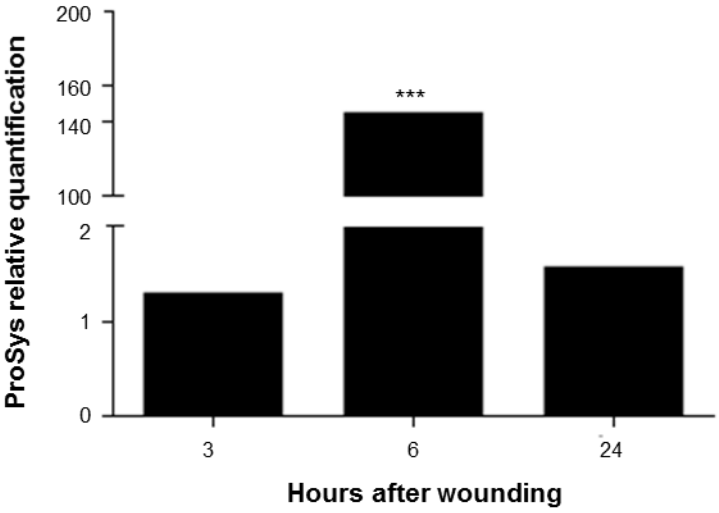

Figure 4.3: Expression analysis of ProSys by qRT-PCR. ProSys relative quantification 3, 6 and 24 hours after wounding. Data are calibrated on unwounding samples. Asterisks indicate data statistical significance (T-Test; *** p<0.001).

Based on these results, recombinant ProSys was applied on wounded leaves 24 hours after wounding and the transcripts of the selected genes were quantified at 6 and 24 hours after treatment. Relative quantification data were calibrated on controls represented by wounded leaves treated with PBS 1X. Notably, as shown in Figure 4.4, all genes resulted significantly overexpressed following the exogenous ProSys application, indicating that recombinant ProSys is biologically active.

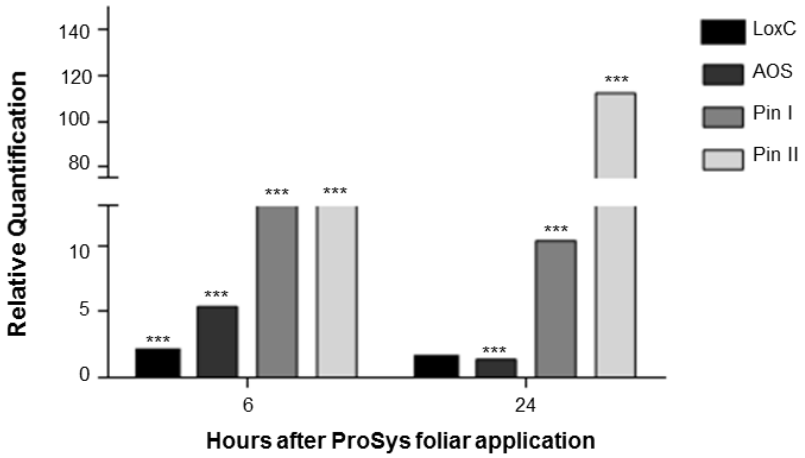

Figure 4.4: Expression analysis of ProSys related genes by RT-PCR. Relative quantification of early (*LoxC* and *AOS*) and late (*Pin I* and *Pin II*) defense genes in plants treated with 100 pM ProSys as indicated in the text. Data are calibrated on controls treated with PBS 1X after wounding. Asterisks indicate data statistical significance (T-Test; *** p<0.001).

Then we tested the ability to trigger the transcription of the selected genes by the four ProSys fragments. In these experiments we decided to perform the assays applying the fragments on unwounding tomato leaves (Figure 4.5).

As already observed with the recombinant ProSys, the induction of these genes under the described experimental conditions propose that all ProSys fragments are biologically active suggesting their perception or internalization in not damaged leaves, by molecular mechanisms still unknown, that leads to the activation of defense genes.

4.2.3 Induction of resistance against pests

4.2.3.1 *Spodoptera littoralis* assays

Then we investigated if the exogenous application of the recombinant products is able to protect the treated plants from pests. For this purpose, growth and survival rate of *Spodoptera littoralis* larvae fed with treated or untreated leaves were monitored. Larvae fed with ProSys treated leaves showed a significant reduction of their weight (18 mg for WC and 7 mg for W ProSys treated leaves at the 15th feeding day, $p<0.0001$, Tukey-Kramer HSD test) and a significantly reduced survival rate compared with larvae fed on control leaves (90.6% for WC control, 31.5% W ProSys at the 15th feeding day, $p<0.0001$, Log-rank test) (Figure 4.6). Moreover, in order to evaluate if the treatment of the plant with the recombinant protein was also able to induce a systemic defense response in untreated leaves of the same plant, the same feeding bioassay was carried out with distal leaves. A similar significant reduction of weight (14 mg for WS and 10 mg for S ProSys treated leaves at the 15th feeding day, $p<0.0001$, Tukey-Kramer HSD test) and survival rate was observed (96.8% for systemic control SC, 56.25% for S ProSys at the 15th feeding day, $p<0.0001$, Log-rank test) (Figure 4.6).

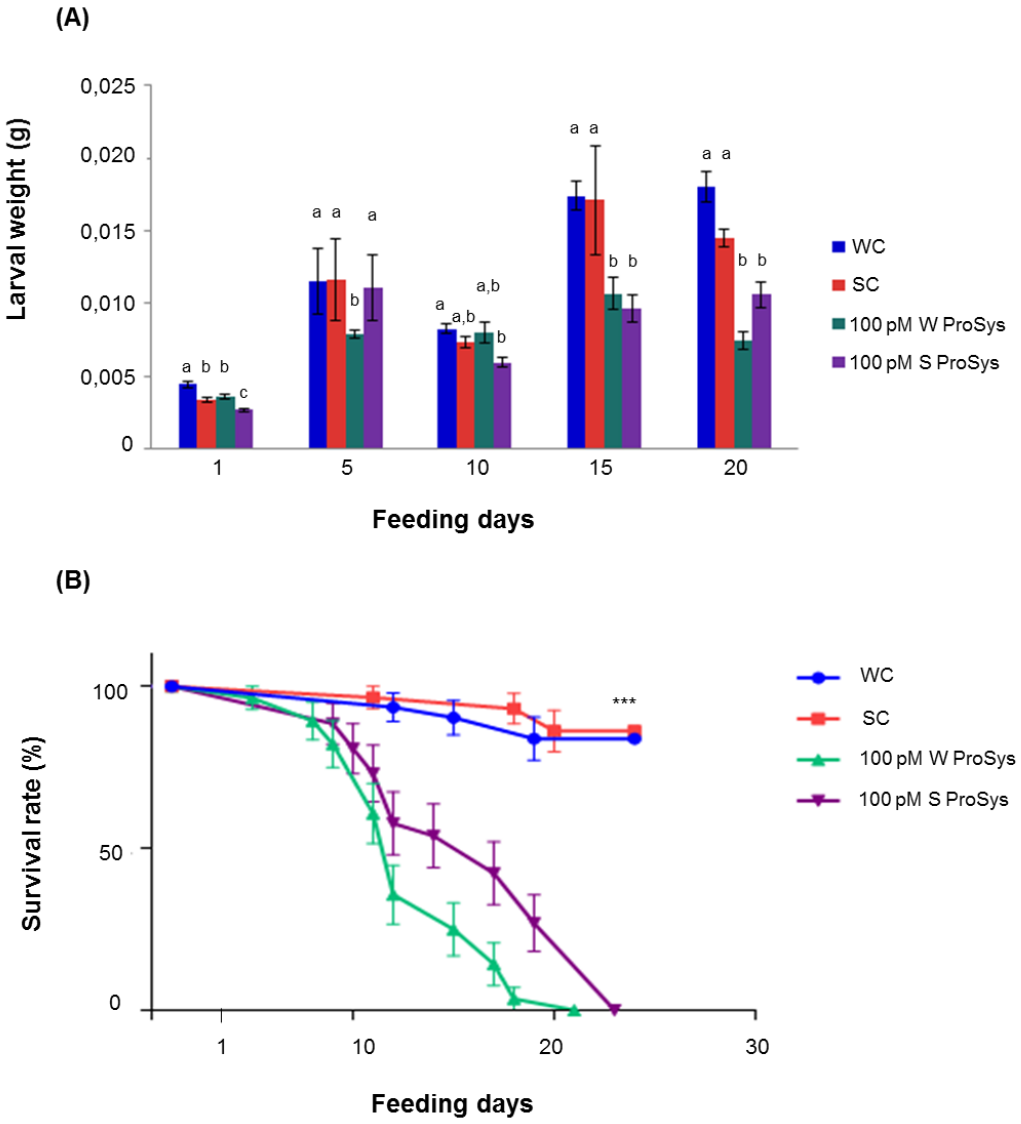

Figure 4.6: Weight increase and survival rates of *S. littoralis* larvae fed with untreated or treated leaves. WC, wounded leaves used as control; SC, leaves distal from wounded leaf used as systemic control; W ProSys, wounded leaves treated with ProSys; S ProSys, leaves distal from the ProSys treated leaf. (A): weights registered for treated samples were compared to controls by One-Way ANOVA followed by the Tukey Kramer Honestly Significant Difference (HSD) multiple range test ($P<0.05$) Letters indicate different statistical groups. (B): survival percentages of tests and controls were compared by One-Way ANOVA coupled with Log-Rank test. Asterisks indicate data statistical significance (One-Way ANOVA and Log-rank test; *** $p<0.001$).

These results confirmed that the recombinant ProSys is biologically active and that its exogenous application is associated with resistance against herbivore insects, as previously observed with transgenic plants overexpressing the natural pro-hormone (Coppola *et al*., 2015).

4.2.3.2 *Botrytis cinerea* assay

Transgenic tomato plants overexpressing ProSys also showed increased resistance against fungi infestations; in order to assess if the exogenous application of the recombinant ProSys could mimic the same results, a bioassay against *Botrytis cinerea* was carried out. The experiment was performed using treated and untreated detached wound leaves 6 hours after the application.

