PROSYSTEMIN: A HUB OF TOMATO PLANT DEFENSE RESPONSES

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A mia mamma, mio padre, a mia sorella, ai miei fratelli e a tutta la mia splendida famiglia, che mi hanno supportato ed hanno sempre creduto in me.

A Teresa, che ha vissuto in pieno questo mio percorso anche e soprattutto nella lontananza.

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Vi porterò sempre nei miei ricordi

INDEX

SUMMARY

Plants live in a complex environment suffering various stress constraints. To counteract stress condition plants have evolved sophisticated defense systems. In tomato plants a key role in defense is played by systemin (Sys), an octadecapeptide, released upon leaf damage from a larger precursor, prosystemin (Prosys). Considering the need to reduce the agro-chemicals we investigated foliar and hydroponic application of Sys to tomato plants that increased both direct and indirect defenses (Chapter 1): treated plants strongly reduce growth and vitality of *Spodoptera littoralis* larvae also damaging the development of future insect generations. In addition, Sys treated plants reduce leaves colonization of the necrotrophic fungus *Botrytis cinerea* and have an increased level of attractiveness of natural herbivores antagonists. In order to investigate the molecular mechanism underpinning Prosys' defence activation, a prediction study of protein-protein interactions (PPIs) was done (Chapter 2). More than 16000 interactions were captured from the interactome query and, among them, 98 Prosys direct interactors were catalogued using GO terms. Prosys sub-network evidenced that Prosys links with two large groups of kinases and transcription factors confirming that the precursor is associated with the very early steps of plant stress perception. Prosys PPIs were also investigated *in vitro* and *in vivo* (Chapter 3). Affinity Purification Mass Spectrometry (AP-MS) detected more than 300 Prosys interactors, including two molecular partners identified *in silico*, a heat shock protein 70 (Sl-HSP70-1), which plays a key role in stress responses, and NAD-dependent epimerase\dehydratase (NaDED), likely associated with both sugar and hormonal plant defense signalling. Some PPIs were validated through BiFC that confirmed the interaction with an ATP-dependent clp protease, detected with AP-MS, and with the NaDED, detected both *in silico* and *in vitro.* BiFC also confirmed two interactors of the *in silico* network, MYB transcription factor and a MAP-Kinase. Overall the results proved that Sys is a very effective plant protectant, and its use could reduce the application of chemical pesticide while Prosys is involved in a large number of interactions possibly due to its ID structure and consequent biological function.

RIASSUNTO

Le produzioni agricole vivono in complessi ecosistemi dove sono costantemente esposte a diversi agenti sfavorevoli che ne possono influenzare e compromettere lo sviluppo e la sopravvivenza. Il recente rapporto della FAO del 2021 ha analizzato le cause delle perdite legate al settore agricolo dal 2008 al 2018, stimate in 108 miliardi di dollari, e dovute a fattori sia biotici che abiotici. Nel corso degli anni sono state sviluppate diverse strategie di controllo degli agenti dello stress biotico che includono l'utilizzo di nuove varietà vegetali, tra cui anche piante geneticamente modificate (PGM) e soprattutto agrofarmaci (Schut *et al*., 2014). Quest'ultimi sono però velocemente diventati un problema a causa del loro massiccio utilizzo che determina molteplici effetti negativi quali l'insorgenza di resistenza nelle popolazioni target, effetti tossici negli organismi non-target, problemi ambientali e problemi legati alla salute dell'uomo (Parameswari *et al.*, 2020). Le PGM sono utilizzate in molte nazioni; USA, Brasile, Argentina, Canada, e India rimangono i primi cinque paesi in cui si coltivano colture GM. Tuttavia, nella gran parte dei paesi europei, Italia inclusa, la coltivazione delle PGM è bandita e l'uso dei pesticidi è il principale strumento di controllo dei parassiti delle piante. Dati i limiti di questo approccio la ricerca di strategie di controllo integrato (Integrated Pest Management, IPM) che prevedono l'utilizzo di vari mezzi di controllo disponibili (chimici, biologici, genetici ecc.) è attualmente considerata di grande interesse. Pertanto, tra gli obiettivi di rilievo delle biotecnologie vegetali rientra l'identificazione di nuovi composti naturali utili per la protezione delle colture, con conseguente riduzione dell'uso di pesticidi chimici.

Nel 1991 Pearce ed i suoi colleghi hanno identificato in pomodoro la systemina (Sys) come segnale primario per l'attivazione dei geni di difesa. Sys è un ormone peptidico di 18 amminoacidi localizzato all'estremità della regione C-terminale di un precursore di 200 amminoacidi chiamato prosystemina (Prosys). Il gene *Prosys* è presente in singola copia nel genoma di pomodoro; la regione codificante conta 4176 coppie di basi suddivise in 11 esoni di cui l'ultimo codifica per la Sys. In condizioni fisiologiche è noto che il gene

Prosys è espresso a livelli femtomolari nelle foglie, nei petali e nei fusti delle piante, ma non nelle radici (Pearce *et al*., 1991; Narváez-Vásquez e Ryan, 2004). Si ritiene che a seguito di danno della foglia il precursore sia sottoposto ad una azione proteolitica mediata probabilmente da una fitaspasi, (Beloshistov *et al*., 2018), determinando il rilascio di Sys nell'apoplasto. A seguito dell'interazione con recettori di membrana, Sys innesca i segnali di difesa che portano all'attivazione di geni difesa-relati (Narvàez-Vàsquez e Orozco-Càrdenas, 2008). È stato dimostrato che la percezione della Sys dipende da SYR1 e SYR2, due recettori di membrana (Wang *et al*., 2018). L'interazione Sys-recettore innesca una serie di eventi a cascata che attivano la via di segnalazione degli octadecanoidi che porta alla produzione di acido jasmonico (JA) (Ryan, 2000). La sovra-espressione del gene della *Prosys* in piante di pomodoro attiva sistemi di difesa diretti e indiretti. I primi includono l'incremento di inibitori di proteasi, che interferiscono con i sistemi di assorbimento nell'intestino degli insetti fitofagi (McGurl *et al*., 1994) mentre i secondi sono la conseguenza dell'aumento di emissione di composti volatili che attirano i nemici naturali degli insetti fitofagi, (Coppola *et al*., 2015; Corrado *et al*., 2007). Le stesse piante sono resistenti all'attacco di funghi necrotrofi e di afidi (El Oirdi *et al*., 2011; Coppola *et al*., 2015) e tolleranti allo stress salino (Orsini *et al*., 2010). Al contrario, il silenziamento del gene determina la quasi completa soppressione della produzione degli inibitori di proteasi (McGurl *et al*., 1992), e una maggiore suscettibilità della pianta nei confronti di larve di *Manduca sexta* (Orozco-Cardenas *et al*., 1993). La funzione biologica di Prosys è sempre stata attribuita al peptide Sys, tuttavia, dati recenti hanno dimostarto che il precursore privo di Sys attiva geni e proteine di difesa in piante di tabacco (Corrado *et al*., 2016). Lo studio strutturale di Prosys (Buonanno *et al*., 2018), ha dimostrato che la proteina è intrinsecamente disordinata (IDP) e quindi in grado di interagire con diversi partner molecolari (Sun *et al.*, 2012; Sun *et al.*, 2013). Partendo da questi presupposti, ho studiato l'impatto della somministrazione esogena di Sys su piante di pomodoro sottoposte ad attacco di vari agenti dello stress biotico (Capitolo 1), e ho identificato numerose proteine candidati interattori molecolari di Prosys attraverso approcci *in silico* (Capitolo 2), *in vitro* ed *in vivo*

(Capitolo 3). I risultati mostrano che larve di *Spodoptera littoralis*, alimentate con foglie di piante di pomodoro trattate con il peptide Sys, applicato su foglia o in idroponica hanno ridotta crescita e vitalità rispetto a individui alimentati con foglie non trattate (Capitolo 1). Inoltre, le piante trattate hanno un aumentato livello di attrattività nei confronti degli insetti antagonisti dei fitofagi, dovuto all'emissione di una miscela modificata di Composti Organici Volatili. Infine, le piante trattate riducono la colonizzazione delle foglie da parte del fungo patogeno *Botrytis cinerea*. L'induzione di queste risposte di difesa è stata associata a cambiamenti molecolari e biochimici controllati dalla cascata di segnalazioni innescata dalla Sys confermate monitorando i livelli di espressione di geni con ruoli chiave nella difesa. Sono stati quindi identificati gli interattori di Prosys attraverso un approccio bioinformatico (Capitolo 2). Per questo studio si è partiti dai risultati di analisi microarray di piante di pomodoro sovraesprimenti Prosys (piante RSYS) (Coppola *et al*., 2015). Sono stati individuati oltre 500 geni differenzialmente espressi (DEGs), provenienti da 695 *expressed sequence tags* (ESTs). La conversione delle ESTs, tramite BlastX, nelle corrispondenti proteine di arabidopsis e pomodoro, ha identificato 309 proteine. Queste, sono state sottoposte ad analisi di interazione proteina-proteina sfruttando due database: Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), e Predicted Tomato Interactome Resource (PTIR). Le interazioni identificate per tutte le proteine derivanti dai DEGs sono state oltre 160.000. Da queste sono state estrapolate le interazioni dirette della Prosys. Sono stati individuati 98 possibili interattori della proteina, undici dei quali provenienti dai DEGs. Le 98 proteine sono state importate in Cytoscape, per la visualizzazione grafica della network, di cui sono stati catalogati i vari gruppi mediante la classificazione e la nomenclatura Gene Ontology (GO). I gruppi più rappresentativi riguardano enzimi indotti da acido jasmonico e acido salicilico, con ruoli sia nelle difese dirette che indirette (Li *et al.*, 2019); enzimi con attività chinasica (MAP-K) e fattori di trascrizione, fondamentali nella trasmissione del segnale di stress e nell'attivazione della risposta genica. Di particolare rilevanza è il fattore di trascrizione MYB (Solyc06g053610), associato all'attivazione di alcuni geni della difesa contro stress biotici e abiotici (Baldoni *et al*., 2015). Sono stati definiti

poi gruppi associati al *burst* ossidativo e alla via di segnalazione del calcio, attivi nelle prime fasi delle risposte di difesa. Le predizioni fatte e mostrate nella network ci hanno spinto ad approfondire lo studio sulle interazioni della Prosys utilizzando approcci differenti con metodi *in vitro* ed *in vivo* (Capitolo 3): Affinity Purification Mass Spectrometry (AP-MS), e Bimolecular Fluorescent Complementation (BiFC). L'AP-MS ha individuato più di 300 probabili interattori della Prosys tra cui due presenti nella network con uno valore di confidenza (Fold Change abundance score, FC-A) significativo, la NAD dependent epimerase/dehydratase (NaDED) (Solyc09g065180) e la heat shock protein 70 (Sl-HSP70-1) (Solyc06g076020). L'interazione Prosys-NaDED è stata poi validata tramite BiFC. La NaDED è un membro di una famiglia di proteine, con attività catalitica, localizzata nel citosol e coinvolta in diversi processi biologici, tra cui il metabolismo dei carboidrati (Cao *et al*., 2013). È noto che gli zuccheri possono stimolare l'immunità delle piante e up-regolare l'espressione dei geni di difesa (Bolouri-Moghaddam e Van Den Ende, 2012), infatti, un alto livello di zuccheri nei tessuti vegetali aumenta la resistenza delle piante contro i funghi patogeni (Morkunas e Ratajczak, 2014). Gli zuccheri possono anche regolare il sistema immunitario delle piante (Morkunas e Ratajczak, 2014), ad esempio, diversi fitormoni, tra cui etilene e jasmonato, interagiscono con la via di segnalazione del saccarosio (Tauzin e Giardina, 2014). L'interazione Prosys-NaDED potrebbe per questo essere associata sia alla difesa tramite il metabolismo dei carboidrati che alla segnalazione ormonale delle piante. Tra gli interattori ottenuti con l'AP-MS ci sono diverse heat shock protein, espresse in particolari condizioni di stress (Kiang and Tsokos, 1998), tra cui anche la Sl-HSP70-1 già predetta *in silico.* Un altro interessante interattore individuato con l'AP-MS è il WRKY-43 (Solyc12g042590), appartenente ad una famiglia di fattori di trascrizione coinvolti in diversi processi biologici, tra cui difesa da patogeni (Huang *et al.*, 2012). L'interazione Prosys-WRKY 43 potrebbe avere un ruolo nella difesa di pomodoro. L'analisi BiFC ha confermato l'interazione tra una proteasi citoplasmatica (Solyc12g042060) e la Prosys, (interazione ottenuta anche con l'AP-MS) e due interazioni predette dallo studio *in silico* ovvero SlMYB14 transcription factor (Solyc06g053610) e una MAPK6