4.3 Discussion

In this study, gene expression and behavioral approaches were used to demonstrate that the recombinant pro-hormone is biologically active. It was monitored the effect of ProSys foliar application on the expression of some defense-related genes in order to understand if the recombinant pro-hormone was perceived at cellular level. The expression analyses established that the perception occurred, both early (*LoxC* and *AOS*) and late (*Pin I* and *Pin II*) JA related defense genes were overexpressed in comparison with their expression in the untreated leaves (Figure 4.4).
LoxC gene, belong to the Lipoxygenase gene family, encodes enzymes which convert polyunsaturated fatty acids in unsaturated hydroperoxide signal molecules. These products have specific roles in signaling and plant defense response to herbivore attack (Porta and Rocha-Sosa, 2002). In plant kingdom, multiple isoforms of Lox have been identified (Feussner and Wasternack, 2002) and in particular in tomato plant five Lox genes were detected (Zhang *et al.,* 2006). It is well known that *LoxC* is constitutively expressed in leaf even if its transcript increased with mechanical damage suggesting its involvement in leaf damage responses (Halitschke and Baldwin, 2003).
AOS gene encodes for a functional member of the CYP74A subfamilies of P450 enzymes in tomato (Song *et al.,* 1993). It commits 13S-hydroperoxy-9(Z), 11 (E), 15(Z)-octadecatrienoic acid (13-HPOT) to the formation of JA (Creelman and Mullet, 1997). The expression profile of this gene increases in response to mechanical injury and herbivore attacks in proximal and distal tissues (Howe and Schilmiller, 2002).
Pin I and *Pin II* encode protease inhibitors which interfere with the activity of digestive enzymes in the gut of the herbivore reducing insect growth and vitality (McGurl *et al.,* 1994).
The induction of these defense related genes is associated, as expected, with the resistance against herbivore insects both locally and systemically (Figure 4.6), as also previously observed with transgenic plants overexpressing the natural pro-hormone (Coppola *et al.,* 2015).
The biological activity is achieved by the final consequence of the release of the hormone Sys peptide from its precursor. These results are in agreement with the theories that identify Sys as a primary signal at a wound site responsible of the JA biosynthesis, an essential component of wound-systemic defense response on plant (Howe, 2005; Sun *et al.,* 2011).
Moreover, the fungal bioassay indicated that the exogenous application of the recombinant ProSys protein is also associated with the resistance against the necrotrophic pathogen, *B. cinerea*. These results were also expected considering the enhanced tolerance observed in the transgenic plant overexpressing ProSys protein. In particular, these plants showed an induction of other systemin-activated genes, not directly related to the JA-pathway, which contribute to enhanced performance against this necrotrophic fungus (Figure 4.8).

4.4 Materials and Methods

4.4.1.1 Materials

QuantiTect Reverse Transcription Kit for the synthesis of the first strand-cDNA was from Qiagen (Germantown, MD, USA). Enzymes and other reagents for DNA manipulation were from Promega (Madison, WI, USA) and from Qiagen (Germantown, MD, USA). All other chemicals were from Sigma-Aldrich (Milano, Italy).

4.4.1.2 Plant materials and growth conditions

Tomato seeds (*Solanum lycopersicum* L. cultivar “Red Setter”) were germinated in Petri dishes on wet sterile paper and placed in a growth chamber at 24±1℃ and 60% RH in total darkness. Upon root emergence, plantlets were transferred to a polystyrene plateau with barren substrate S-type (Floragard) in a growth chamber at 26±2°C and 60% RH under 16 hours light/ 8 hours dark, with brightness of 5000 lux. After 2 weeks, plants were grown in pots of diameter of 9 cm with sterile soil mixture using the same growth conditions.

4.4.2 Gene expression analysis

4.4.2.1 Tomato plants treatment with ProSys protein and its fragments

For ProSys assay, three leaves of 5 weeks old plants were excised with a sterile razor and the expression of the ProSys gene was monitored at 3, 6 and 24 hours after wounding. The latter time point after wounding was selected for leave treatments with aliquots of 2 µl of 100 pM purified recombinant protein gently placed

at wound sites. ProSys fragments were assayed at the same final concentration by spraying or applying aliquots on fully expanded healthy leaves. Leaf samples were collected after 6 and 24 hours from ProSys and ProSys fragment foliar application and use for total RNA extraction. All experiments had three biological replicated for control and treated plants.

4.4.2.1 Total RNA extraction

Total RNA was obtained from leaves using a standard method based on phenol/chloroform purification and lithium chloride precipitation protocol. Leaves were harvested and immediately frozen in liquid nitrogen. Five hundred mg of plant material was powdered in nitrogen liquid using mortars and pestles.
Leaf powder was added to a 2 ml tube containing 750 µl of RNA extraction buffer (100 mM Tris-HCl pH 8.5, 100 mM NaCl, 20 mM EDTA pH 8.0 and 1% SDS) and 750 µl phenol/chloroform (1:1).
After thorough mixing of samples with a vortex shaker, the tubes were centrifugated at 13000 rpm for 5 minutes at 4°C. The supernatant was collected, and phenol/chloroform extraction was repeated using the same conditions. After centrifugation the supernatant was transferred to a 2 ml tube containing 1 ml of chloroform and then centrifugated at 13000 rpm for 5 minutes at 4°C. The Nucleic acid precipitation was obtained by adding 750 µl of isopropanol, mixed by inversion, incubated on ice for 5 minutes and centrifugated at 13000 rpm for 10 minutes at 4°C. Supernatant was removed and well dried pellet was suspended in 400 µl of DEPC-treated water (DEPC- Diethylpyrocarbonate, SIGMA).
RNA selective precipitation was obtained by adding 400 µl of 4M LiCl (Sigma Aldrich) and incubating on ice over-night. RNA pellet was collected by centrifugation at 13000 rpm for 20 minutes at room temperature. Pellets were suspended in 400 µl of DEPC treated water. RNA precipitation was promoted through the addition of 40 µl of 3M Sodium Acetate pH 7.2 and 1 ml of 96% ethanol. Afterwards, samples were incubated for 10 minutes at -80°C and then they were centrifuged at 13000 rpm for 10 minutes at 4°C. Total RNA was suspended in 42 µl of DEPC-treated water.

4.4.2.2 Control of the RNA

Total RNA was analyzed quantitatively and qualitatively using NanoDrop ND-100 Spectrophotometer (NanoDrop Tecnologies, Termo Scientific). RNA integrity was checked by electrophoresis on a 1.2% agarose gel in a denaturing loading buffer (10X RNA Loading Buffer, 400 µl formamide, 120 µl 37% formaldehyde, 16 µl 50% TAE 5 µl loading Dye 10X). Twenty µl of 10X Loading buffer together with 2 µg of total RNA was treated for 5 minutes at 65°C before electrophoresis. After denaturation, samples were loaded on agarose gel applying a 70V potential difference for 30 minutes. RNA bands were checked using UV light (UV Gel Doc BIORAD).

4.4.2.3 Reverse transcription and RT-PCR

First strand-cDNA was synthetized from 2 µg of total RNA using QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's protocol. All cDNA samples were used as template for a PCR reaction performed as control of cDNA synthesis and of DNA contaminant presence using StEF Fw and LeEF Rv as primers (table

4.1) for amplification of the ubiquitously expressed Elongation Factor-*1α* gene (*EF-1α*) (Shewmaker *et al.,* 1990). The PCR reaction was prepared in 20 µl containing 1 µl of cDNA template, 1X GoTaq Buffer (Promega), 0.5 µM primers, 0.2 µM primers and 0.5 U GoTaq (Promega). The thermal cycling program begin with a step of 5 minutes at 94°C, 30 cycles of 30 seconds at 94°C, 30 seconds at 53°C, 30 seconds at 72°C, followed by a final polymerization step for 7 minutes at 72°C using Veriti Thermal Cycler (Applied Biosystem).
PCR products were loaded onto a 1.5% (w/v) agarose gel prepared with the addition of GelRed in 1X TAE Buffer (40 mM Tris-Acetate, 1mM EDTA) (Sambrook *et al.,* 1989). The electrophoresis was performed applying an 80V potential difference for 30 minutes. DNA bands were visualized using UV light (UV Gel Doc BORAD).

4.4.2.4 Real Time RT-PCR

Real Time RT-PCR for the relative gene expression analysis was performed using Corbett Rotor Gene 6000 (Corbett Research). Reactions were prepared with 2X QuantiFastSybr Green PCR Master Mix (Qiagen), 0.3 µM of each primer (table 4.1) and 1 µl of 1:20 dilution of first strand cDNA template in a final volume of 10 µl. All the experiments were carried out using three biological replicates and two technical replicates for each sample. The thermal cycling program begin with a step of 10 minutes at 95°C, 45 cycles of 30 seconds at 95°C, 30 seconds at Tm value of the primers (showed in table 4.1), 15 seconds at 72°C, followed by a dissociation step useful to assess the specify of the amplification reaction.
Data were analyzed with the 6000 Rotor-Gene System Software, version 1.7 (Corbett Research) using the Comparative Ct method with the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001), where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{endogenous control}}$ $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}}$. The housekeeping *EF-1α* gene was the endogenous reference gene used for the normalization of the expression levels of the target genes.
The statistical significance of the results was evaluated using the t-Student's test and One-Way ANOVA analysis.
Genes under investigation are listed in table 4.1.

Table 4.1: List of primers and amplification conditions

Primer	Sequence (5'-3')	Tm[1]	Gene name	Accession number	LA	NR
StbEF Fw	AAGCTGCTGAGATGAACAAG	58	*EF-1α*	X14449.1	687[2]	30
LeEF Rv	GTCAAACCAGTAGGGCCAAA	54		X53043.1	767[3]	
Pin I Fw	GAAACTCTCATGGCACGAAAAG	64	*Pin I*	K03290	114	40
Pin I Rv	CACCAATAAGTTCTGGCCACAT	64				
Pin II Fw	CCAAAAAGGCCAAATGCTTG	58	*Pin II*	K03291	116	40
Pin II Rv	TGTGCAACACGTGGTACATCC	64				
LoxC Fw	TTGCCTATGGTGCTGAATGGA	62	*LoxC*	U37839	101	40
LoxC Rv	CAAGCCATGTGGTTCATTTGG	62				
AOS Fw	GATCGGTTCGTCGGAGAAGAA	68	*AOS*	AF230371	101	40
AOS RV	GCGCACTGTTTATTCCCCACT	66				
EF FwRt	CTCCATTGGGTCGTTTTGCT	62	*EF-1α*	X53043	101	40
EF RvRt	GGTCACCTTGGCACCAGTTG	64				

LA: length amplicon. NR: number of cycles. Tm: melting temperature 1 calculated on according to the rule of Wallace: 4ºC for G and C, 2 ° C for the A and T (Wallace *et al.,* 1999); 2 Produced obtained by amplifying the transcribed mRNA; 3 Produced obtained by amplifying genomic DNA.

4.4.3 Bioassays

4.4.3.1 *Spodoptera littoralis* bioassay

Spodoptera littoralis larvae were reared in an environmental chamber at 25±2°C, 70±5% RH on artificial diet composed by 41.4 g/l wheat germ, 59.2 g/l brewer's yeast, 165 g/l corn meal, 5.9 g/l ascorbic acid, 1.8 g/l methyl 4-hydroxybenzoate, 29.6 g/l agar under a 16: 8 hours light/dark period. Newborn larvae were allowed to grow on this artificial diet until the second instar.
Uniform third instar larvae were singly transferred, under the same environmental conditions, in trays with wells (Bio-Ba-32, Color Dec, Italy) containing a thin layer of a 2% agar solution (w/v) and covered by perforated plastic lids (Bio-Cv-4, Color-Dec, Italy).
For ProSys assay, wounding and unwounding leaf discs of control and treated tomato plants (WC: wounded control leaf spotted with PBS 1X; SC: unwounded leaf of the same control plant; W Prosys: wounded treated leaf spotted with 100 pM ProSys; S Prosys: unwounded leaf of the same treated plant) were daily supplied to experimental group of 32 newly hatched larvae and maintained at 28°C. On the contrary, for ProSys fragments only unwounding leaf discs of control and treated plants were used for the feeding assay starting from first instar larvae. Larvae were weighted three times per week and mortality was daily checked during the whole larvae feeding period. Mature larvae (6^{th} instar) were transferred for pupation into plastic boxes containing vermiculite. This set up was replicated in two independent experiments. Statistical analysis was performed with the Graphpad Instar 3.0 software. Differences in larval weights were analyzed by One-Way ANOVA followed by the Tukey-Kramer Honestly Significant Difference (HSD) multiple range test ($P<0.05$). Differences in survival rate were compared by using Kaplan-Meier and long-rank analysis.

4.4.3.2 *Botrytis cinerea* bioassay

Botrytis cinerea spores were cultivated on MEP solid medium (30 g/l malt extract, Oxoid; 5 g/L mycological peptone, Oxoid; 8 g/L bacto agar, Applichem) spreading on the media plates 20 µl of conidial suspension with the concentration of $1 \cdot 10^6$conidia/mL and incubating at 22 °C under diffused light for 15 days.
Spores were suspended in sterile distilled water, filtered through sterile Kimwipes (Kimberly-Clark), useful to remove fragments of hyphae, and adjusted to a concentration of $1 \cdot 10^6/10^7$ conidia/ml.
Ten µl of the spore suspension were applied between tomato leaf veins, at 3 different inoculation points per wounded detached leaves for ProSys and unwounded detached leaves Prosys fragments 6 hours after treatment. This assay was carried out using three compound leaves from three different plants per each thesis. Control and treated 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% RH. The size of the lesions was measured at 1, 3, 5 and 8 days after infestation. Lesion dimensions were measured using a digital caliber; diameters measured were used to calculated necrosis areas as elliptic areas and data significance was evaluated by One-Way ANOVA analysis. Statistical analysis was performed with the Graphpad Instar 3.0 software.

Chapter 5

General conclusions

The detection of invading organisms is a crucial step of plant immunity, which initiates the activation of defense responses. Herbivore-associated elicitors (HAE) are molecules recognized by the plant, which induce different defense reactions, selectively associated with distinct components of the HAE (Xu et al., 2015). Anti-herbivore defenses are induced not only by molecules produced by the invading organisms, but also by endogenous plant molecules, that are released following the damage caused by the feeding insect and, therefore, are also referred as damage-associated molecular patterns or DAMP (Krautz *et al.,* 2014; Savatin *et al.,* 2014). These molecules, such as reactive oxygen species (ROS), oligosaccharide and protein fragments (Chai and Doke*,* 1987; Albersheim and Anderson, 1971; Pearce *et al.,* 1991), act as warning signals. The effective amplification of the danger signal and of the triggered defense responses is under control of enzymatic cascades, which are up-regulated by feeding damages. For example, ROS signals are produced by NADPH oxidase (Chen *et al.,* 2008), while cell wall fragments originate through the activity of polygalacturonase; both enzymes are induced by mechanical wounding or by biotic stress agents (Bergey *et al.,* 1999; Torres *et al.,* 2002), which also induce genes encoding the precursors of endogenous peptide elicitors (Pearce *et al.,* 1991; Huffaker *et al.,* 2006).

Systemin (Sys) was the first peptide signal discovered in tomato plants produced following the processing of its precursor, ProSystemin (ProSys) (Pearce *et al.*, 1991). The observation that ProSys overexpression in tomato elicits multiple defense pathways conferring protection against a wide range of environmental stresses agents, suggested that it may play a role in plant defense broader than expected.

A successful cloning and purification strategy allowed to obtain the purified recombinant protein for a deeper investigation of its structure.

The results, described in Chapter 2, revealed that ProSys is an Intrinsically Disordered Protein (IDP). The dynamic and interactive coordination of various signalling networks underpins the phenotypic plasticity required to integrate external cues with developmental programs (Covarrubias *et al.,* 2017). Considering that IDP interactions with different molecular partners are mediated by disordered regions (Dyson and Wright, 2005), it is proposed that ProSys may exert its multifaceted biological activity as a consequence of its intrinsic disorder. This observation suggests new interesting insights on the role ProSys in plant response against biotic and, possibly, abiotic stressors.

Notably, specific bioassays proved that the recombinant ProSys was biologically active being very effective in the induction of tomato defense-related genes, which confer protection against *S. littoralis* larvae both locally and systemically and also against the infection of the phytopatogenic fungus *B. cinerea* (Chapter 4). The observed biological activity is worth of consideration from an applied perspective, since it nicely substantiates the use of recombinant proteins as innovative tools in insect control, which act not by exerting a toxic action but by triggering plant defense responses.

Sys peptide is traditionally considered as the principal actor of the modulation of the resistance towards pests. However, recent evidences (Corrado *et al.,* 2016) suggest that ProSys N-terminal region, deprived of Sys, could have a role in plant defense responses. This observation was underpinned by bioinformatics tools that identified regions along ProSys sequence characterized by intriguing conformation features that leaded to the hypothesis that they may be biologically active. This hypothesis was verified by producing the recombinant fragments corresponding to these regions. Four purified fragments were produced: Fragment I and III which cover the N-

terminal region of Prosys protein and Fragment II and Fragment IV that cover the C-terminal region of the pro-hormone and include the Sys peptide (Chapter 3, Figure 3.1). The biophysical and the biochemical characterization of these regions showed that all the recombinant fragments are disordered in agreement with what previously shown for the whole precursor (Chapter 3).

The exogenous supply of the recombinant ProSys fragments to tomato plant was investigated for the ability in triggering the expression of defense-related genes. It turned out that all the fragments induced early and late defense gene. Fragment I and III were considered the most interesting ones as they do not include Sys and therefore represent novel peptides likely associated with plant defense responses. Interestingly leaf treatments with picomolar solution of both fragments counteracted the growth and survival of *S. littoralis* larvae and leaf colonization of *B. cinerea* (Chapter 4). Finally, more recent studies have shown that also femtomolar solution of these recombinant proteins are able to induce the expression of defense-related genes thus greatly minimizing protein production costs.

From the outcome of our investigation it is possible to conclude that plant endogenous peptides prompting defense responses against invaders represent a very safe approach to plant protection, due the expected low or null toxicity of these molecules on humans and non-target organisms. Here we described two novel peptides, whose direct delivery to tomato leaves effectively protect the plants against two major biotic stress agents. They are very promising from an applied perspective, representing an exploitable biotechnological tool for IPM strategies.

As described in Chapter 1 in different evolutionary lineages, peptides evolved as defense signals involved in the finely tuned orchestration of gene expression underlying plant immune responses. The development of control strategies of biotic stresses implying their direct delivery to the plants represents a very powerful tool for sustainable agriculture, minimizing the use of chemical inputs while providing food quality and safety.

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STAGE

Eight months stage (04/07/2016 - 31/03/2017) at Department of Biology, Institute of Biostructures and Bioimaging (IBB)-CNR, University of Naples “Federico II”. Italy: group of Dr. Simona Maria Monti to perform protein purification and characterization.

Three months stage (26/06/2017 – 26/08/2017; 12/03/2018 – 17/04/2018) at Sequentia Biotech, a bioinformatics company, Barcelona. Spain: group of Dr. Riccardo Aiese Cigliano and Dr. Walter Sanseverino to perform bioinformatic analysis.

Publications

Papers: Martina Buonanno, Mariangela Coppola, Ilaria Di Lelio, Donata Molisso, Marilisa Leone, Francesco Pennacchio, Emma Langella, Rosa Rao, Simona Maria Monti (2018). Prosystemin, a prohormone that modulates plant defense barriers, is an intrinsically disordered protein. Protein Science.

Poster comunication: Alessia Vitiello, Donata Molisso, Nunzio D'Agostino, Maria Cristina Digilio, Francesco Pennacchio, Giandomenico Corrado Rosa Rao (2016). Transcriptional reprogramming of zucchini plant during aphid infestation. Proceedings of the LX SIGA Annual Congress Catania, Italy – 13/16 September, 2016 ISBN 978-88-904570-6-7.

Poster comunication: Donata Molisso, Martina Buonanno, Mariangela Coppola, Ilaria Di Lelio, Emma Langella, Rosa Rao, Simona Maria Monti (2017). Tomato Prosystemin: structural and functional characterization. Proceedings of the Joint Congress SIBV-SIGA Pisa, Italy - 19/22 September, 2017 ISBN 978-88-904570-7-4.

Oral comunication: Mariangela Coppola, Ilaria Di Lelio, Alessandra Romanelli, Donata Molisso, Michelina Ruocco, Giandomenico Corrado, Francesco Pennacchio, Rosa Rao. (2017). Exogenous supply of the systemin peptide protects plants to pathogens and insect pests and enhances plant-to-plant communication. Proceedings of the Joint Congress SIBV-SIGA Pisa, Italy - 19/22 September, 2017 ISBN 978-88-904570-7-4.

Poster comunication: Donata Molisso, Martina Buonanno, Mariangela Coppola, Ilaria Di Lelio, Emma Langella, Rosa Rao, Simona Maria Monti (2018). Tomato Prosystemin: structural and functional characterization. XI European Congress of Enthomolgy Napoli, Italy – 9/6 July 2018.

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Poster Communication Abstract – 1.37

TRANSCRIPTIONAL REPROGRAMMING OF ZUCCHINI PLANT DURING APHID INFESTATION

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Cucurbita pepo, RNA-seq, Aphis gossypii, zucchini protection, digital gene expression profiling

Cucurbita pepo is an economically important species within the genus *Cucurbita*. Zucchini is widely cultivated in temperate regions and it is one of the most variable species in terms of fruit shape. In addition to immature fruits, also flowers and seeds are consumed and are considered an important sources of nutrients. The melon aphid *Aphis gossypii* (Homottera: Aphididae) is a serious pest of zucchini. Its feeding behaviour causes leaf curling and chlorosis, hindering plant photosynthetic capacity. *A. gossypii* is also effectively able to transmit several plant viruses, resulting in a significant yield loss. The aim of this study is to investigate, through a time-course transcriptomic analysis based on RNA-seq, the transcriptional response of zucchini plants (cv. "San Pasquale") during *Aphis gossypii* infestation. Zucchini plants were grown in controlled conditions until the 3rd leaf stage. Ten adult aphids were transferred on the 1st and 2nd leaves, which were collected after 24, 48 and 96 hours post infestation (pi). The same leaves were collected from un-infested plants and used as controls. Total RNA was extracted from each sample and sequenced using the Illumina HiSeq 2500 platform. The sequencing resulted in ~34 million of paired-end reads per sample. After quality assessment and processing, high quality reads were *de novo* assembled into 71,648 transcripts with an average length of 1331 nucleotides. About 94% of the assembled transcripts contains coding sequences that could be translated into proteins. Over 70% of the transcripts was functionally annotated using Blast2GO. It was possible to assign one or more Gene Ontology (GO) terms to 51,398 transcripts. The transcriptome was used as high quality reference for read alignment. To ensure that each locus was represented only once in the dataset, we filtered out the longest transcript for each gene locus. Then, we employed this dataset, which includes 42,517 sequences, for read mapping and differentially expressed genes (DEG) identification. The filtering criteria used for DEG call were: a logFold change in expression greater than 2 and a FDR< 0.05. Considering the three time points, 766 transcripts were differentially expressed. After 24 hours pi, 158 transcripts (115 up and 45 down) were influenced by aphid infestation. The number of affected transcripts increased to 565 after 48 hours (420 up and 145 down), and declined to 179 transcripts (62 up and 117 down) after 96 hours from the infestation. Major categories involved in zucchini response are "photosynthesis", "response to stress", "primary metabolism" and "protein metabolism". Transcripts upregulated at 24 hours pi include serine/threonine protein kinases, involved in signalling response, 2-alkenal reductase proteins, involved in detoxification mechanism of toxic aldehydes and cysteine proteinases, involved in plant-insect pest interaction. In-depth analysis of transcriptional reprogramming will help elucidating, for the first time, the zucchini response to aphid infestations.