(Solyc05g049970). Le proteasi citoplasmatiche e intra-plastidiche delle piante, come quella validata, hanno molteplici ruoli, tra cui uno nella difesa, agendo nel riconoscimento di patogeni e parassiti e nell'induzione di risposte di difesa (Van der Hoorn e Jones, 2004). In questo scenario l'interazione tra Prosys e la proteina ha un valore importante come candidato nel potenziamento delle risposte di difesa di pomodoro. Il fattore di trascrizione SlMYB14 appartiene ad una tra le più grandi famiglie di fattori di trascrizione; le proteine MYB sono coinvolte in vari processi compresa la partecipazione alle risposte di difesa agli stress biotici e abiotici, alla sintesi ormonale e alla trasduzione di vari segnali (Dubos *et al*., 2010; Zhao *et al*., 2014). SlMYB14 è un gene JA-responsivo che gioca ruoli positivi nell'accumulo di flavonoidi e nella tolleranza allo stress ossidativo (Li *et al.*, 2021). L'interazione Prosys-SlMYB14 potrebbe essere responsabile dell'attivazione della trascrizione dei geni di difesa JA-responsive, della riduzione dell'accumulo di ROS e della promozione della biosintesi dei flavonoidi. Infine, l'interazione Prosys-MAPK6 potrebbe innescare altre chinasi per la trasmissione del segnale di difesa, come suggerito dalla presenza di diverse altre chinasi nella Prosys-subnetwork ottenuta con approcci bioinformatici. La MAPK6, localizzata nel citosol e/o nel nucleo, è associata alla trasduzione del segnale intracellulare e alla regolazione dell'espressione genica (Eulgem e Somssich, 2007). Questa interazione probabilmente è uno dei primi attori nella trasmissione del segnale di difesa con la conseguente attivazione delle vie di biosintesi dei fitormoni. Ulteriori approfondimenti saranno necessari per le altre interazioni predette ed ottenute con l'AP-MS, per fare chiarezza sul complesso meccanismo che regola la difesa delle piante ed essere utile ai fini biotecnologici. In conclusione, i biosaggi sviluppati per verificare la risposta delle piante di pomodoro trattate con il peptide Sys contro vari invasori, hanno dimostrato che i trattamenti incrementano le difese dirette e indirette (Capitolo 1) provando che SYS può essere uno strumento valido in strategie IPM, con conseguente riduzione dell'uso di pesticidi. Le interazioni predette (Capitolo 2) e poi validate (Capitolo 3) hanno inoltre confermato l'ipotesi che Prosys ha un ruolo nei meccanismi di difesa, interagendo con diversi partner molecolari.

Questo risultato la esclude dall’essere considerata un semplice carrier per la Sys, rendendola una proteina chiave nelle risposte di difesa.

CHAPTER 1

SYSTEMIN EXOGENOUS DELIVERY ON TOMATO PLANTS ENHANCED THE LEVEL OF DIRECT AND INDIRECT DEFENSES ASSOCIATED WITH INCREASED EXPRESSION OF DEFENSE-RELATED GENES

In collaboration with M. Coppola, M.Ruocco, P.Cascone and E.Guerrieri and R. Rao

ABSTRACT

Prosystemin (Prosys) is a pro-hormone of 200 aminoacidic residues that releases a bioactive peptide of 18 amino acids, from the C-terminal region, called Systemin (Sys). Sys is involved in the activation of defense genes in tomato plants, in response to mechanical damage, herbivore feeding and pathogen attacks (Zhang *et al*., 2020). In this chapter we report the results of the effects of Sys application on healthy tomato plants by foliar spotting or hydroponic solution. The results show that the noctuid moth larvae of *Spodoptera littoralis*, fed on treated plants have a reduced growth and development, in addition the results show that treated plants have an increased level of attractiveness of natural herbivores antagonists caused by the emission of a changed blend of Volatile Organic Compounds (VOCs). Finally, the data show that treated plants reduce leaf colonization of the fungal pathogen *Botrytis cinerea.* The induction of these defense responses was associated with the increased expression of defense related genes known to be triggered by the activation the *Prosys* gene. Our results indicate that the direct delivery of Sys represents an innovative biotechnological tool for the sustainable protection of tomato plants, in particular due to the expected low or null toxicity of the peptide on non-target organisms.

1. INTRODUCTION

1.1 SYSTEMIN MEDIATED DEFENSE SIGNALLING

The use of pesticides in agriculture has been widespread since the 1950s to reduce production losses due to pests and pathogens, and

to satisfy the increasing food demand. Several commercial formulations have been used in crops protection as insecticides, herbicides, and fungicides but, over the decades excessive use caused environmental problems, bio-accumulation in the food chain and undesirable effects on non-target organisms, including humans (Kumar and Kumar, 2019). Several formulations have been banned in many countries, such as DDT (dichlorodiphenyl trichloroethane) that is presently allowed, in some countries, only to control vectors of diseases. Hence, there is an urgent need to develop alternative pest-control strategies able to reduce the amount of pesticides. For this reason, the enhancement of plant endogenous defense, through the identification of genes and molecules able to contain harmful insect population, is considered a suitable tool for crop protection (Le Mire *et al.*, 2016).

Bio-pesticides are environmentally sound with minimum or non-toxic effects on humans and non-target animals. They could be developed from the study of the natural barriers used by plants; in fact, under attack, plants release molecules called damage-associated molecular patterns (DAMP), that mainly include cell wall or protein fragments, peptides, nucleotides, and amino acids. DAMPs are detected by plasma membrane receptors of nearby cells, regulating immune responses against the invading organisms, and promoting damage repair (Hou *et al*., 2019). In tomato plants, after wounding or pathogens attack, a small peptide, Sys, triggers tomato defense responses via the octadecanoid pathway (Ryan *et al*., 1994). Sys was the first plant bioactive peptide discovered in 1991 by Ryan's group, during a pioneering study demonstrated that the peptide is a potent inducer of proteinase inhibitors (Pin) in tomato and potato plants. Sys is an 18 amino acids peptide, released from a large precursor protein of 200 aa called prosystemin, after mechanical damages or insect attacks (McGurl and Ryan, 1992). The *Prosys* gene (4526 bp) is located on chromosome 5 in a single copy; the gene is composed by eleven exons, five homologous pairs and one non-homologous exon at C-terminus that encode for Sys. The gene structure suggest that it evolved by several gene duplication-elongation events. In fact, the study of the nucleotide and amino acid sequence homologies

suggests that a small ancestral gene was duplicated to form two tandem repeats, followed by subsequent duplication-elongation events (McGurl and Ryan, 1992). *Prosys* homologs have only been found in species of the *Solanaceae* family, including tomato, potato, bell pepper, and nightshade, but not in tobacco (Constabel *et al*., 1998). *Prosys* gene is induced by wounding, chewing insects, JA application and pathogen attacks, and its over-expression, regulated by the CaMV 35s promoter, in transgenic tomato plants determined the constitutive accumulation of high levels of several defensive proteins in leaves (McGurl *et al*., 1994), in addition, the expression of tomato Prosys gene in Arabidopsis reveals systemic translocation of its mRNA and confers necrotrophic fungal resistance (Zhang *et al*., 2017). This suggests the mobility of Prosys mRNA and its fuction in distal leaves where probably is processed and Sys released. After its release, Sys induces the production of JA that activate the systemic response (Schilmiller and Howe, 2005; Sun *et al*., 2011). After binding its receptor, SYSTEMIN RECEPTOR1 (SYR1) (Wang *et al.*, 2018), Sys promotes the depolarization of plasma membrane, the alkalinisation of apoplast, Ca^{2+} influxes and H_2O_2 release. These events determine the activation of MAPK and phospholipase A2 (PLA2) which releases α-linolenic acid (LA) from plastid membranes promoting the octadecanoid pathway which leads to the biosynthesis of jasmonic acid, a powerful inducer of defense genes (Ryan, 2000). Sys peptide, Prosys mRNA and JA derivates could move from local to distal tissues as demonstrated in several experiments (Zhang *et al*., 2020). Furthermore, Sys triggers the production of defensive compounds such as protease inhibitors not only in the wounded plant but also in neighbouring plants (Farmer and Ryan, 1990), suggesting that the peptide promotes plant-to-plant communication with a consequent priming of defense responses. In fact, tomato plants, in response to insect feeding, release different compounds: monoterpenes and sesquiterpenes, locally and systemically, while C6 green leaf volatiles are released only from damaged leaves (Farag and Pare, 2002). In addition, Coppola and collaborators in 2017 demonstrated that tomato plants exogenously treated with Sys induced a defense reaction in neighbouring plants likely through the emission of VOCs. Several groups studied the function of *Prosys*

gene, by producing transgenic tomato plants that constitutively overexpressed or silenced the full-length cDNA. In 1992, McGurl and co-workers, shown that tomato plants transformed with an antisense *Prosys* cDNA, exhibited significantly suppressed systemic wound induction of *Pin I* and *II* synthesis in leaves. The same group demonstrated that the overexpression of the *Prosys* gene in transgenic tomato plants generates a systemic signal that constitutively induces proteinase inhibitor synthesis. In addition, Coppola and co-workers showed that tomato transgenic plants, promoted 503 differentially expressed genes indicating that several biological functions were affected. Transgenic lines were more resistant against different biotic stressors such as aphids (*Macrosiphum euphorbiae*), phytopathogenic fungi (*Botrytis cinerea* and *Alternaria alternata*) and phytophagous larvae (*Spodoptera littoralis*), indicating that a single peptide may provide a wide resistance against several biotic stress agents. The use of transgenic lines was one interesting example of tomato crop protection likely achievable without or with reduced use of chemicals. Unfortunately, the European rules on plant genetically modified adoption prevented the spread of these plants into agricultural systems (UE 412/2015). One of the first example of transgenic crop resistant to insect, successfully introduced on the market and still produced in several non-European countries, is corn (*Zea mays*) engineered to express *Bacillus thuringiensis* toxins (de Maagd *et al.*, 1999). Furthermore, several herbicide-resistant crops have been developed and commercialized, for example maize and soybean resistant to glyphosate and glufosinate, two of the most used herbicides worldwide (Green and Castle, 2010; Green, 2009). Given the current legislative situation, regarding the restriction in the utilization of transgenic organism in EU countries, alternative approaches to the use of chemical pesticides are widely pursued with the principal aim to reduce the amount of chemicals and the consequent effects on environmental pollution, damages of useful insects, impact on human health, according to the invitation of the EU directive (2009/128) on sustainable use of pesticides. One possibility is to learn from plants the strategies that they evolved to defend themselves by environmental treats. The use of the exogenous supply of peptide as

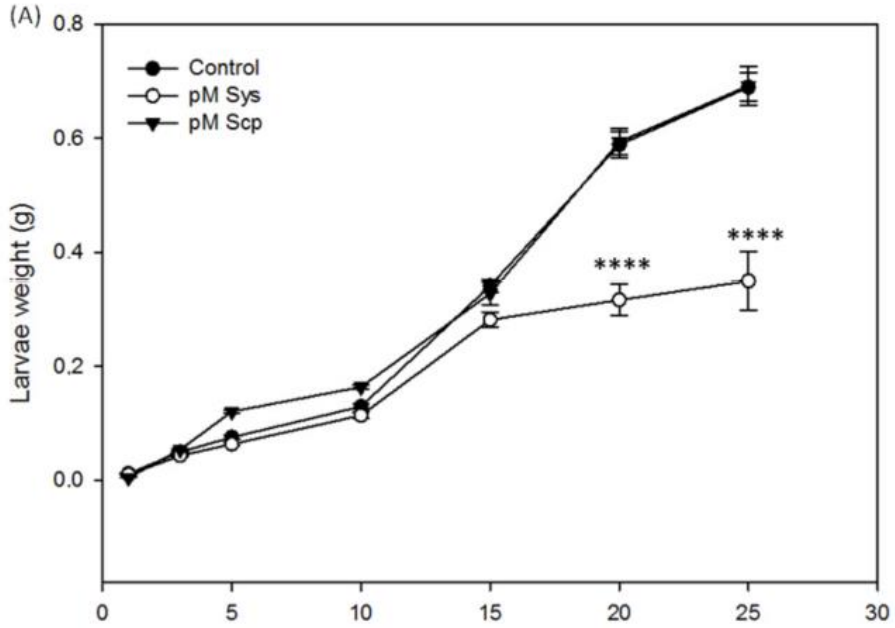
Sys on tomato plant may represent an interesting approach for the protection of the crop, investigating novel strategies for the use of plant molecular weapons developed in a long co-evolutionary history regarding plant and parasites. In this context, we evaluated if the exogenous supply of Sys peptide to tomato plants was able to trigger the plant endogenous defense and protect the plants in a similar way as occurs in transgenic plants. Here we demonstrate that healthy tomato plants, treated by spotting Sys on intact leaves or by supplying the peptide through hydroponic cultures, are resistant to the noctuid moth *S. littoralis* and to the fungal pathogen *B. cinerea.* Moreover, treated plants shown an increased emission of volatile compounds, known to be able to attract insect natural enemies (Dicke, 2015; Strapasson *et al*., 2016). The resistant phenotype of treated plants is associated with the expression of an array of defense-related genes induced upon Sys treatment. The results obtained prove that this approach is very interesting and innovative for crop protection.