This work was carried out in the frame of the "GenHORT - OR1 "Qualità e sostenibilità delle produzioni mediante strumenti di genomica strutturale e funzionale" project (PON. 02 00395 3215002).

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Poster Communication Abstract – 6.33

TOMATO PROSYSTEMIN: STRUCTURAL AND FUNCTIONAL CHARACTERIZATION

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tomato, plant defense, precursor, functional domains, structural characterization

Systemin (Sys) is a 18-amino-acid peptide hormone which, upon insect attack, is released from its precursor protein, Prosystemin (Prosys) to initiate a complex signaling cascade that leads to the production of defense compounds (1). The tomato genome contains only one copy of the *Prosys* gene; it is composed of 4176 bp and is structured into 11 exons, of which the last one codes for Sys. Sys is considered one of the key component of the tomato plant molecular defense against insect herbivores. Despite that, it was recently demonstrated that the precursor, deprived of the Sys aminoacidic sequence, promotes defense responses (2). This observation prompts us to investigate the biochemical and structural features of the Prosys protein. To this purpose *Prosys* cDNA was amplified (using site-specific primers), cloned into pETM11 vector (useful to link His-tag at N-terminal region of these proteins) and expressed in BL21 (DE3) E. coli strain. The recombinant protein was purified by three chromatographic steps: Immobilized Metal Affinity Chromatography (IMAC), Ion Exchange Chromatography (IEC) and Size Exclusion Chromatography (SEC). After each step of purification, protein purity was assessed on 15% SDS-PAGE. Since the beginning Prosys showed peculiar behaviours (3) as observed by SDS-PAGE and chromatographic tools, further analyses have been performed. Bioinformatic tools allowed us to evaluate the intrinsic features of the protein and structure prediction servers have been used to analyze the secondary and tertiary structure of the prohormone. Finally, functional experiments in plants have been carried out to evaluate its biological activity.

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Oral Communication Abstract – 3.04

EXOGENOUS SUPPLY OF THE SYSTEMIN PEPTIDE PROTECTS PLANTS TO PATHOGENS AND INSECT PESTS AND ENHANCES PLANT-TO-PLANT COMMUNICATION

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DAMP, priming, crop protection

Due to their sessile nature, plants evolved a myriad of responses to counteract the damages imposed by a different array of enemies. Among the plant defence responses, endogenous peptides represent a promising tool for crop protection due to the rapid onset of their accumulation and the low or null toxicity on non-target organisms.

Systemin (Sys) is a tomato octadecapeptide hormone that promotes plant defense against different biotic stressors. Upon fungi and insect attacks, the peptide is released by a larger precursor protein, the Prosystemin (ProSys), triggering complex intracellular signalling events that lead to the production of defense compounds. We previously demonstrated that ProSys constitutive expression increases tomato tolerance to insect damages and severely interferes with phytopathogenic fungi colonization. These observations were coupled by a wide transcriptome reprogramming affecting several defence hormone-related pathways. Here we report the results of the investigation on alternative delivery strategies, not relying upon transgenic plants, in which the Sys peptide was exogenously supplied to tomato plants through foliar application, hydroponic growth and spray.

Treated plants perceived the exogenous Sys and activated, both locally and systemically, the expression of defense genes able to protect the plant against phytopathogenic fungi as *Alternaria solani* and *Botrytis cinerea* and insect herbivores as *Spodoptera littoralis*. In addition, Sys-treated plants affected plan-to-plant communications, inducing a primed state in neighbour undamaged plants mainly associated with an elevated transcription of pattern-recognition receptors, signalling enzymes and transcription factors. Compared to naïve plants, systemin-primed plants were significantly more resistant to herbivorous pests, more attractive for parasitoids and displayed an augmented response to wounding.

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New approaches for the management of agricultural and forest pests

TOMATO PROSYSTEMIN: STRUCTURAL AND FUNCTIONAL CHARACTERIZATION

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Systemin (Sys) is a 18-amino-acid peptide hormone which, upon insect attack, is released from its precursor protein, Prosystemin (Prosys) to initiate a complex signaling cascade that leads to the production of defense compounds (Ryan, 2000). The tomato genome contains only one copy of the Prosys gene; it is composed of 4176 bp and is structured into 11 exons, of which the last one codes for Sys. Sys peptide was traditionally considered as the principal actor of the resistance towards pests observed in tomato plant overexpressing the ProSys. However, recent evidences (Corrado et al., 2016) suggest that ProSys N-terminal region could contribute to defense response. This observation prompts us to investigate the biochemical and structural features of the Prosys protein. To this purpose Prosys cDNA was amplified, cloned into pETM11 vector and expressed in BL21 (DE3) E. coli strain. The recombinant protein was purified by three chromatographic steps: Immobilized Metal Affinity Chromatography, Ion Exchange Chromatography and Size Exclusion Chromatography. After each step of purification, protein purity was assessed on 15% SDS-PAGE. Since the beginning Prosys showed peculiar behavior (Délano et al., 1999) as observed by SDS-PAGE and chromatographic approches. Moreover, bioinformatics and structure prediction tools allowed us to evaluate the intrinsic features of the protein and to analyze the secondary and tertiary structure of the prohormone.
The results show, for the first time, that Prosys is an Intrinsically Disordered Protein (IDP) (Buonanno et al., 2017). Finally, plant assays revealed that the recombinant pro-hormone is biologically active being very effective in the induction of tomato defence-related genes, which confer protection against S. littoralis larvae both locally and sistemically (Buonanno et al., 2017). To our knowledge, this is the first biotic stress related IDP identified in plants. Studies aimed at a deeper characterization of ProSys function are presently in progress.

THE PROTEIN SOCIETY

Prosystemin, a prohormone that modulates plant defense barriers, is an intrinsically disordered protein

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Abstract: Prosystemin, originally isolated from *Lycopersicon esculentum*, is a tomato pro-hormone of 200 aminoacid residues which releases a bioactive peptide of 18 aminoacids called Systemin. This signaling peptide is involved in the activation of defense genes in *solanaceous* plants in response to herbivore feeding damage. Using biochemical, biophysical and bioinformatics approaches we characterized Prosystemin, showing that it is an intrinsically disordered protein possessing a few secondary structure elements within the sequence. Plant treatment with recombinant Prosystemin promotes early and late plant defense genes, which limit the development and survival of *Spodoptera littoralis* larvae fed with treated plants.

Keywords: Prosystemin; natively unfolded; solanaceae; *Spodoptera littoralis*; plant defense

Introduction

Plants, as sessile organisms, cannot escape adverse environmental changes and have developed very efficient defense responses against stress agents. The molecular mechanisms underlying defense responses against insect attack are activated locally, at the damaged site, and promote a long-distance signaling largely modulated by jasmonic acid (JA).[1]

The pioneering work on tomato plants showed how wound damage results both in local and systemic expression of proteinase inhibitors (PIs), disrupting the digestion of feeding insects.[2,3] In tomato, these events are triggered by a peptide hormone named Systemin (Sys).[4] The signaling molecule is an octadecapeptide intensely investigated over the years[5–9] which, upon wounding, is released from the C-terminal region of a larger pro-hormone of 200 amino acids called Prosystemin (Prosys) by means of a molecular mechanism still unknown.[8]

Prosys gene is transcribed at low level in physiological conditions, while its expression is enhanced by mechanical wounding or feeding by herbivore insects.[6,10] The key role of Prosys in plant defense was discovered by Ryan's group through the analysis of transgenic tomato plants expressing the coding gene in sense and anti-sense orientation. The overexpression of Prosys was associated with the constitutive production of PIs connected with a significant increase of resistance against herbivore insects.[6,10,11] Conversely, plants expressing Prosys cDNA in anti-

Additional Supporting Information may be found in the online version of this article.

Martina Buonanno and Mariangela Coppola have contributed equally to this work.

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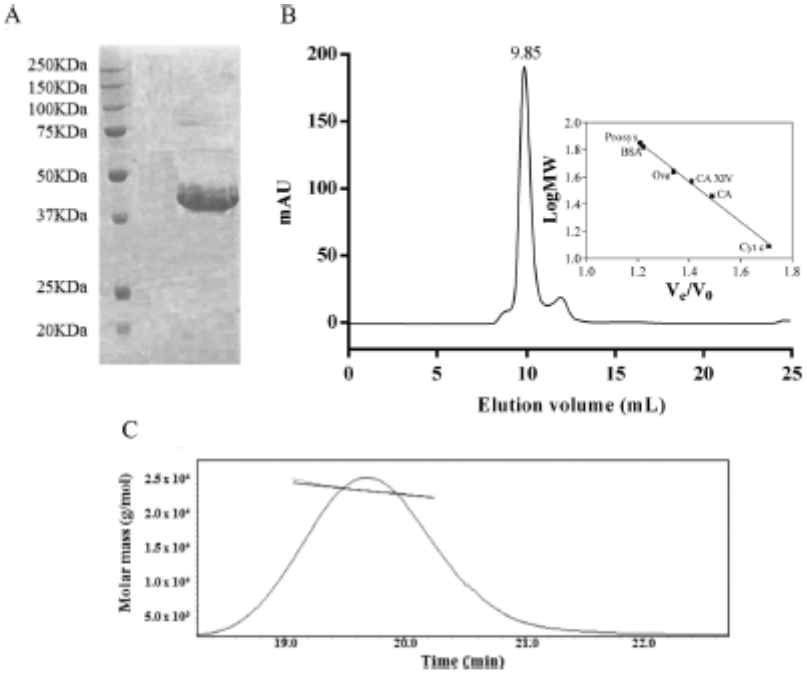

Figure 1. Recombinant Prosys has an hydrodynamic dimension typical of intrinsically disordered proteins. (A) 15% SDS-PAGE stained with Coomassie Brilliant Blue. Molecular masses of broad range protein marker (20–250 kDa) (BIORAD) are indicated in kDa. (B) Elution profile of Prosys on a Superdex 75 10/16 size exclusion chromatography column. Inset, molecular mass deduced from the calibration curve. (C) molecular mass value of Prosys determined by light scattering analysis.

sense orientation showed a nearly complete suppression of PIs production upon wounding[12] associated with a reduced capacity to limit the feeding damage by *Manduca sexta* larvae.[13] The constitutive production of Prosys in tomato plants resulted in an increased resistance not only against chewing larvae but also against phytopathogenic fungi and aphids.[9,14,15] Moreover, transgenic plants showed indirect defenses reinforced, being more attractive towards parasitoids and predators of their insect pests[16,17] and show an increased plant resistance to saline stress.[18] Finally, it was recently demonstrated that the pro-hormone, deprived of the Sys aminoacidic sequence, promotes defense responses which are not induced by the release of Sys peptide.[19]

The observation that Prosys elicits multiple defense pathways to protect tomato plants against a wide range of stress agents suggests that it may play a role in plant defense broader than expected. Due to the lack of structural information on the whole Sys precursor, we performed a structural and biological characterization of it. The gathered experimental evidence on the full length recombinant protein, along with predictions by bioinformatics analysis, proved that Prosys is a member of the Intrinsically Disordered Proteins (IDPs) family. IDPs are a class of proteins completely or only partially unstructured but still functionally active.[20] These results suggest novel hints for the understanding of the multiple roles of Prosys in the tomato defense mechanisms.

Results

Recombinant Prosys has an hydrodynamic dimension typical of intrinsically disordered proteins

Prosys has no sequence similarity with any structurally characterized protein. To investigate its structure-function features, pETM11-Prosys was produced in *E. coli* BL21(DE3) bacterial strain by expressing a PCR-amplified cDNA in NcoI/XhoI sites. Prosys, recovered from the soluble part of lysate, was purified at a high degree (above 98%) upon three purification steps with a final yield of 4 mg/L colture. As assessed by SDS-PAGE, Prosys showed an aberrant migration with an apparent molecular mass of 40 kDa, exceeding the expected molecular mass of 26 kDa (inclusive of the His-tag) [(Fig. 1(A)] and its identity was confirmed only by LC-ESI-MS analysis (data not shown). Elution from the SEC column occurred as a sharp peak with an apparent molecular mass (MM_{app}) of about 71 kDa [Fig. 1(B)], as estimated by the calibration curve [inset Fig. 1(B)]. This value was well above the

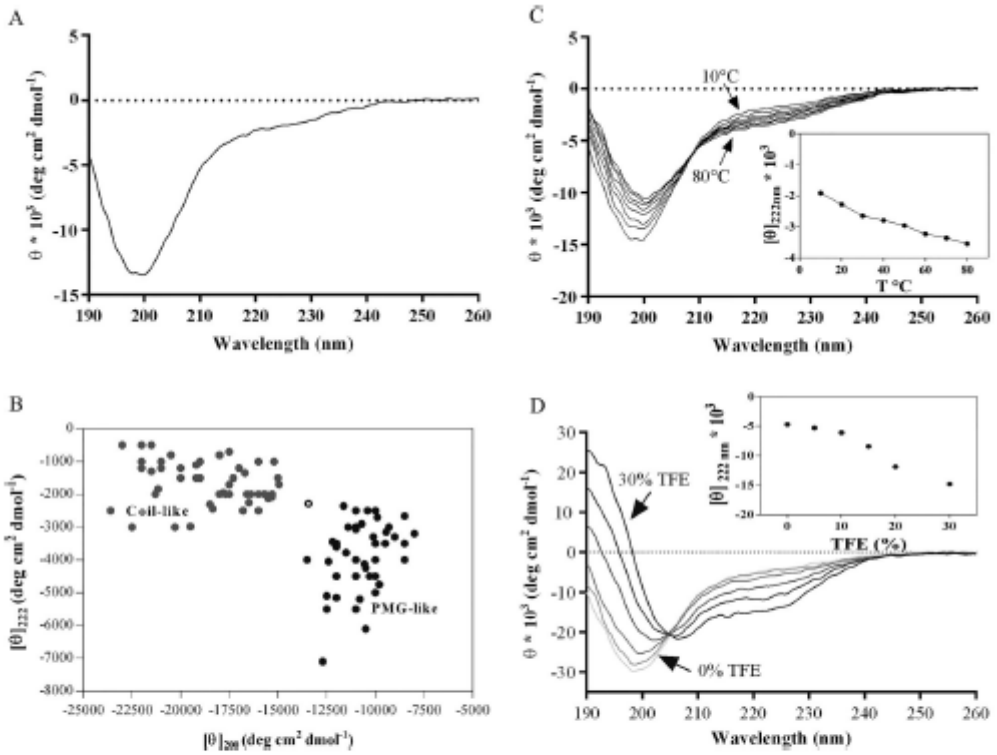

Figure 2. Far-UV CD spectra of Prosys. (A) CD spectrum was recorded in 10 m*M* phosphate buffer, pH 7.4 at a protein concentration of 4 μM. (B) $[\theta]_{222}$ versus $[\theta]_{200}$ ellipticity plot modified from Uversky;[25] $[\theta]_{222}$ of a set of well-characterized coil-like (gray circles) and premolten globule-like subclasses (black circles) has been plotted against $[\theta]_{200}$. The position of Prosys is indicated with an empty circle. (C) Temperature effect on folding of Prosys (at 10°C, 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C). The ellipticity $[\theta]_{222}$ versus temperature is shown in the inset. (D) TFE induced folding of Prosys (at 0%, 5%, 10%, 15%, 20%, and 30%). The ellipticity $[\theta]_{222}$ versus TFE percentage is shown in the inset.

expected value (MM_{theo}) and was not compatible with a monomeric globular structure. Indeed, the elution volume suggested either a folded trimeric oligomerization or a flexible conformation with scarce compactness. By means of light scattering studies, the hydrodynamic properties of Prosys were elucidated, showing that, in solution, the prohormone occurs as a monomeric protein, with a molecular mass of 23.7 ± 0.1 kDa [Fig. 1(C)], and an apparent hydrodynamic radius of 5.61 ± 0.01 nm of the monodisperse peak, which is indicative of a protein with low compactness. The same measurements, carried out in presence of urea, showed an increase of the hydrodynamic radius to 8.6 ± 3.3 nm, suggesting that the protein in native conditions contains a residual structural content which is lost in presence of a denaturing agent.[21]

Protease sensitivity

A poorly compact protein is more sensitive to protease activity as cleavage sites are better exposed than in globular structures. Then, to further corroborate the low compactness of the recombinant protein, Prosys was subjected to proteolytic digestion using a protease with a broad substrate specificity such as trypsin.[22] Prosys, incubated with different E:S ratio and at different time intervals, was readily digested already after 1 h of incubation (Fig. S1). In contrast, a well-folded and structured protein, such as carbonic anhydrase II, was not digested after 24 h incubation time (data not shown).

CD spectra of Prosys and temperature effects

The secondary structure content was investigated by far UV-CD spectroscopy [Fig. 2(A)]; Prosys spectrum at neutral pH showed low ellipticity at 190 nm and a large negative ellipticity at 198 nm, typically observed for proteins in a largely disordered conformation. Some residual secondary structure was consistent with the observed ellipticity values at 200 and 222 nm.[21,23,24] Indeed, according to Uversky, extended disordered proteins can be assigned to two structurally different groups, premolten globule-like (PMG-like) group and random coil-like (RC-like) group, depending on the ratio of the ellipticity values at 200 and 222 nm.[25] Notably, Prosys falls in

the twilight zone between RC-like and PMG-like, suggesting that it exhibits some amount of residual secondary structure [Fig. 2(B)]. This result is in accordance with DLS data, which showed the presence of residual intramolecular interactions in native conditions, a behavior shared by proteins having a PMG-like conformation.[21] Prosys undergoes temperature-induced changes; as the temperature increases, modest but discernible far-UV CD spectral changes are evident, due to the formation of secondary structure [Fig. 2(C)]. The structural changes induced by heating are completely reversible and are not driven by a cooperative behavior [inset Fig. 2(C)]. Heat-induced structuring is a typical feature of IDPs, in contrast to globular proteins, which undergo unfolding upon heating.[26] The peculiar effect is likely due to the increased strength of the hydrophobic interactions occurring at high temperature, which act as a driving force for hydrophobic folding.[23,26]

Induced folding of Prosys

We tested the propensity of Prosys to fold in water/trifluoroethanol (TFE) mixtures. TFE as a cosolvent is often used as a probe to investigate hidden structural propensities of proteins and peptides,[27] since it can mimic a more hydrophobic environment which occurs during protein-protein interactions.[28] Thus, in order to evaluate the potential folding of Prosys upon partner binding, CD spectra were recorded at increasing concentrations of TFE. Prosys showed an increased α-helical content upon addition of TFE, as indicated by the characteristic maximum at 190 nm and the occurrence of a double minima at 208 and 222 nm [Fig. 2(D)]. A slow increase of α-helical content gradually occurred from 5 to 15% TFE, whereas most of the unstructured-to-structured transitions arose between 20% and 30% TFE, reaching a plateau at higher concentrations. It is worth noting that the existence of an isodichroic point at 204 nm is consistent with a two-state coil-helix transition.[21,29]

Conformational analysis by NMR spectroscopy

Conformational analysis of Prosys in solution was further conducted by means of 1D [^{1}H] and 2D [^{1}H, ^{1}H] NMR spectroscopy. The 1D [^{1}H] NMR spectrum together with the 2D [^{1}H, ^{1}H] TOCSY and 2D [^{1}H, ^{1}H] NOESY experiments of Prosys presented the canonical features of the unstructured proteins, being dominated by a poor spectral dispersion, typical of IDPs. In 1D [^{1}H] spectrum, a strong methyl peak in the random coil range around 0.85 ppm, that arises from the 18 isoleucines, 6 leucines, and 11 valines contained in Prosys, could be observed [Fig. 3(A)]. This evidence highlights the absence in Prosys of the canonical hydrophobic core of a folded system that would have generated upfield-shifts for the resonances of these methyl protons. Similarly, as

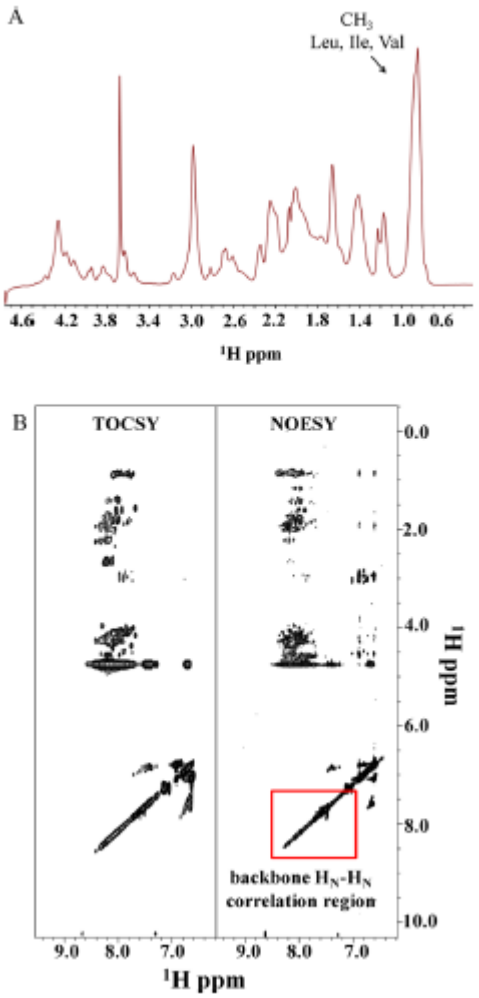

Figure 3. Conformational analysis by NMR spectroscopy. (A) Expansion of the 1D [^{1}H] NMR spectrum of Prosys containing signals from backbone Hα and side chain protons. The strong methyl peak generated by Leu, Ile, and Val residues is highlighted. (B) Comparison of 2D [^{1}H, ^{1}H] TOCSY and NOESY spectra of Prosys. Panels show the spectral regions where peaks from H_N and aromatic protons can be seen. 1D and 2D NMR spectra were acquired with a 2 mg/mL protein sample at 298 K on a 600 MHz spectrometer equipped with a cold probe (see Experimental Section for further details).

can be easily seen in 2D [^{1}H, ^{1}H] TOCSY and NOESY spectra [Fig. 3(B)], the backbone amide protons fall in a rather narrow chemical shift range, that is, 7.7–8.4 ppm; the absence of backbone amide H_N resonances around 9 ppm and of Hα protons provided with chemical shifts around 5–6 ppm [Fig. 3(B)], let us to speculate the absence of extended β-structures in the protein. In general, the lack of H_N–H_N NOE connectivities between amide protons [Fig. 3(B)] further confirms the absence of ordered secondary structure elements in a relevant amount.