1.2 RESULTS

1.2.1 *SPODOPTERA LITTORALIS* ASSAY: *SYS* APPLICATION PROMOTES DIRECT DEFENSES

We firstly evaluated the impact of Sys foliar application on growth and mortality of *S. littoralis* larvae, using a feeding bioassay comparing Sys-treated plants with untreated or Scp treated controls (Sys-Scramble peptide contains the same amino acids but in random order). Since Scp was synthesized and purified in the same way it was done for Sys, the introduction of this control was important to exclude effects due to peptide preparation. We selected the concentration of 100 pM Sys solution, based on the results obtained from the gene expression study illustrated below. Sys solution was spotted on tomato fully expanded leaves. The larvae were fed directly with Sys-treated leaves, with Scp-treated leaves and with untreated fresh leaves . The larval weight was monitored for a period of twenty five days, but after five days of feeding, the reduced weight was already evident, and this consistent trend over time generated significant differences after day fifteen (One Way ANOVA test: $P<$

0.0001, F = 14.9) (figure 1.1A). Moreover, the survival rate of experimental larvae was monitored, and it clearly shown the effect of the peptide. In fact, the larvae fed on treated leaves had a survival rate significantly lower than larvae fed on controls (Log-rank (Mantel-Cox) test: $P< 0.0001$, $dF = 2$, $X2 = 51.16$) (figure 1.1B). The survival rate of larvae fed with Sys treated plants, after twenty five days of feeding, was 25% compared to 90% and 97% respectively for Scp and control plants (figure 1.1B). Thus, the bioassay shown that the foliar application of Sys peptide impairs both growth and survival of *S. littoralis* larvae. To determine the peptide concentration for the bioassay, it was carried out a gene expression analysis, monitoring two classes of genes activated following Sys perception: early defense genes: *Prosys* and *allene oxide synthase (AOS*), and late defense genes: *wound-induced proteinase inhibitors I and II (Pin I and Pin II*), on Sys and Scp treated plants (results shown below in the paragraph 1.2.4).



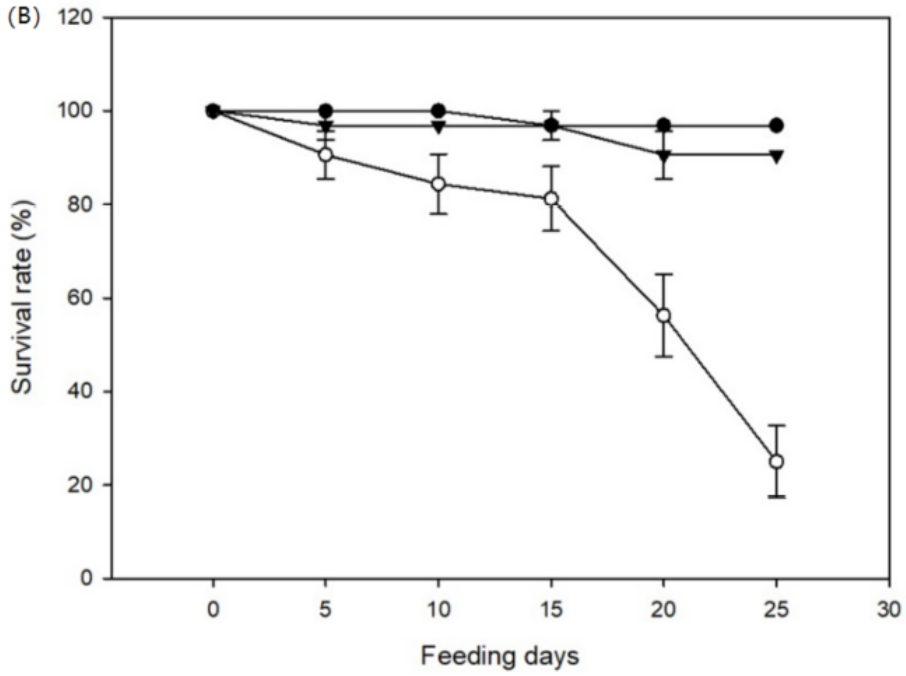


Figure 1.1 A-B. Effect of Sys foliar application on *S. littoralis* larvae. (A) Mean weight (± S.E., standard error) of *S. littoralis* larvae feeding on control and treated leaves. (B) Survival rate of experimental *S. littoralis* larvae. Asterisks denote statistically significant differences (one-way Analysis of Variance, ANOVA: **** *P*< 0.00001).

Then, we evaluated the effect of Sys supply in hydroponic cultures on larval growth. The results showed a negative effect of Sys peptide on larval growth and survival rate (figure 1.2): larvae fed with leaves from tomato plants grown on Sys-enriched hydroponics had a significant reduction in growth after five days of feeding (one-way ANOVA: *P*< 0.0001, F(2.93) = 67.837) (figure 1.2A); the survival rate of larvae fed on hydroponics was significantly reduced if compared with the other two control groups (Log-rank (Mantel-Cox) test: $P < 0.023$; df = 1; $\chi 2 = 5.164$) (figure 1.2B).

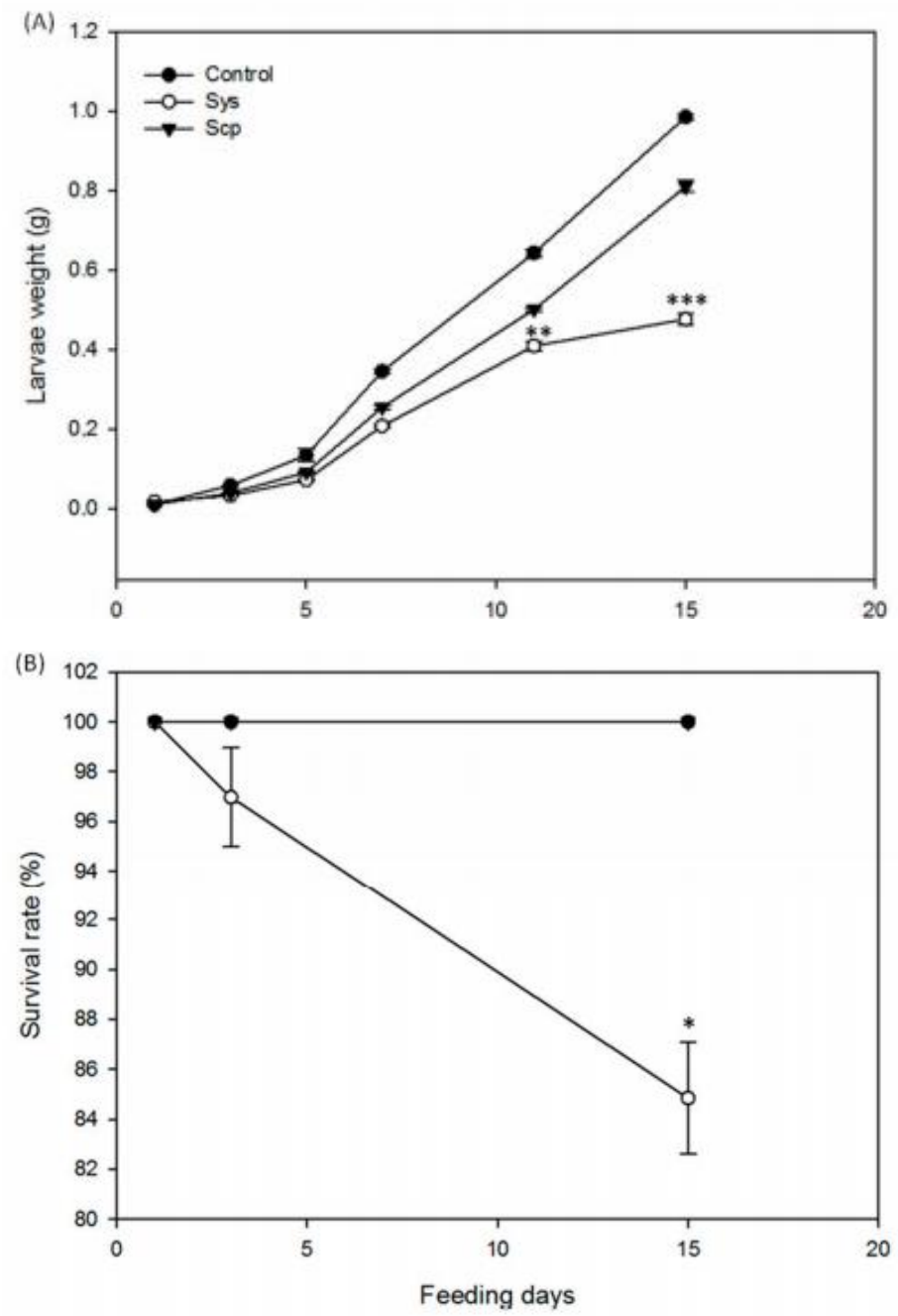
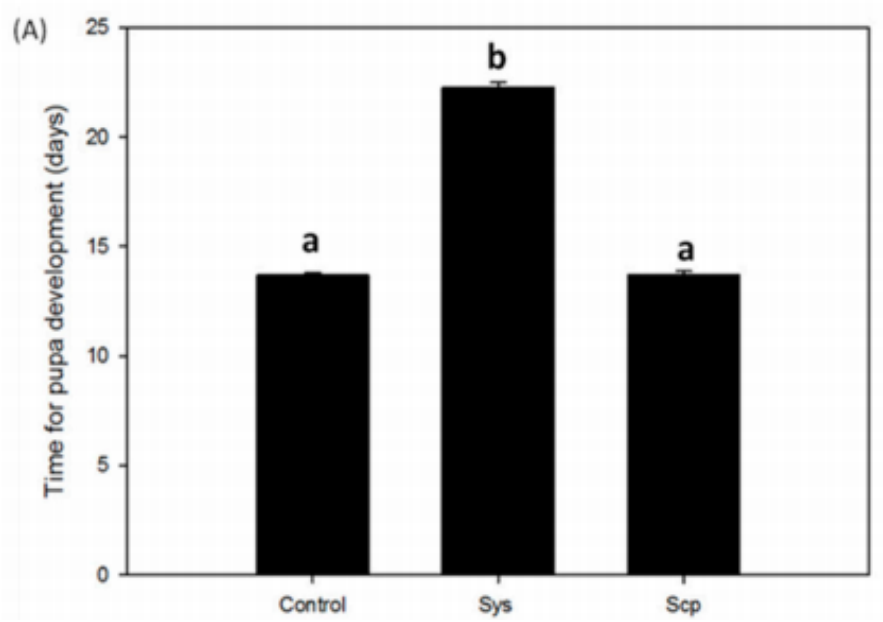


Figure 1.2 A-B. Effect on insect performance of Sys peptide supplied via hydroponics. Tomato plants were grown in hydroponic culture and supplied with 100 pM Sys or 100 pM Sys-scramble (Scp) or PBS1X. (A) Mean weight (± S.E.) of *S. littoralis* larvae feeding on tomato leaves. (B) Survival rate of

experimental *S. littoralis* larvae. Asterisks denote statistically significant differences (one-way ANOVA: *P< 0.05; **p < 0.01; ***P< 0.001).

Survived larvae were used to monitor pupal development, adult survival, and reproduction. The time required by the experimental larvae, fed on Sys treated plants, to pupate was significantly higher (Kruskal-Wallis Test: P< 0.0001; KW = 71.170; n = 32) (figure 1.3A). In addition, the emerged adults shown a significantly reduced survival rate (log-rank (Mantel-Cox) test: P < 0.0001, dF = 2, $\chi 2$ = 45.04) (figure 1.3B) and a significantly lower fecundity (one-way ANOVA test P< 0.0001; F(2.37) = 37.496) (figure 1.3C).



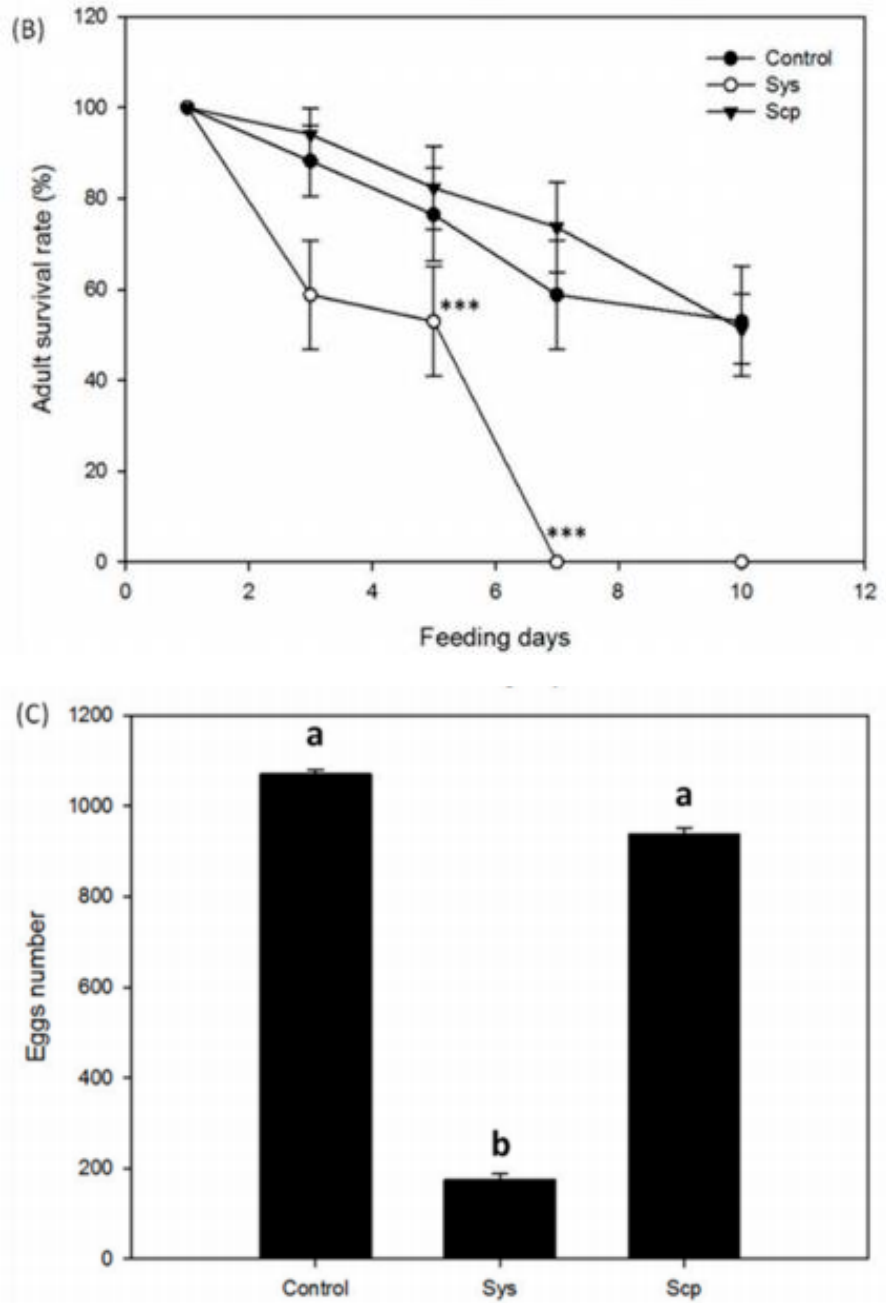


Figure 1.3 A-B-C. Sys effect on development and reproduction of *S.littoralis* larvae. Tomato plants supplied with 100 pM Sys, or 100 pM Scp or PBS1X in hydroponics were used to feed *S. littoralis* larvae, on which the following parameters were scored: duration of pupal development (A), adult survival rate (B) and number of laid eggs (C). Letters and asterisks denote statistically significant differences (*** P< 0.001; one-way ANOVA).

1.2.2 SYS APPLICATION ENHANCES PLANT TOLERANCE AGAINST *BOTRYTIS CINEREA*

Coppola and collegues in 2015 demonstrated that the over-expression of *Prosys* in tomato plants determined an increased resistance to *B. cinerea*. So, based on these data, we decided to evaluate the performance of Sys-treated plants against this necrotrophic fungus, at four different time points (one, three, six and nine days post-inoculum, pi). Disease severity was quantified by measuring necrotic areas. *Sys*-treated leaves shown a marked reduction of *B. cinerea* induced lesions at all the time points considered (highest significant differences at six and nine days pi with $P< 0.00001$) (figures 1.4 and 1.6), the same results were observed following the fungal inoculum on plant grown in hydroponic media, where the same concentration of Sys was applied ($P< 0.05$) (figure 1.5). The hydroponic application of Scp did not show any difference with control. These results proved that the hydroponic application of the Sys peptide interferes with fungal growth and colonization thus reducing disease severity.

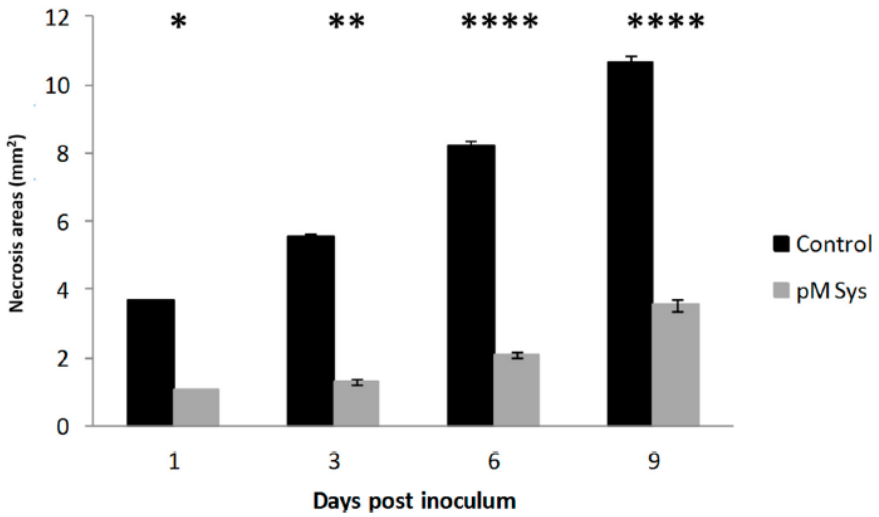


Figure 1.4. Enhanced resistance to *B. Cinerea* of Sys treated leaves. Response to *B. Cinerea* infection by leaves treated with 100 pM *Sys*. The graphs display the average (± S.D.) of the lesion size at one, three, six and nine days pi. Asterisks denote statistically significant differences (T-test: *P <0.05; **P < 0.01; ***P < 0.001; ****P < 0.00001).

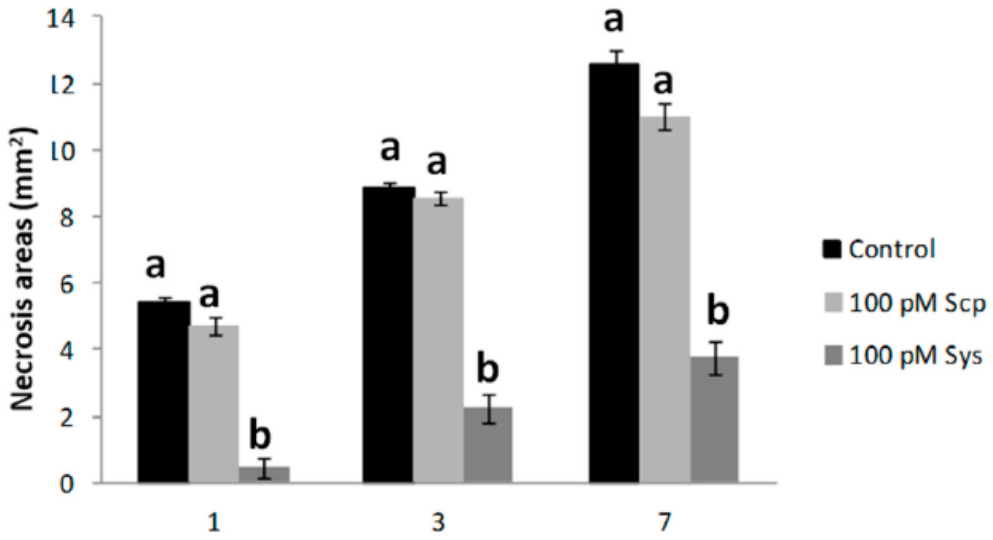


Figure 1.5. Enhanced resistance to *B. cinerea* of tomato plants supplied with Sys via hydroponics. Response to *B. cinerea* infection by leaves from plants treated with 100 pM Sys, or 100 pM Scp or PBS1X in hydroponics. The graphs display the average (±S.D.) of the lesion size one, three and seven days pi. Letters denote statistically significant differences (One-way ANOVA, Tukey test).

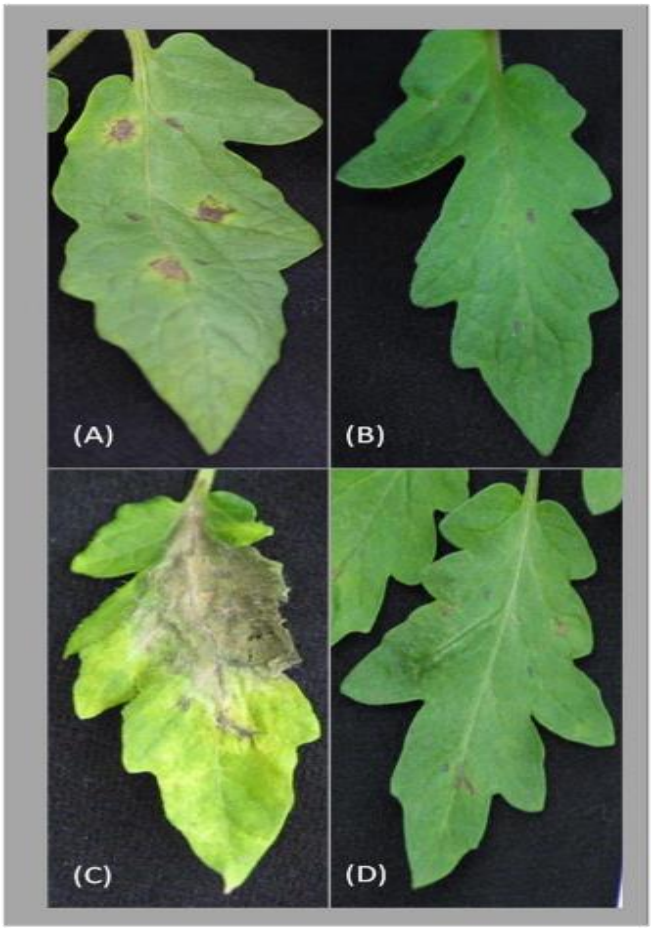


Figure 1.6 A-B-C-D. Symptoms of *B. cinerea* infection on tomato leaves. Necrosis caused by *B. cinerea* spores three and ninedayspi are shown in control (A, C) and Sys-treated (B, D) leaves.

1.2.3 SYS APPLICATION PROMOTES INDIRECT DEFENSES BY INCREASING THE EMISSION OF VOC

In several publications as in Howe, 2004, Corrado *et al*., 2007, Degenhardt *et al*., 2010 and more recently in Coppola *et al*., 2017 and 2019, it was demonstrated that Sys plays a key and complex role in the regulation of indirect defense responses. For example, tomato plants overexpressing *Prosys* produced an increased amount of VOCs that are able to alert neighboring plants, priming their defense responses (Coppola *et al.*, 2017). In our experiment, healthy tomato plants treated with Sys showed an increased attractiveness towards

Aphidius ervi females compared to the controls (figure1.7A). *A. ervi (*Hymenoptera*, Braconidae)* is an endophagous parasitoid of tomato aphid *Macrosiphum euphorbiae* and of various cereal aphids which use host for oviposition. *A. ervi* females shown 45% of oriented flights and 40% of landings on Sys-treated plants in comparison to 9.5% (G test, $\chi 2$ = 31.35, *df* = 1, *P*< 0.01) and 4.8% (G test, $\chi 2$= 27.60, *df* = 1, *P*< 0.01) observed for controls, respectively. Similarly, plants grown in the presence of Sys-enriched hydroponic solution elicited 46.2% of oriented flights and 31.6% of landings on targets in comparison to 20% (G test, $\chi 2$= 17.01, *df*= 1, *P*< 0.01) and 9.6% (G test, $\chi 2$= 15.72, *df*=1, *P*< 0.01) recorded for the controls (figure 1.7B). No significant difference in parasitoid attraction was registered for Scp-treated plants, respect to controls (figure 1.7A,B). After these results we decide to analyze the volatile blend emitted by leaves of treated plants, to support the observed increased attractiveness towards the parasitoid with a specific experiment. The objective was to identify the volatile signals known to be involved in indirect defense, and we registered a quantitative variation in volatile blends released by treated plants (table 1).