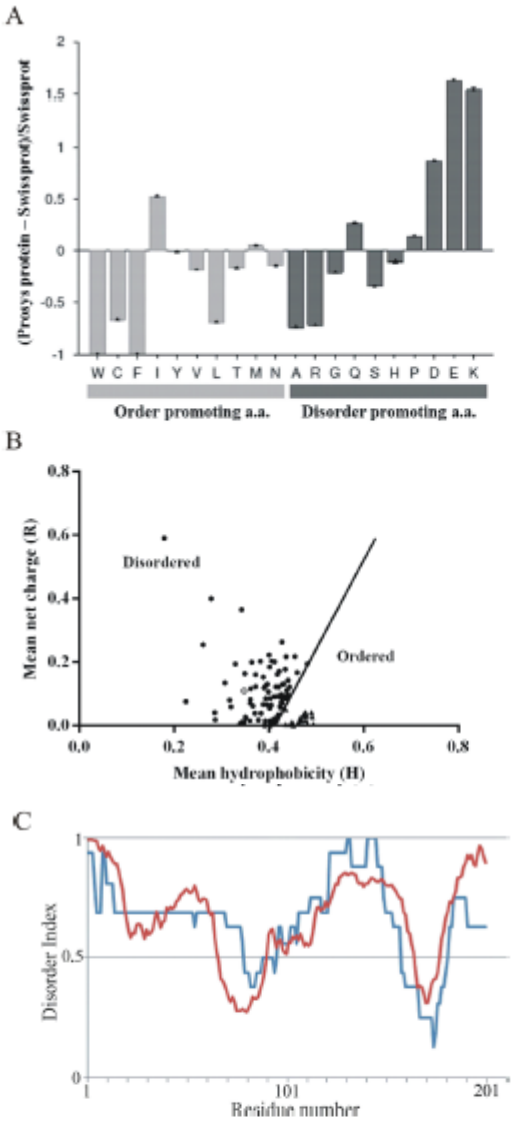

Figure 4. Sequence properties of Prosys. (A) Amino acid compositional analysis performed by means of Composition Profiler tool. Prosys sequence is compared to the reference value of the average amino acid frequencies of the Swiss-Prot database.[31] (B) Charge-Hydrophobicity plot generated as described by Uversky.[25] Black dots, intrinsically disordered protein reported in literature (data partially taken from Ref. 26), black triangles, natively folded proteins randomly taken from PDB. Solid black line, border between the ID and the natively folded proteins, described by the equation $H = (R + 1.151)/2.785$, where H and R are the mean hydrophobicity and the mean net charge, respectively. Empty red circle, Prosys. (C) Predictions of intrinsic disorder by PONDR-fit (red line) and DisMETA (blue line) predictors. Values higher than 0.5 indicate a propensity for disorder, and lower than 0.5 indicate a propensity for order.

In silico *analysis of Prosys*

Prosys has a unique amino acid sequence, rich of charged residues. This peculiarity was already underlined in previous papers together with the observation of repetitive sequence elements.[30] However, an in-depth sequence analysis aiming at studying the sequence-structure relationship of Prosys is lacking. For this reason, we performed a bioinformatics analysis of Prosys primary sequence using a wide range of *in silico* tools. We used first Composition Profiler, which allows to identify statistically significant patterns of amino acid enrichment or depletion along the sequence.[31] To this aim, the sequence composition of Prosys was compared to that of proteins within the Swiss-Prot database.[21] Prosys showed a peculiar amino acid composition, depleted in the so-called "order promoting" residues, that include Cys, Asn, Leu, Val,Trp, Phe, and Tyr which are regularly represented in the hydrophobic core of folded globular proteins.[20] On the contrary, it is enriched in most "disorder promoting" residues such as Gln, Asp, Glu, Lys and Pro [Fig. 4(A)]. These sequence features suggested that Prosys behaves as an intrinsically disordered protein. Notably, the abundance of charged residues (Asp, Glu, and Lys) and the lack of hydrophobic residues (Trp, Phe), or their scarceness (Tyr), are indicative of a high net charge and low mean hydrophobicity, respectively. When correlating these two parameters[32] Prosys is predicted to be a disordered protein [Fig. 4(B)].

In addition, since previously discussed experimental data provide evidence that Prosys is a PMG-like protein not completely unfolded, but with a certain degree of compactness and a residual secondary structure (Fig. 2), we used *in silico* tools to obtain further insights into its structural features. To this aim we performed disorder predictions and calculations of its secondary and tertiary structure. PONDR-FIT[33] and DisMeta[34] predictors were employed to assess the degree of disorder in the sequence. Results suggest that Prosys is mainly disordered [Fig. 4(C)] with local propensities to order, as indicated by the disorder score close or below 0.5 [Fig. 4(C)] in regions 75–105 and 160–180 which belong to the Central and the C-terminal part of Prosys, respectively.

Secondary structure calculations were performed using different predictors: PSIPRED,[35] SSPro,[36] and QUARK.[37] Results shown in Figure 5(A) indicate that most of the sequence is random coil with local tendencies to assume a regular secondary structure (α-helix or β-strand), mainly in the Central and C-terminal region of the protein, consistently with the above discussed disorder predictions [Fig. 4(C)].

Finally, we carried out predictions of the three-dimensional structure of Prosys using QUARK *ab initio* server[37] [Fig. 5(B), S2]. The models generated

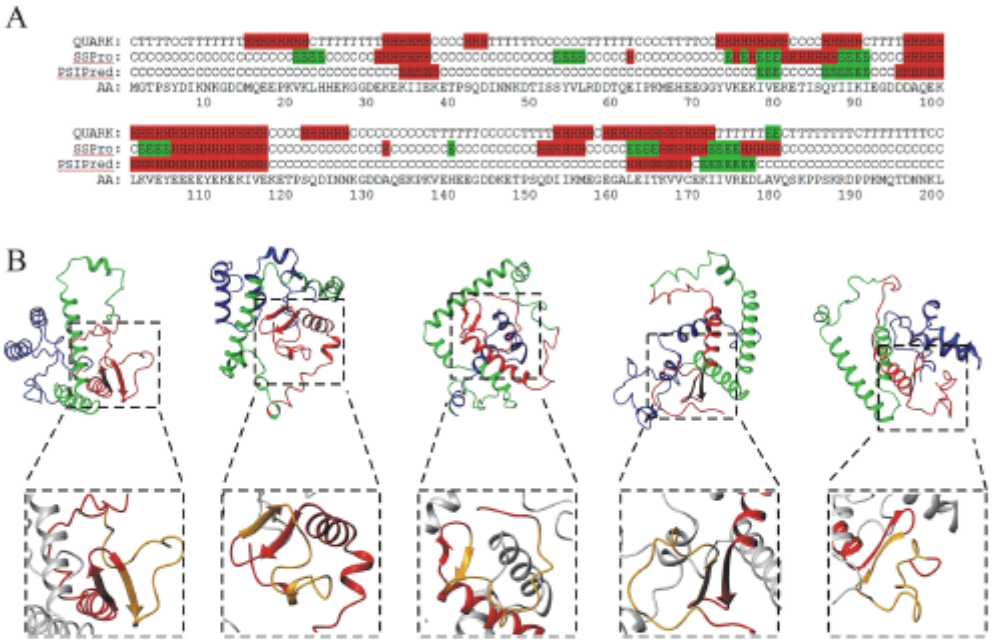

Figure 5. Structure predictions of Prosys. (A) Secondary structure predictions by QUARK, SSPro and PSIPred. The amino acid (AA) sequence is also shown. H: helix, E: strand, C: coil, T: turn. (B) Ribbon representation of five out of the 10 3D-structure models predicted by QUARK *ab initio* server for Prosys full-length (the entire ensemble of 10 models is reported in Figure S1). N-terminal region (1–70) is in blue; Central region (71–140) is in green; C-terminal region (140–200) is in red. Details of beta-hairpin (172–180) which precedes the Sys polypeptide in the C-terminal region (140–200) of Prosys in each model have been highlighted in dashed boxes. Region corresponding to Sys polypeptide is in orange.

by QUARK have a quite low TM-score value (TM-score = 3.1), which indicates no single-high probability model for a specific fold but an ensemble of energetically similar conformations. This finding further suggested that Prosys is an IDP, devoid of a unique well defined global fold, possessing long disordered regions. Moreover, the analysis of the structural models provided insights into the local propensity of protein regions to assume a regular secondary structure, as expected for a PMG-like protein. In details, in all the models we found that: (i) the N-terminal region [blue ribbon in Fig. 5(B)] is highly disordered, having long random coil segments; (ii) the central part of the protein [green ribbon in Fig. 5(B)] consists of two long V-shaped α-helices with a varying inter-helices angle; (iii) the C-terminal region [red ribbon in Fig. 5(B)] displays a long random coil segment and an α-helix/β-hairpin motif which precedes the disordered Sys region. According to the QUARK models [Fig. 5(B)], these three regions can be reciprocally oriented in many different ways, leading to an ensemble of possible conformations.

Notably, there is a good agreement between the different *in silico* predictors used, indicating that Prosys is mainly unstructured and possesses some residual secondary structure elements in the Central and C-terminal region of the protein.