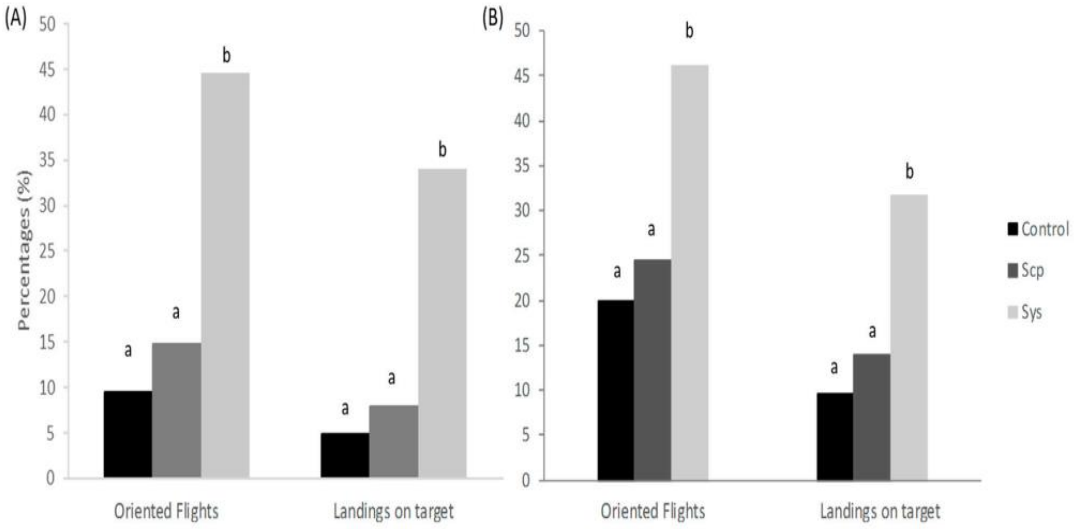


Figure 1.7 A-B. Flight behavior of the aphid parasitoid *Aphidius ervi* towards tomato plants treated with Sys, Scp, and untreated (control) on intact leaves (A) or in hydroponics (B). Values indicate the percentage of females showing

oriented flights and landings on source. Each assay was conducted using at least 100 females tested against 9 plants. Different letters indicate significant differences (G-test, $P < 0.05$).

Table 1.1. Volatile organic compounds (VOCs) increase upon treatment with the Sys peptide. List of VOCs significantly improved by Sys foliar application in comparison to VOCs blend released by mock- and Scp-treated plants (*$P < 0.05$, Kruskal-Wallis One Way ANOVA).

			Concentration (PPb)		
Name	**Molecular Formula**	**Molecular Weight g\mol**	**Control**	**Sys**	**Scp**
Benzaldehyde *	C_7H_6O	106.124	$1.54 \times 10^6 \pm 1.2 \times 10^5$	$3.09 \times 10^6 \pm 2.8 \times 10^5$	$1.81 \times 10^6 \pm 1.18 \times 10^5$
Ethylbenzene, p-Xylene *	C_8H_{10}	106.168	$1.53 \times 10^6 \pm 1.43 \times 10^5$	$3.06 \times 10^6 \pm 2.7 \times 10^5$	$1.81 \times 10^6 \pm 1.16 \times 10^5$
β-Ocimene, α-pinene, Limonene *	$C_{10}H_{16}$	136.238	$1.01 \times 10^6 \pm 8.1 \times 10^4$	$1.22 \times 10^7 \pm 1.46 \times 10^5$	$1.19 \times 10^6 \pm 0.97 \times 10^5$
Methyl Jasmonate *	$C_{13}H_{20}O_3$	224.300	$6.4 \times 10^5 \pm 4.2 \times 10^4$	$1.16 \times 10^6 \pm 5.17 \times 10^4$	$5.68 \times 10^5 \pm 4.02 \times 10^4$
B-caryophyllene *	$C_{15}H_{24}$	204.357	$1.75 \times 10^5 \pm 2.5 \times 10^4$	$0.95 \times 10^6 \pm 7.6 \times 10^4$	$1.16 \times 10^5 \pm 1.37 \times 10^4$

The compounds associated with attractiveness towards insect natural enemies, as benzaldehyde, ethylbenzene and p-xylene, then β-ocimene, α-pinene and limonene (grouped in monoterpenes), methyl-jasmonate and β-caryophyllene, were detected to be strongly increased (around 10 folds) after Sys application, while no differences were observed in control, mock- and Scp-treated plants (table 1.1). In order to determine the direct effect of Sys exogenous application on the production of MeJA, its absolute quantification was carried out (figure 1.8). Sys-treated plants released 2.57×10^8 ppbv of MeJA, significantly higher in comparison to control and Scp (around 1×10^8 ppbv).

1.2.4 SYSTEMIN APPLICATION ON LEAVES OF INTACT PLANTS INDUCED THE EXPRESSION OF DEFENSE GENES

The effects of exogenous application of Sys on the expression of defense-related genes was also monitored. Sys solution was spotted on the abaxial face of fully expanded healthy leaves or, as in previous experiment, added in the hydroponic medium. Transcripts of early signaling related genes (*Prosys* and *AOS*), and of late defense-related genes (*Pin I* and *Pin II*), were quantified for treated and control leaves. Gene expression was analyzed in a time-course assay (ninety min, three and six hours pi) by qRT-PCR, on plants exposed to two different concentrations of the experimental peptides. Relative quantification of treated samples was referred to the mock-treated control (relative quantification; RQ= 1). The results shown an enhanced transcription of the selected genes, in the treated leaves (figure 1.8) and in distal leaves (untreated leaves of treated plants) (figure1.9). In particular, Prosys transcripts significantly increased in the treated leaves (figure 1.9), with a maximal accumulation occurring within three h (F= 0.0124; P = 0.00276), while AOS transcripts doubled after ninety min and remained constantly transcribed at higher levels at all experimental time-points. A different transcript profile was registered for Pin I (F= 0.00813; P= 0.00312) and Pin II (F= 0.047; P= 0.00272), which showed a gradual increase, to reach a peak after six h. Pin II transcription, after six h, showed a dose-dependent effect of Sys. In the distal leaves (figure 1.9), no Prosys transcripts up-regulation was observed, while AOS transcripts greatly increased after six h. Pin I and Pin II transcripts showed a moderate up-regulation after three h and a high increase after six h. Similarly, for the early genes, that showed the same expression profile following the application of the two different Sys concentrations: a different expression level was registered for the late genes: 100 pM concentrations had the strongest induction effect on gene transcription. No significant variation in the transcript levels of the tested genes was registered in leaves treated with Scp (figures 1.8\1.9). Thus, the expression analysis confirmed that the enhancement of selected genes is nassociated with the leaf application of the Sys peptide. The same analysis was carried out in

the leaves of plants grown under hydroponics enriched with 100 pM Sys. All the transcripts were significantly up-regulated (*P*-value: Prosys, *P*= 0.0219; AOS, *P*= 0.02037; Pin I, *P*= 0.0001; Pin II, *P*= 0.0038) as shown in figure 1.10, while no significant transcript increase was observed following Scp application (figure 1.11). These results proved that hydroponic delivery of Sys is able to induce the expression of defense-related genes associated with the Sys signaling pathway.

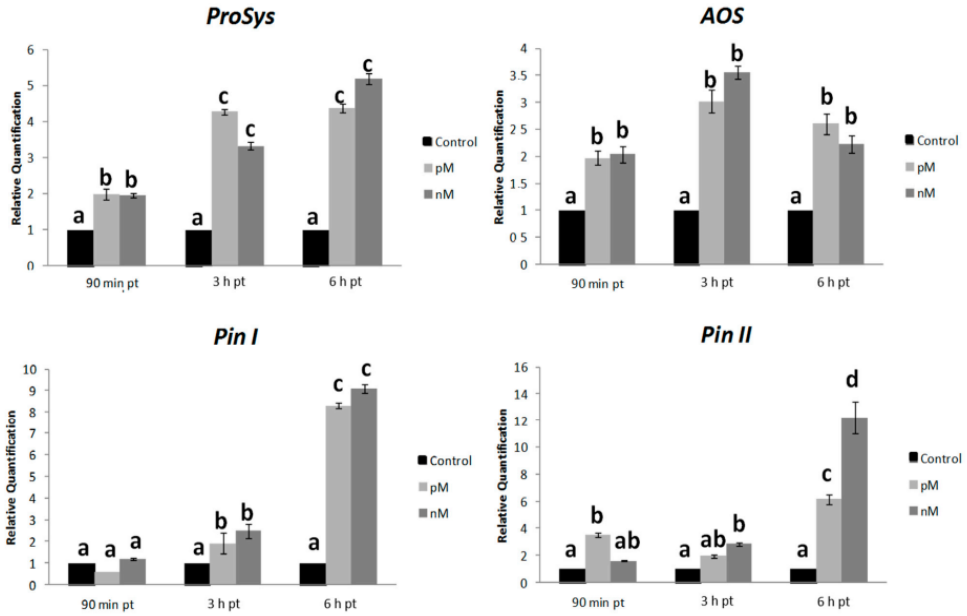


Figure 1.8. Gene expression analysis in leaf treated with Sys (local). Quantification of transcripts of early (*Prosys, AOS*) and late genes (*Pin I, Pin II*) by Real-Time Polymerase Chain Reaction (RT-PCR) after ninety min, three h and six h following 100 pM and 100 nM systemin peptide treatment. Relative quantities are calibrated on samples obtained from tomato leaves spotted with PBS1X (Control). For each gene, relative quantification (*RQ*) variations have been analyzed by two-way ANOVA. Different letters denote significantly different values (*P*< 0.01). Error bars indicate standard error.

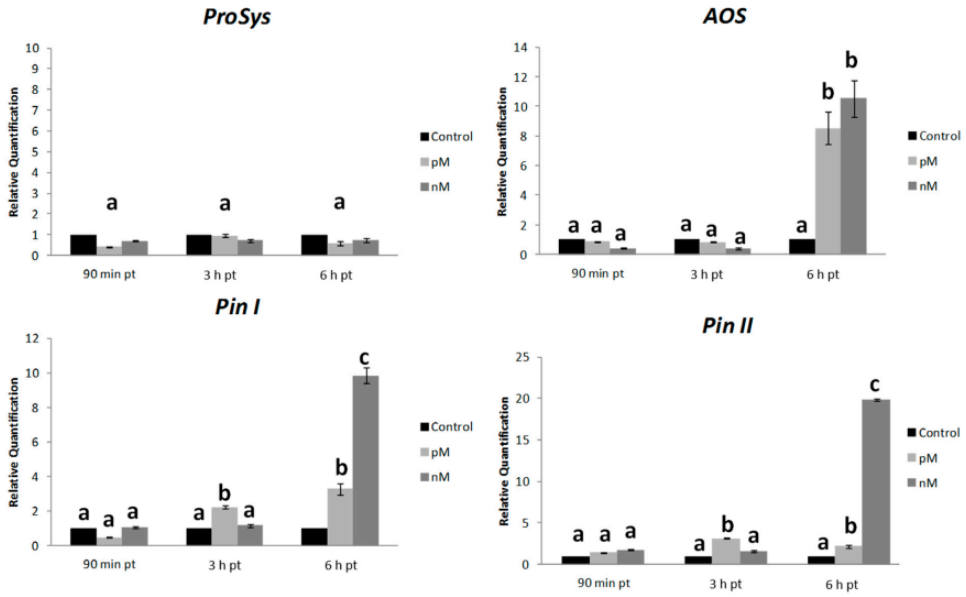


Figure 1.9. Systemic gene expression analysis in leaves upon Sys foliar treatment (pt). Quantification of transcripts of early (*Prosys, AOS*) and late genes (*Pin I, Pin II*) in leaves distal from the treated ones by real time RT-PCR after ninety min, three h and six h following 100 pM and 100 nM systemin peptide treatment. Relative quantities are calibrated on samples obtained from tomato leaves spotted with PBS1X (Control). For each gene, *RQ* variations have been analyzed by two-way ANOVA. Different letters denote significantly different values (*Prosys*: $P< 0.05$; *AOS*: $P< 0.01$ six h pt; *Pin I* and *Pin II*: $P< 0.05$ three h pt, $P< 0.01$ six h pt). Error bars indicate standard error.

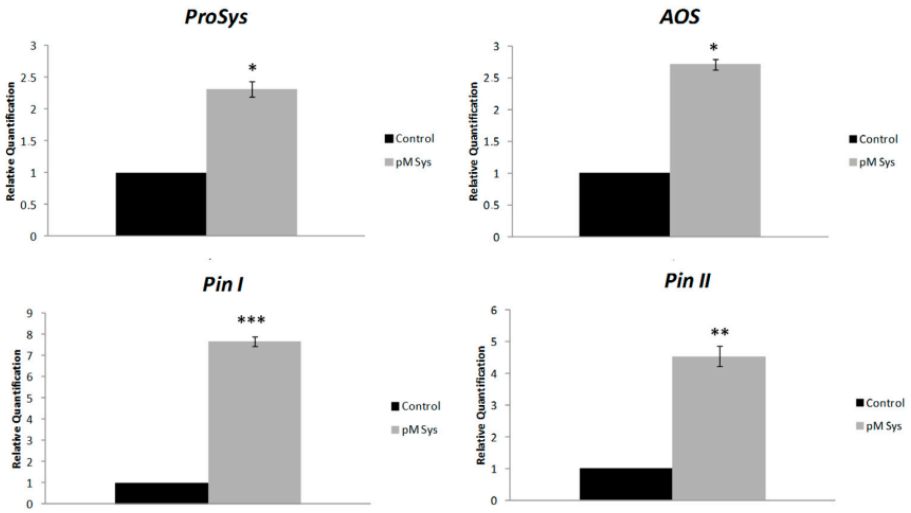


Figure 1.10. Gene expression in plants grown in hydroponic solution containing Sys. Quantification of transcripts of *Prosys, AOS, Pin I* and *Pin II* by Real Time RT-PCR detected in leaves of plants grown in a hydroponic system, three h after the addition of 100 pM systemin. Relative quantities are calibrated on samples obtained from tomato leaves of plant grown in a hydroponic system supplied with PBS1X. Asterisks denote statistically significant differences (*P< 0.05, **P< 0.001, ***P< 0.0001; T-test). Error bars indicate standard error.

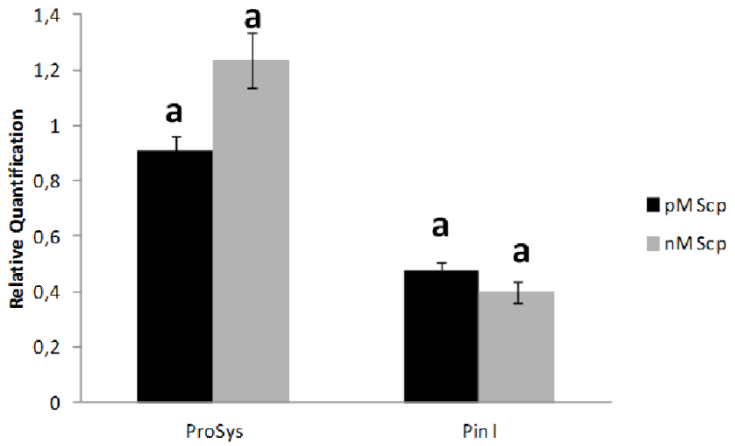


Figure 1.11. Relative quantification of defense transcripts upon Scp foliar treatment. Expression analysis of *Prosys* and *Pin I* by Real Time RT-PCR six h following Scp treatment. Relative quantities are calibrated on samples obtained from Red Setter leaves spotted with PBS1X. No significant

differences were registered (One-way ANOVA). Error bars indicate standard error.

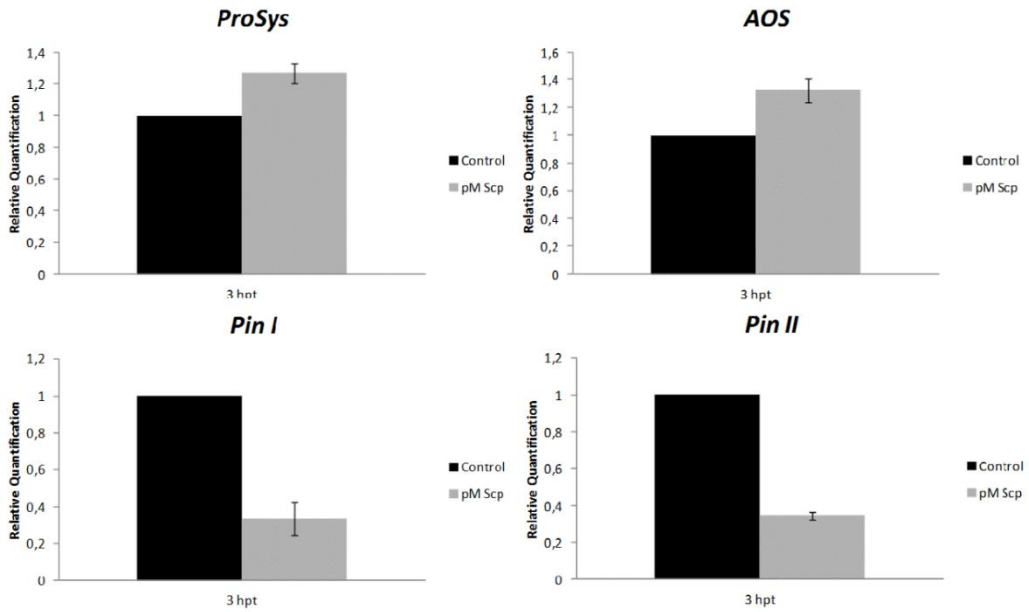


Figure 1.12. Effect of 100 pM Scp added in hydroponics. Relative quantities of defense transcripts by Real Time RT-PCR detected in leaves after 3 h of hydroponics. Relative quantities are calibrated on samples obtained from tomato leaves of plant grown in a hydroponic system supplied with PBS1X. No significant differences were registered (One-way ANOVA). Error bars indicate standard error.

1.3 DISCUSSION

Plant defense signal peptides have been identified in different species as soybean (Yamaguchi *et al*., 2011) and Arabidopsis (Yamaguchi *et al.*, 2010); while Sys homologues have been described only in solanaceous plants belonging to the subtribe Solaneae, like tomato, potato, black nightshade, and pepper (Constabel *et al*., 1998). In *Arabidopsis,* a peptide named Pep1 is released from the C-terminus of a longer precursor protein (ProPep) and is perceived as a DAMP by specific receptors with the consequent amplification of the plant innate immune responses against pathogens. The constitutive expression of the precursor confers resistance to Arabidopsis plants against the

oomycete plant pathogen *Pythium irregulare* (*P. irregulare*) and resistance to the necrotrophic fungus Botrytis cinerea (Huffaker *et al*., 2006; Zhang *et al*., 2017). Conversely, *Z. mays Pep3* regulates direct and indirect anti-herbivore defenses, likely by modulating the downstream signaling response to insect oral secretions (Huffaker *et al.*, 2013). ProPep orthologous were identified in numerous species (Huffaker *et al*., 2006) and interestingly, a functional orthologous was also found in tomato, where it is involved in defense against a root pathogen (Trivilin *et al.*, 2014). In this chapter different approaches were used to evaluate the effects of Sys supply on tomato plants, a DAMP peptide produced in this plant species following leaves damage. This information is fundamental to exploit the full potential of this peptide, in the Integrated Pest Management (IPM) strategies for agricultural practice. The IPM strategies are focused on prevention of pest or their damage through a combination of different methods as biological control, modification of cultural practices, use of resistant varieties and consequently, reduce the use of pesticides (Flint, 2012). Systemin is one of the best studied peptides and represent a good candidate for pest control due to its ability in triggering plant endogenous defenses active against different agents of biotic stress (Coppola *et al*., 2015; Diaz *et al*., 2002; El Oirdi *et al*., 2011). Our results demonstrated that the exogenous Sys supply using different delivery system conferred measurable protection against *S. littoralis* and *B. cinerea,* making this peptide potentially suitable for pest control. As controls in *S. littoralis* bioassay fresh leaves from healthy untreated or Scp-treated plants were used. The mechanical demage caused by the harvesting was equal in controls and treated plants, but despite this Sys-treatment determined higher mortality, reduction of eggs number and higher time to pupate. The observed protection is likely the consequence of Sys activation of the expression of defense-related genes, both early and late involved in plant defense responses. Then, *AOS*, the first enzyme in the JA biosynthetic pathway contributes to JA production triggering the systemic activation of defense in treated plants (Chauvin *et al*., 2013). This was confirmed by the increased production of Methyl-Jasmonate (MeJA) detected in treated plants. JA is a powerful inducer of *Pin I* and *Pin II* (Pearce *et al.*, 1991) similarly to what was observed in our

experimental plants. An increased accumulation of Pin I and Pin II transcripts, likely produced an increased level of protease inhibitor, compounds that are known to inhibit insect digestion with a consequent reduction of nutrients assimilation that causes the larvae reduced weights and vitality (Chen *et al*., 2005). *Pin I* and *II* play a key role even against *B. Cinerea*, in fact the expression of these genes, known as JA-dependent genes, determined the immune response against necrotrophic fungi (El Oirdi *et al*., 2011). The resistance to *B. cinerea* and *S. littoralis* is likely affected by the expression of pepr1/2 ortholog receptor-like kinase1 (PORK1) as this protein showed biological functions in Sys signalling and tomato immune responses, against necrotrophic fungi and herbivory insect (AbuQamar *et al.*, 2008; Xu *et al.*, 2018).

Currently, it´s known that the plant cell wall is semi-permeable and perhaps Sys is able to pass through it decreasing the plasma membrane–cell wall adhesion, a mechanism which appears to be used by pathogen to penetrate plant cell (Mellersh and Heath, 2001). Once passed the cell wall Sys interacts with its receptor with the subsequent activation of the signalling cascade. Previous work showed that soil drenched with a solution containing Sys, at nM concentration, induced defense genes and metabolites in tomato (de la Noval *et al*., 2007; Pastor *et al*., 2018), while in Arabidopsis, 4-week-old plants grown in soil sprayed with Pep1 (nM), a 23–amino acid peptide that enhances resistance to a root pathogen and its an endogenous amplifier of innate immunity, showed an increased expression of a gene encoding a defensin (Yamaguchi *et al.*, 2010). In addition, *A. thaliana* treatment with the bacterial peptide flagellin induced the expression of several defense-related genes and triggers resistance to pathogenic bacteria (Zipfel *et al.*, 2004). Sys is also perceived by roots, in fact, previous work observed root elongation in response to Sys in *Solanum pimpinellifolium* (Holton *et al*.,2007). Our results nicely complement these observations: both foliar spotting and hydroponic supply of Sys, determined an increase of direct and indirect defense response. In addition, the treatment had a strong impact on the fitness of the insect population by reducing fecundity of the F1 generation obtained by larvae fed on treated leaves. Sys-

treated plants modified the blend of VOCs emitted; in particular a strong increase of benzaldehyde, ethylbenzene, monoterpenes as β-ocimene, α-pinene and limonene, methyl-jasmonate and β-caryophyllene was observed. These compounds are known to be signals for pest natural enemy, that used VOC to catch their prey (Corrado *et al.*, 2007; Webster *et al.*, 2008). β-caryophyllene is one of these compounds and it is identified at antennal level by *A. ervi* in a concentration as low as 0.01 mg/ml and determining a significant higher attractiveness towards this parasitoid compared to control solvent tested as purified compounds in wind tunnel bioassay (Sasso *et al*., 2009). In our experiment it was demonstrated that Sys treatment of healthy tomato plants increased the attraction of *A. ervi*, a natural antagonist of the aphid *Macrosiphum euphorbiae*, thus inducing an increasing of the indirect defense barriers. The results of behavioural bioassay with *A. ervi* are consistent with the volatile blend released by Sys-treated plants. To exploit at the best the potential of the peptide, it was necessary to investigate different Sys concentrations in order to define the minimum peptide levels able to confer effective protection in tomato and other *Solanaceae* crops. The experimental concentration was then selected (100 pM) and used for all the experiments. On preliminary base, the results indicated that a much lower concentration is also effective (Rao, unpublished). This is very important as the use of the peptide in agricultural practices may be costly and therefore smallest concentrations should be preferred. However, the cost of the treatment is presently an issue. The recombinant production of the peptide may help to solve this problem. Another important aspect is the evaluation of the cost of the treatment on plant physiology, and this issue is presently being investigated in the lab.