Recombinant Prosys activates the expression of tomato defense genes

The biological activity of Prosys was monitored by evaluating the expression of defense-related gene in response to the application of purified recombinant pro-hormone on wounded leaves. Four defense related genes were selected: two early genes of the octadecanoid pathway, that lead to the formation of JA, *Lipoxygenase* (*Lox C*) and *Allene oxide synthase* (*AOS*), and two late JA related defense genes, encoding for proteinase inhibitors I and II (*Pin I* and *Pin II*, respectively). In order to circumvent the effect of endogenous Prosys on the activation of defense genes following wounding, we experimentally identified the time point for its exogenous application. To this aim, a wounding experiment was carried out as previously described (see experimental procedures), in which leaves of wealthy tomato plants were wounded and endogenous Prosys transcripts quantified through qRT-PCR, 3, 6, and 24 h after wounding. As shown in Figure 6(A), the expression of the endogenous Prosys largely decreased 24 h after wounding. Therefore, we selected this time point for leaf treatments with the recombinant protein and quantified, by qRT-PCR, the transcripts of *Lox*, *Aos*, *Pin I* and *Pin II*, at 6 and 24 h after treatment. Relative quantification data were calibrated on controls represented by wounded

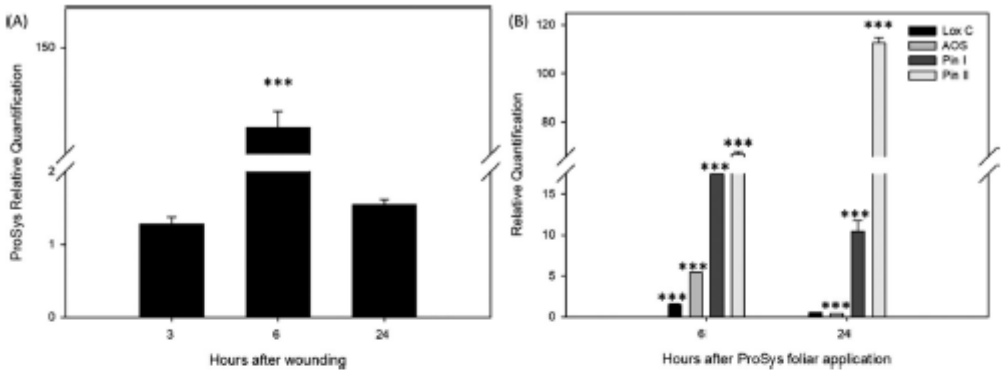

Figure 6. Expression analysis of Prosys and Prosys related genes by RT-PCR. (A) ProSys relative quantification 3, 6, and 24 h after wounding. Data are calibrated on unwounded samples. (B) Relative quantification of early (*LoxC* and *AOS*) and late (*Pin I* and *Pin II*) defense genes in plants treated with 100 pM Prosys as indicated in the text. Data are calibrated on controls treated with PBS after wounding. Asterisks indicate data statistical significance (T-test; $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$). Error bars indicate standard error.

leaves treated with PBS. As shown in Figure 6(B) all genes resulted significantly overexpressed (min P value $3.26E^{-18}$, max p value $1.2E^{-9}$) following the exogenous Prosys application, indicating that recombinant Prosys is biologically active.

Leaf treatment with recombinant Prosys strongly counteracts larval growth and survival

Since Prosys overexpression is associated with plant resistance to insects,[9] we investigated if the exogenous application of the recombinant pro-hormone could mimic the overexpression of the endogenous gene. For this purpose we monitored growth and survival of *S. littoralis* larvae fed with treated or untreated leaves. Larvae fed with Prosys treated leaves had a significant reduction of their weight (18 mg for WC and 7 mg for W Prosys at the 15th feeding day, $P < 0.0001$, Tukey-Kramer HSD test) and showed a significantly reduced survival rate compared with larvae fed with untreated leaves used as control (90.6% for WC control, 31.25% W Prosys at the 15th feeding day, $P < 0.0001$ Log-rank test) (Fig. 7). In order to evaluate if the treatment of the plant with Prosys was able to induce a systemic defense response in untreated leaves of the same plant, the same feeding bioassay was carried out with distal leaves. A similar significant reduction of weight increase (14 mg for SC and 10 mg for S Prosys at the 15th feeding day, $P < 0.0001$, Tukey-Kramer HSD test) and survival (96.8% for systemic control SC and 56.25% for S Prosys, at the 15th feeding day, $P < 0.0001$ Log-rank test) was observed (Fig. 7). Taken together, these results confirmed that recombinant Prosys is biologically active and that its exogenous application is associated with resistance against herbivore insects, as observed with transgenic plants overexpressing the natural pro-hormone.[9]

Discussion

The present study provides a multidisciplinary characterization of plant pro-hormone Prosys. The pro-hormone, which does not contain N-linked glycosylation sites, was produced as bacterial recombinant protein, overcoming the expression problems encountered in earlier studies.[38] Indeed, previous attempts of bacterial expression of the full-length pro-hormone were poorly efficient, basically due to the presence of a translation start site (Shine-Dalgarno sequence) just upstream an internal ATG codon.[38] This sequence, which provides a common bacterial start site, likely directed the translation of a truncated form (Prosys 185) missing the first 14 initial amino-acids. In addition, an intramolecular association of the ribosome binding site of the pET 11d expression vector with the 5′ coding region of the pro-hormone contributed to the low production of the recombinant protein. These problems were only partially overcome by producing a mutated protein (Met15Ala) and by introducing conservative mutations in the 5′ coding region of the Prosys nucleotide sequence.[38] Here we show that the use of pETM11 vector allows the expression of the full-length protein in *E. coli* with an His tag at the N-ter part of the protein. The protein has a unique amino acid composition with a considerable number of acidic residues (20 Asp and 35 Glu over 200 amino acid) which are responsible for an aberrant migration on SDS-PAGE[21,39] [Fig. 1(A)]. Accordingly, the apparent molecular mass of Prosys was 1.5 times higher than that calculated from sequence data or measured by mass spectrometry [Fig. 1(B)]. The high protease-sensitivity, the DLS results and the analysis of the 1D [^{1}H] NMR spectrum confirmed the lack of a packed core within the protein. On the basis of CD spectra, Prosys is characterized by a largely disordered conformation, with a residual secondary

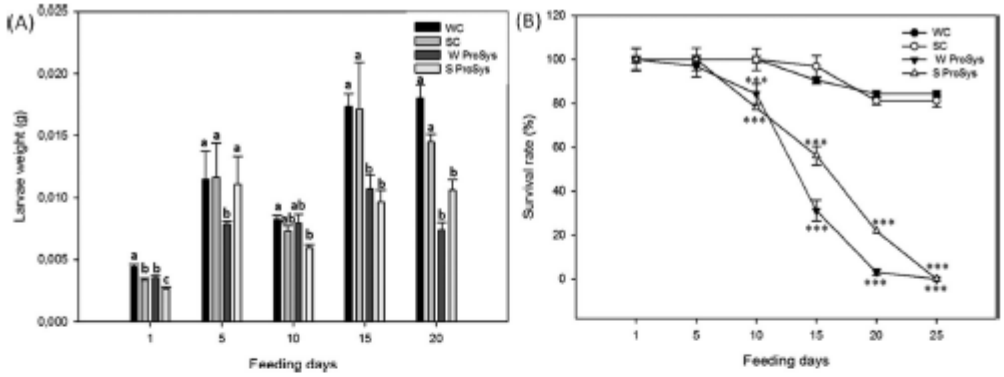

Figure 7. Weight increase and survival rates of *S. littoralis* larvae fed with untreated or treated leaves. WC, wounded leaves used as control; SC, leaves distal from wounded leaf used as systemic control; W Prosys, wounded leaves treated with ProSys; S Prosys, leaves distal from the Prosys treated leaf. (A) Weights registered for treated samples were compared to controls by One-Way ANOVA followed by the Tukey–Kramer Honestly Significant Difference (HSD) multiple range test ($P < 0.05$). Letters indicate different statistical groups. (B) Survival percentages of tests and controls were compared by One-Way ANOVA coupled with Log-Rank test. Asterisks indicate data statistical significance (One-Way ANOVA and Longrank test; ***$P < 0.001$).

structure content which increases upon heating. 1D and 2D NMR spectra exhibited low chemical shift dispersion that is distinctive of IDPs and revealed, in agreement with CD spectroscopy, that Prosys is mostly unstructured. All together, these data indicate that Prosys belongs to the IDP family, having residual secondary structure which is characteristic of PMG-like proteins. *In silico* results further corroborated the gathered experimental evidence showing that Prosys is a disordered protein containing few regular structural elements in the Central (sequence 75–110) and C-terminal region (sequence 160–180). IDPs are a very large class of proteins lacking a stable or ordered three-dimensional structure which play a central role in regulation of signaling pathways and in crucial cellular processes, including regulation of transcription, and translation.[40] Their flexibility allows them to assume a wide spectrum of states from fully unstructured to partially structured. Specific recognition may occur by binding plasticity, which allows IDPs to interact promiscuously with different macromolecular partners.[20,39] When binding to a partner, IDPs may undergo a conformational transition known as induced folding. In order to investigate the eventual induced folding of Prosys we performed CD analysis in presence of TFE thus mimicking the mainly hydrophobic environment sensed in a protein-protein interaction. CD results show that the protein gains an ordered three-dimensional structure following TFE addition, exhibiting α-helices formation. This leads to hypothesize that a large part of Prosys consists of intrinsically disordered regions (IDRs), which may undergo a disorder-to-order transition upon binding with a partner.[25,41]

Interestingly, the *in silico* study predicts the presence of a β-hairpin motif which precedes the disordered Sys sequence at the carboxy-terminal end of Prosys [Fig. 5(B)]. It is tempting to speculate that this structural motif could represent a recognition site for hormone-releasing enzymes responsible for the cleavage and release of Sys. This is a fascinating hypothesis to be tested since, to date, very little is known about the molecular mechanism of Sys release from the precursor.

Finally, plant assays revealed that the recombinant pro-hormone is biologically active being very effective in the induction of tomato defense-related genes, which confer protection against *S. littoralis* larvae both locally and systemically. This evidence further corroborated that the observed intrinsic disorder of recombinant protein is a functional innate feature of Prosys and is not due to experimental conditions. The registered biological activity is likely due to the release of the Sys peptide from the precursor.[8] Although little information on the mechanism of Sys release from the precursor is available,[42] it has been suggested that, upon environmental cues such as insect attacks, the prohormone is processed and the released peptide initiates a signal transduction pathway that leads to the induction of PIs and other defense-related genes.[6] The observed biological activity is worth of consideration from an applied perspective, since it nicely substantiates the use of recombinant proteins and synthetic peptides as innovative tools in insect control, which act not by exerting a toxic action but by triggering plant defense responses.

Many regulatory proteins involved in signal transduction and transcription regulation belong to the IDP family,[40,43] which has been mainly investigated in the animal kingdom,[44–46] rather than in plants.[47] In the latter case, IDPs have a broad impact on many biological functions, being involved

mainly in plant responses to abiotic stress, transcription regulation, plant immunity, light perception and development.[48,49] As plants are sessile organisms they have to constantly respond to the perturbations occurring in the environment. This phenotypic plasticity is supported also by a wide diversity of defense strategies that plants select in response to the environmental cues based on an efficient coordination of various signaling networks. Consequently, protein disorder can play an extremely important role in plant defense, providing a fast mechanism to obtain complex, interconnected and versatile molecular networks. For example, LEA (Late Embryogenesis Abundant) are a group of entirely or partially disordered proteins that coordinate different signals and pathways involved in the plant molecular response to abiotic stresses.[50] These multiple functions appear to be strictly correlated with the IDP nature of LEA that work as a hub interacting with multiple partners.[48] Similarly, the disordered nature of Prosys may promote selective binding of several partners as well as integrate signals from multiple stress agents[9] supporting the hypothesis that Prosys has a biological function other than being an intermediate in the synthesis of Sys. Interestingly, as reported for genes encoding IDPs or disordered domains that originated by duplication and module exchange,[51] Prosys gene is thought to be originated through duplication and elongation events.[30]

In conclusion, the detailed characterization of Prosys by means of a multidisciplinary approach reveals that this precursor is an intrinsically disordered protein, suggesting new interesting insights on the role of IDPs into plant response against biotic stressors. Considering that protein-protein interactions mediated by disordered regions are involved in molecular recognition and signaling events, it is proposed that Prosys may exert its multifaceted biological activity as a consequence of its intrinsic disorder.