The use of peptides, such as Sys, in agricultural practice is an interesting safe and sustainable crop protection strategy that could be included in IPM. In addition, the null effect of Scp peptide confirmed that the correct Sys sequence is fundamental for the defense activation. Application of plant endogenous peptides, not directly toxic for the insect, such as Sys (Rao, unpublished) but able to activate plant defense responses affecting the fitness and behaviour of

herbivores and pathogens, represents a very safe approach of plant protection, in particular for the expected low or null toxicity of these molecules on non-target organisms. The prospects, based on these results, are very encouraging, pushing us to deepen the topic; but with new questions to be answered regarding applicability and degradation of the peptide in open field.

1.4 CONCLUSIONS

The achieved results showed the potential of used approaches. The developed bioassays to verify the response of tomato plants treated with Sys by foliar spotting or hydroponic solution, against various invaders, allowed to analyse its effects on direct and indirect defense responses. The results proved that the peptide defense system, evolved by plants, could be a powerful tool for sustainable agriculture in IPM strategies, which could have, consequently, the reduction of the use of pesticides. The results obtained in this work, with foliar and hydroponic supplies that directly counteract *S. littoralis* larvae and the necrotrophic fungus *B. cinerea*, proved for the first time that the peptide is an excellent candidate for tomato protection. The efficacy of different delivery strategies is very promising from an applied perspective, representing a significant addition towards the use of DAMPs in open field. However, there are still fundamental aspects, in peptide utilization to be evaluated such as the analysis of eventual environmental degradation with consequent loss of biological activity, best application mode, selection of the minimal useful concentration and, no less important the costs. An interesting example of the use of proteins in agricultural is Messenger®, a commercial formulation containing a natural non-toxic protein that enhances disease and pest resistance in treated plants, triggering natural defense systems and increase yield and quality (Wei and Betz, 2007). Recently, in 2019 Vestaron® Company commercialized a new peptide-based bio-insecticide, Spear®-Lep, a biological insecticide that targets lepidopteran pests, that is effective for vegetables, fruits, and high-value field crops. Regarding the peptides production costs, the use of yeast as bioreactors (Vandermies and Fickers, 2019) for the synthesis of recombinant Sys can greatly reduce the prices and increase the

feasibility of the proposed approaches. In addition, despite the continuous exposition of pests to Sys within the naturally occurring tomato-pest interaction, no pest resistance to the peptide was observed, thus suggesting a good durability of the proposed approaches. Regarding the application mode, hydroponics is largely used for tomato plants and other *Solanaceae,* and the use of Sys solution for watering plants, could be an interesting option for protection against pests and pathogens, similarly to foliar application, that can be developed as open field strategy. The results obtained in this work represent, to our knowledge, the first demonstration that the treatment of healthy unwounded tomato leaves with Sys confers resistance against pests. From an applied perspective it represents a very promising strategy that could promote the reduction of chemical pesticide for pest control.

1.5 MATERIALS AND METHODS

1.5.1 PEPTIDES PREPARATION

To carry out these experiments, two different peptides were produced: Sys and Sys-scramble (Scp), used as control. Sys was obtained by solid phase synthesis following standard protocols (Avitabile *et al*., 2013) using the Rink Amide MBHA resin, (loading 0.65 mmol/g). The purification of the peptides was carried out by Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) (Shimadzu LC-8A, equipped with an SPD-M10 AV) on a semipreparative column (Jupiter 10_Proteo 90A, 250 × 10.0 mm, Phenomenex, Torrance, CA, USA) using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 5 to 50% in 30 min at 5 ml/min. Peptides were characterized by mass spectrometry (LC-MS ESI-TOF 6230 Agilent Technologies, Milan, Italy) obtaining crucial information. Sys sequence: AVQSKPPSKRDPPKMQTD; Mass calculated (Da): 2009.3. Mass spectrum fragmentation data (Da): 670.94 $[M + 3H]^{3+}$; 1005.60 $[M + 2H]^{2+}$. Sys scramble sequence: KSKMDRQPVQAPDKPSPT. Mass calculated: 2009.3. Mass found:670.96 $[M + 3H]^{3+}$; 1005.53 $[M + 2H]^{2+}$. Peptide stability was tested as described in Coppola *et al*., 2017. The analysis of the HPLC (Shimadzu LC-8A, equipped with an

SPD-M10 AV) profiles and of the mass spectra collected indicates that the peptide isstable in all the tested conditions. Stock solutions of the synthesized peptides were prepared as described in Czyzewicz *et al*., 2017. Peptide synthesis, purification and stability process are described in detail in Coppola *et al*., 2017.

1.5.2 PLANT MATERIALS

In all these experiments carried out the tomato (*Solanum lycopersicum*) cultivar “Red Setter” was used. Seeds were germinated on sterile paper disks moistened with water and kept in the dark for three days in a climate chamber at 24 ± 1 °C. At the break of cotyledons, tomato seeds were exposed to a 16:8 h light:dark photoperiod, for 48 h. After germination, the seeds were transferred to sterile soil in a climate chamber, at 26 ± 1 °C, under a 16:8 h light:dark photoperiod. Four weeks-old plants were used for biological and molecular investigations, unless otherwise indicated. 2 µL of 100 pM and 100 nM Sys or Scp were applied on intact leaves by spotting the abaxial surface. Both peptides were dissolved in phosphate buffer solution (PBS). Control plants were treated with the buffer using the same procedure. The expression analysis and bioassays with pests were carried out on treated leaves. For hydroponics, tomato seeds, 5 days after sowing at two-cotyledon stage, were transferred into a hydroponic system, and grown for 4 weeks in a 5 L solution, containing: $Mg(NO_3)_2$, $6H_2O$ (384.0 mg/l), $Ca(NO_3)_2$, $4H_2O$ (812.9mg/l), KNO_3 (101.5mg/l), K_2SO_4 (319.3mg/l), KH_2PO_4 (204.8mg/l), Hydromix (14.0 mg/l), and the experimental peptides to a final concentration of 100 pM.

1.5.3 BIOASSAY WITH *SPODOPTERA LITTORALIS*

Feeding bioassays with the phytophagous insect *S. littoralis* larvae were carried out as described in Coppola *et al*., 2015. The larvae were obtained from a laboratory population maintained at Isagro Ricerca (Novara, Italy) and reared in a climate chamber at 25 ± 2°C; 70 ± 5% relative humidity (RH); 16:8 h light:dark photoperiod. The larvae have been fed an artificial diet composed as follows: 41.4 g/l wheat germ,

59.2 g/l brewer's yeast and 165 g/l maize flour, supplemented with 5.9 g/l ascorbic acid, 1.8 g/l methyl 4-hydroxybenzoate and 29.6 g/l agar. Larvae grown with this artificial diet up to the 2nd instar. Uniform second instar larvae were divided into groups of 32 individuals, and each group was monitored to assess larval weight and survival rate as a result of treatment with 100 pM Sys compared to mock-treated controls (phosphate buffer; PBS) or supplied with 100 pM Scp. The experimental larvae were fed with leaf discs obtained from similar leaves, in terms of size and position on the plant. The leaves were collected daily from five control or treated plants (biological replicas). The tomato leaf discs were stored on 2% agar (w/v) to create a humid environment necessary to keep them turgid in a tray well (Bio-Ba-32, Color-Dec, Lucca, Italy) covered with perforated plastic lids (Bio-Cv-4, Color-Dec, Lucca, Italy). The larvae have been individually separated in each box and fed with the corresponding leaf disc (control or treated). The leaf discs were replaced daily, increasing the size (initially 2 cm^2, then 3, 4 and 5 cm^2), to satisfy the food requirements of the growing larvae. The plastic trays were incubated under controlled conditions (28 ± 1°C; 70 ± 5% RH; 16:8 h light:dark photoperiod). During this period, the larval weight and mortality were recorded until pupation, which took place in plastic boxes containing vermiculite (25 x 10 x 15 cm). For the experiment in hydroponics condition where was supplied Sys, were used 3rd instar larvae, for which larval weight and longevity were recorded. In addition, the following reproduction parameters were registered: pupa development time (from the beginning of the bioassay to pupation), longevity and fecundity of the adult. The pupae were collected, washed in a 50% solution of bleach (0.05% sodium hypochlorite), rinsed with distilled water and air-dried, then they were sexed under a stereo microscope (40X) observing the morphological characters, as described (Sannino *et al*., 2001), separated into aerated plastic boxes (25 x 10 x 15 cm) and analyzed daily until the adult's emergence. After they emerged, the adults received a 50% aqueous solution of honey to allow mating; in fact, males and females were kept together (1 female:2 males) for 24 hours at 25 °C. Subsequently, the mating females were separated from the males, suitably marked, and transferred individually into a plastic cylinder (diameter 8 cm, height 9 cm), covered with paper,

where their egg laying activity was evaluated daily, throughout their life, counting the number of eggs laid on paper, under a stereomicroscope operating at 40X magnification. The longevity of adults was also recorded. To obtain a significance of the data obtained, each experiment was repeated twice.

1.5.4 BIOASSAY WITH *BOTRYTIS CINEREA*

In this bioassay four week-old tomato plants, treated with 100 pM Sys directly delivered on the leaf surface or dissolved in the hydroponic solution (final Sys concentration was 100 pM) has been used and tested the resistance to *B. cinerea*.
Spores of the fungus were obtained with a suspension in sterile distilled water, filtered through sterile Kim wipes (Kimberly-Clark) to remove fragments of hyphae, and adjustment to a concentration of $1 \cdot 10^6$ conidia per ml. Six hours after Sys application, an aliquot of 10 µL of the fungus spore suspension was applied by spotting on the leaves. The assay was carried out using four plants per treatment, which were incubated in a growth chamber at 23 ± 1°C, for a 16 h photoperiod and under 90% RH. The size of the lesions was measured at different days post inoculums (pi), using a digital caliber (Neiko 01407A).

1.5.5 *APHIDIUS ERVI* FLIGHT BEHAVIOR

Bioassays with parasitic wasps *A. ervi* were conducted in a wind tunnel with a dimension of 100 x 50 x 50 cm, as described in detail by Guerrieri *et al*., in 1993. Plants were tested 24 h after the treatment with the experimental peptides Sys and Scp (100 nM), and control buffer applied directly on leaves oradded in the hydroponic growth solution. *A. ervi* native females, 1–2 days old, mated and fed, were released singularly in the wind tunnel, 50 cm down wind from the target plant and observed up to 5 min to determine their flight orientations and landings on the plant. Insect behavior was recorded as “Oriented flight” when the females flew within 5 cm of plant or landed on it. Similarly, it was recordedas “Landing on target” when females landed on plant. Bioassays were conducted by observing at

least 100 females on six different plants for each treatment on six different days. Plants were presentedin random order each day to avoid any daily bias. The experimental conditions were a temperature of 20 ± 1°C; 65 ± 5% RH; wind speed, 25 ± 5 cm/s; Photosynthetic Photon Flux Density (PPFD) at releasing point, 700 µmol m^2/s.