Materials and Methods

Molecular cloning, expression, and purification of Prosystemin

Prosystemin cDNA (GenBank: AAA34184.1) was PCR amplified and cloned in NcoI-XhoI site of pETM11 (a kind gift from EMBL, Heidelberg) using site-specific primers:

Forward:5′-CGCGCGCCATGGGAACTCCTTC-ATATGATATCA

Reverse:5′-CGCGCGCTCGAGTTACTAGAGT-TTATTATTGTCTGTTTGCATTTTGG-3′

The generated plasmid was checked by sequencing and appropriate digestion with restriction enzymes. The expression of the recombinant construct was induced in *E. coli* BL21(DE3) cells by exposure to 2.0 m*M* IPTG (isopropyl-β-D-1-tiogalattopiranoside), for 16 h at 22°C. After centrifugation (20 min at 4°C at 6000*g*), the pellet was lysed in 20 m*M* Tris, 20 m*M* imidazole, 50 m*M* NaCl, 1 m*M* DTT pH 8.0, in presence of 0.1 m*M* phenylmethanesulfonyl fluoride, 5 mg/mL DNaseI, 0.1 mg/mL lysozyme and 1× protease inhibitors (Sigma-Aldrich). Cells were disrupted by sonication and after centrifugation (30 min at 4°C at 30,000*g*) the soluble protein was purified by an ÄKTA FPLC, on a 1 mL HisTrap FF column (GE Healthcare), according to manufacturer's instruction (GE Healthcare). After elution, Prosys was dialyzed in 20 m*M* Tris, 50 m*M* NaCl, 100 μ*M* PMSF,1 m*M* DTT, pH 8.0 and purified by means of ionic exchange chromatography on a 1 mL Mono Q HR 5/5 column and subsequently on a size exclusion chromatography (SEC) column. LC-ESI-MS analysis of the protein, performed as previously described,[52] confirmed its identity. Biological assays were performed after extensive dialysis in PBS 1×.

Size exclusion chromatography

Size exclusion chromatography (SEC) was performed using a Superdex 75 10/300 GL column (GE Healthcare) in 20 m*M* Tris, 150 m*M* NaCl, 100 μM PMSF, 1 m*M* DTT buffer, pH 8.0. Calibration was carried out using the following standards (Sigma Aldrich, St. Louis, MO, USA): horse cytochrome c (12,400 Da), chicken ovalbumin (45,000 Da), bovine serum albumin (66,400 Da), carbonic anhydrase from bovine erythrocytes (29,000 Da) and recombinant carbonic anhydrase XIV (37,000 Da, homemade).

Circular dichroism

Circular dichroism (CD) spectra were recorded with a Jasco J-715 spectropolarimeter equipped with a Peltier temperature control system (Model PTC-423-S) as described[53,54] using a 4 μ*M* sample in 10 m*M* sodium phosphate buffer pH 7.4. The same parameters were applied to perform measurements in the temperature range of 10–80°C and titration with increasing concentrations of TFE (from 5% up to 30%). Data were analyzed using the DICHROWEB website. CDSSTR was used as a deconvolution method to evaluate the α-helical content of the protein.

Light scattering

The oligomeric state of the protein was analyzed by size exclusion chromatography equipped with multi-angle light scattering and quasi-elastic light scattering detectors (SEC-MALS-QELS).[55,56] In particular, the experiment was set up by an ÄKTA FPLC, on a Superdex 75 10/300 GL (GE Healthcare) column linked to a multi angle detector (mini-DAWN TREOS, Wyatt Technology) and a refraction index detector (Shodex RI 101). The data were analyzed

with the program ASTRA 5.3.4.14 (Wyatt Technology). Dynamic light scattering (DLS) measurements were carried out using a Malvern nano zetasizer (Malvern, UK) as previously reported.[57,58] Briefly, the sample with a concentration of 0.4 mg/mL was placed in a disposable cuvette and held at 25°C during analysis. The same experiment was carried out in denaturing conditions, in presence of 7.4 M filtered urea (Sigma–Aldrich, Milan).

Tryptic protease sensitivity

TPCK treated trypsin (Sigma-Aldrich, Milan) at an enzyme:substrate ratio of 1:100 and 1:200 (w:w) was added to an aliquot of Prosys (100 μg) in 50 m*M* Tris HCl buffer, pH 7.5. Proteolysis was monitored by SDS-PAGE upon a digestion time of 30 min, 1 h, 2 h, 16 h at 26°C. Carbonic anhydrase II[59] (homemade) was used as a control.

Nuclear magnetic resonance

Nuclear Magnetic Resonance (NMR) experiments were acquired at 298 K on a Varian Unity Inova 600 MHz spectrometer equipped with a cold probe. The NMR sample consisted of Prosys dissolved in a mixture PBS (phosphate buffer saline, 10 m*M* phosphates, 140 m*M* NaCl, 2.7 m*M* KCl, pH 7.4, Sigma-Aldrich, Milan, Italy)/D_2O (98% D, Armar Chemicals, Dottingen, Switzerland) 90/10 v/v with a total volume equal to 600 μL. Prosys sample (2 mg/mL) was analyzed through the 1D [^{1}H] spectrum together with a set of 2D experiments: 2D [^{1}H, ^{1}H] TOCSY[60] (70 ms mixing time), 2D [^{1}H, ^{1}H] NOESY[61] (300 ms mixing time). 1D [^{1}H] spectrum was acquired with a relaxation delay d1 of 1.5 s and 128 scans; 2D experiments were recorded with 32 scans, 128–256 FIDs in t1, 1024 or 2048 data points in t2. Water suppression was achieved by Excitation Sculpting.[62] Chemical shifts were referenced to the water signal (4.75 ppm). Spectra were processed with VNMRJ (Varian by Agilent Technologies, Italy) and analyzed with NEASY[63] comprised in the CARA software package (http://www.nmr.ch/).

Bioinformatics sequence analysis and ab initio *modeling*

Amino acid compositional analysis was performed by means of Composition Profiler tool (http://www.cprofiler.org), comparing the Prosys sequence with the reference value of the average amino acid frequencies of the Swiss-Prot database (http://us.expasy.org.sprot).[31] The Charge-Hydrophobicity (CH) plot was generated as described by Uversky[25] using data reported in literature (data partially taken from Ref. 32 for the intrinsically disordered protein, and randomly taken from PDB for natively folded proteins). In particular the mean net charge <R> and the mean hydrophobicity (H) were calculated using the program protParam at the EXPASY server (http://us.expasy.ch/tools).[21] The CH border is described by the equation H=(R + 1.151)/2.785. The intrinsic disorder profile of Prosys was determined using two meta-predictors PONDR-FIT[33] and DisMeta,[34] which perform a combined consensus prediction from a broad range of different predictors. Secondary structure predictions were performed comparing the results of three different predictors: PSIPRED,[35] SSpro,[64] and QUARK.[37,65] Three-dimensional structure of Prosys full-length was predicted by QUARK *ab initio* server.[37] QUARK program builds 3D structure models by replica-exchange Monte Carlo simulation under the guide of an atomic-level knowledge-based force field.

Plant assays and gene expression analyses

Two set of leaves of 5 weeks-old plants were cut with a sterile razor. The former was used to quantify Prosys expression 3, 6, and 24 h after wounding; the latter, 24 h after wounding, was treated with aliquots of 10 μL of 100 nM purified recombinant Prosys applied at the wound site. Expression of defense genes was monitored 6 and 24 h after Prosys application. Specific transcripts were quantified through qRT-PCR using primers listed in Table S1 as previously described.[66] Briefly, total RNA was obtained from leaves using a phenol/chloroform purification and a litium chloride precipitation based protocol. The synthesis of the first strand cDNA and qRT-PCR were performed as already reported.[66] Three plants and two technical replicates for each of them were used. The housekeeping gene EF-1α was used as endogenous reference gene for the normalization of the expression levels of the target genes.[66] Relative quantification of gene expression was carried out using the $2^{-\Delta\Delta Ct}$ method.[67]

Insect bioassays

A feeding bioassay on *S. littoralis* larvae was carried out as previously described.[9] Briefly, larvae were reared in an environmental chamber at 25 ± 2°C, 70 ± 5% RH and fed with an artificial diet composed by 41.4 g/L wheat germ, 59.2 g/L brewer's yeast and 165 g/L corn meal, supplemented with 5.9 g/L ascorbic acid, 1.8 g/L methyl 4-hydroxybenzoate and 29.6 g/L agar. Newborn larvae were allowed to grow on this artificial diet until the second instar. Uniform second instar larvae were selected to form 4 groups of 15 individuals, and each group was used to evaluate larval weight and survival rate as affected by the following experimental treatments: wounded control spotted with PBS (WC PBS), unwounded leaf of the same plant, to assess any systemic effect (SC PBS), 100 pM Prosys spotted on a wound site (W Prosys), an unwounded leaf of the same treated-plant (S Prosys). Experimental larvae were singly isolated in a tray well (Bio-Ba-8, Color-Dec, Italy) covered by perforated plastic lids (Bio-Cv-

1, Color-Dec Italy), containing 2% agar (w/v) to create a moist environment required to keep turgid the tomato leaf disks, which were daily replaced, adjusting the size (initially of 2 cm^2, later of 3, 4, and 5 cm^2) in order to meet the food needs of growing larvae. Plastic trays were kept at 28°C 16:8 h light/dark photoperiod. Larval weight and mortality were recorded until pupation, which took place into plastic boxes containing vermiculite. Data were collected from two independent experimental replications. Differences in weight and survival rate were analyzed by One-Way ANOVA.

Statistical analysis

Wounding experiment, relative to differences in *Prosys* transcript between wounded samples and unwounded control (three replicates for both of them) were analyzed by T-test. Values of ΔC_t for test and controls were compared using a two-tailed T-test. Similarly, differences in relative quantities of defense transcripts resulted upon Prosys application on the wound-site have been analyzed by comparing ΔC_t values for all replicates of controls and Prosys-treated by a two-tailed T-test. Error bars referring to standard error have been displayed. For insect assay, larval weights were compared by One-Way ANOVA followed by the Tukey–Kramer Honestly Significant Difference (HSD) multiple range test ($P < 0.05$). Survival curves of *S. littoralis* larvae, fed with test and control leaf disks, were compared by using Kaplan–Meier and log-rank analysis.

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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