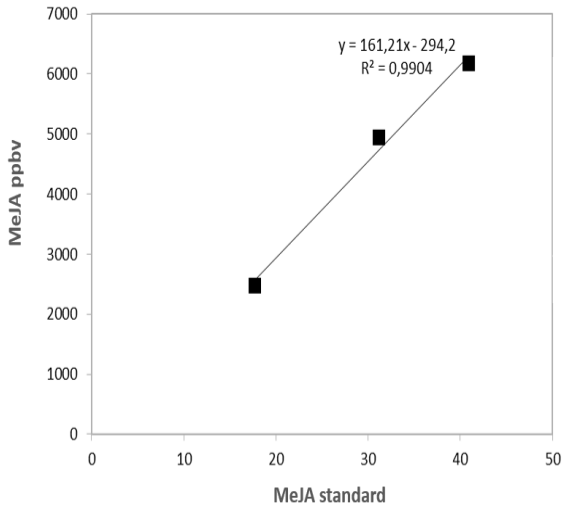
1.5.6 VOLATILE ORGANIC COMPOUNDS (VOCS) COLLECTION AND ANALYSIS

VOCs collection and analyses were performed under controlled temperature, at 25 ±1°C. Leaf treated plants and control (100 pM Sys or 100 pM Scp or buffer) has been used for headspace volatile collection to carry out VOCs analysis. VOCs released by five plants closed into glass box (60 x 60 x60 cm), were sampling into headspace after 1h. The collected headspaces were directly injected into the Proton Transfer Reaction ionization with a Time-of-Flight Mass Spectrometry (PTR-TOF-MS) drifttube hated (110 °C) peek inlet tube with a flow rate of 100 sccm for calculation. This sophisticated machine allows to detect VOCs in real-time through proton transfer reactions, using Proton Transfer Reaction-Quadrupole interface Time of Flight- Mass Spectrometry (PTR-Qi-TOF-MS) apparatus supplied by Ionicon Analytik GmbH (Innsbruck, Austria). The drift tube was under specific controlled conditions: pressure (3.8 mbar), temperature (80 °C) and voltage (1000 V), resulting in a field density ratio (E/N) of 141 Td (E being the electric field strength and N the gas number density; 1Td = 10-17 V cm^{-2}) (Coppola *et al*., 2019).The raw data recorded by the PTR apparatus were acquired by the TofDaq software (TofwerkAG, Thun, Switzerland), normalized per plant and subsequently evaluated with the PTR-MS Viewer 3.2.6 (Ionicon analytic GmbH, Innsbruck, Austria).

1.5.7 CALIBRATION OF METHYL-JASMONATE STANDARD

The machine calibration was necessary to verify the accuracy of the data obtained. The absolute quantification of methyl jasmonate (m/z 152.15) was performed using the IONICON Liquid Calibration Unit (LCU) coupled with PTR-Qi-TOF-MS. LCU evaporates aqueous

standards into a gas stream, resulting in a gas flow containing compounds at exactly know trace concentrations.
The MeJA calibration curve was produced obtaining a gradient flow by nebulizing both the liquid standard (MeJA at concentration of 10^{-6}) and the distilled water and starting from 100% water to 100% MeJA. Nitrogen was utilized as a carrier gas at 1000 sccm (nitrogen with a purity of 5.0i.e., 99.999% purchased from Linde-Vienna-Austria) with a constant flow. The combination of liquid from the two inlets was sprayed and evaporated inside the heated spray chamber at the temperature of 100°C and was introduced in the inlet of PTR-Qi-TOF-MS. Finally, data were filtered to remove all peaks as cribbed to water chemistry (m/z 21.022 and m/z 39.033 corresponding to $H_3{}^{18}O^+$ and H_2O and $H_3{}^{18}O^+$, respectively) or other interfering ions (e.g., oxygen, nitrogen monoxide).



MeJA released (ppbv)		
Control	Sys	Scp
$1{,}03*10^8$	$2{,}57*10^8$	$0{,}91*10^8$

Figure 1.13. Absolute quantification of methyl-jasmonate (MeJA) released by systemin-treated plants. Standard curve and calculation of released amount of MeJA in tomato plants treated with Sys, Scp or mock on intact leaves.

1.5.8 GENE EXPRESSION ANALYSIS

To carry out the gene expression analysis three full-expanded leaves per plant were treated and were used three plants for each treatment (Sys or Scp or buffer) as biological replicates. Treated leaves and un-treated leaves of treated plants (named as distal leaves) were harvested at different time points, immediately frozen in liquid nitrogen, and stored at -80°C until use. The hydroponics experiments were performed growing plants in three different tanks with nutritive solution without peptide (control plants) or with 100 pM *Sys* or 100 pM *Scp* treated plants). Three leaves per plant and three plants per each experimental condition were harvested 3 h after treatment and stored as described above. The isolation of total RNA from leaves, the synthesis of the first strand cDNA and RT-PCRs were performed according to standard procedures, as already described elsewhere (Corrado *et al*., 2012). For the gene expression analysis were used two technical replicates for each ofthe three biological replicates, for each sample. Relative quantification of gene expression was carried out using the 2^{-DDCt} method (Livak and Schmittgen, 2001). The housekeeping gene selected was the Elongation Factor 1α (EF-1α) as endogenous reference gene, for the normalization of the expression level of the target genes (Marum *et al*., 2012) (Muller *et al*., 2015). Primers used and their main features are reported in Table 1.2.

Table 1.2. List of primers and amplification condition. LA: length amplicon. NR: number of cycles. Tm: melting temperature calculated on according to the rule of Wallace: 4°C for G and C, 2°C for the A and T (Wallace *et al*., 1999).

Primer	Sequence (5´-3´)	Tm	Gene name	Accession number	LA	NR
Pin I Fw Pin I Rv	GAAACTCTCATGGCACGAAAAG CACCAATAAGTTCTGGCCACAT	64 64	Pin I	K03290	114	40
Pin II Fw Pin II Rv	CCAAAAAGGCCAAATGCTTG TGTGCAACACGTGGTACATCC	58 64	Pin II	K03291	116	40
AOS Fw AOS RV	GATCGGTTCGTCGGAGAAGAA GCGCACTGTTTATTCCCCACT	68 66	AOS	AF230371	101	40
EF Fw EF Rv	CTCCATTGGGTCGTTTTGCT GGTCACCTTGGCACCAGTTG	62 64	EF-1α	X53043	101	40
Prosys Fw Prosys Rv	GGGAGGGTGCACTAGAAATA TTGCATTTTGGGAGGATCAC	58 58	Prosystemin	M84801.1	110	40

1.5.9 STATISTICAL ANALYSIS

Statistical analysis was performed using different methods. Differences in relative quantities of defense transcripts were analyzed by comparing DCt values by one-way or two-way ANalysis Of VAriance (ANOVA), while for coupled comparisons a two-tailed Student'st-test was used. For the insect assay, larval weights were compared by one-way ANOVA or Kruskal-Wallis non-parametric ANOVA, followed by Tukey-Kramer honestly significant difference (HSD) and Dunn's post test for multiple mean value comparisons. Survival curves of *S. littoralis* larvae and adults were compared by using Kaplan-Meier and log-rank analysis. The time required by larvae to pupate was compared by Kruskal-Wallis non-parametric ANOVA followed by Dunn's post test for multiple mean value comparisons, while the number of laid eggs was compared by one-way ANOVA, coupled with Tukey-Kramer multiple comparisons test. For the evaluation of Sys effect on *B. cinerea* infection, necrosis area

differences between control and 100 pM Sys-treated sample were analyzed by Student's t-test. Size differences of the necrotic areas, induced by fungal inoculum on plants treated with Sys or Scp via root uptake, were analyzed by one-way ANOVA coupled with Tukey-Kramer honestly significant difference (HSD) test.
The number of parasitoids responding, as oriented and non-oriented flight, to each target plant was compared by a G-test for independence, as described in (Sokal and Rohlf, 1995). Differences in VOCs released by treated and control plants were compared using Kruskal-Wallis non-parametric ANOVA.

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CHAPTER 2

IDENTIFICATION OF PROSYSTEMIN SUB NETWORK BY *IN SILICO* APPROACHES

IN COLLABORATION WITH M. COPPOLA AND R. RAO

ABSTRACT

Understanding the molecular mechanisms underlying plant defense responses is essential in order to define an integrated strategy of pest management that includes the use of chemicals, agricultural practices and biological control, which are the basis of Integrated Pest Management (IPM) strategy. The objective of the work illustrated in this chapter was to shed light on the molecular mechanisms through which tomato plants, over-expressing *Prosys,* are able to modulate different defense pathways, that leads to the resistance to biotic and abiotic stresses (Coppola *et al*., 2015; Orsini *et al*., 2010). Protein-protein interaction (PPI) studies presently benefit of the interactomic approach and of protein-protein interaction network at genomic level; in addition, offers new opportunities to link wet lab approaches and *in silico* approaches using bioinformatic tools that may predict PPIs. Here are presented the results of an *in silico* study aimed to identify Prosys interactors. More than 16000 interactions were captured from the interactome query and, among them, 98 Prosys direct interactors, catalogued on the base of Gene Ontology (GO) vocabulary, were underlined. The obtained interactome clearly evidenced that Prosys is linked with the signaling pathways of the three major phytohormones involved in plant defense: jasmonic acid, salicylic acid and ethylene. Among the direct interactors we detected other two large groups, one related to kinases and another one related to several families of transcription factors. These findings nicely confirming that Prosys is active since the very beginning of stress perception.

2.1 INTRODUCTION

Protein-protein interactions (PPIs) handle a wide range of biological processes, including metabolic and developmental control and cell-to-cell interactions (Srinivasa *et al*., 2014). PPIs have always been

considered relevant to shed light on a variety of biological processes including signal transduction, stress responses and plant defense (Uhrig, 2006). At the molecular level PPIs play key roles in post-transcriptional modifications, protein phosphorylation, transcriptional co-factor recruitment, in addition, are considered important to understand gene function (Zhang *et al.*, 2010). Therefore, PPIs are central actors of many physiological and pathological processes, fundamental in all organisms (Barabasi *et al.*, 2004; Von Mering *et al.*, 2002). The study of PPIs includes several methods and improved over the years in different model species (Yuan *et al.*, 2005); these methods can be divided in: *in silico*, *in vitro* and *in vivo*. *In silico* methods use a large number of data that may be recruited from high throughput techniques, sequence alignments and experimental methods. A variety of *in silico* methods have been developed to predict new interactions or to corroborate interactions detected by experimental approaches (Srinivasa *et al*., 2014). The computational methods for *in silico* prediction include sequence-based approaches, structure-based approaches, gene ontology, and gene expression-based approaches (Srinivasa *et al*., 2014). The prediction process starts with the comparison of a sequence, a gene or a protein, with those annotated in other species. Generally, a protein may share significant similarities with proteins of other organisms involved in known functions and it is assumed that the protein has either the same or similar function. The study of protein interactions has undergone a great impulse, taking advantage of the data available from 'omics' approaches, and bioinformatics became indispensable to study the biological functions of PP network (Widlak, 2013). The study of *in silico* PPIs in tomato plants exploits the high number of databases dedicated to the crop as well as databases dedicated to other plants species, in particular Arabidopsis. Several online bioinformatics platforms allow redesigning part of tomato proteome as the Predicted Tomato Interactome Resource (PTIR) and Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), two interactome resources consulted to predict protein-protein interactions in this study. PTIR is based on experimentally determined orthologous interactions in six model organisms. This database covers 357.946 non-redundant PPIs among 10.626 proteins (update in 2015); 12.291

with high-confidence, 226.553 with medium-confidence and 119.102 with low-confidence interactions (Yue *et al*., 2016). These interactions are expected to cover 30.6% of the entire tomato proteome. STRING is a database of known and predicted protein-protein interactions. The interactions include direct (physical) and indirect (functional) associations (Szklarczyk *et al*., 2019). STRING interactions derive from different main sources: genomic context predictions, high-throughput laboratory experiments, interactions aggregated from other (primary) databases. The STRING database currently covers 24.584.628 proteins from 5.090 organisms (Szklarczyk *et al*., 2019). PPIs can be also evaluated by shared GO terms, co-evolution, co-expression, co-localization and domain interactions. Further software allows to analyze a vast amount of data and visualize in an easy manner the biological network, through graphs, nodes and edges. Nodes represent the biological molecules and edges, that connect the nodes, the relationship between them. One of these tools is Cytoscape. Cytoscape is an open-source software for the visualization of interaction networks, applicable to any system of molecular components and interactions (Shannon *et al*., 2003). Cytoscape is generally used in combination with large databases of protein-protein, protein-DNA, and genetic interactions that are increasingly available for humans and model organisms. In addition, Cytoscape's software provides features to layout and query the network, integrating the network with expression profiles, phenotypes, and to link the network to databases of functional annotations (Kohl *et al*., 2011). *In vitro* and *in vivo* techniques are based principally on laboratory experiments: the first ones are based on recombinant and cloning technology; while the second ones are performed in living models to visualize the interaction. These approaches are chosen for specific characteristics, among which the most important are the sensitivity and specificity of the method. A technique with high sensitivity allows to detect many of the occurring interactions. A high specificity indicates that the approach allows to identify most of the interactions that occur into the cell. The laboratory techniques, defined *in vitro*, are based on the physical contact between proteins or between proteins and specific antibodies, before the detection through the Western Blotting, mass spectrometry or fluorescent; for example, Co-immunoprecipitation

(Co-Ip), Yeast Two-Hybrid system (Y2H) and one of the most recently used Affinity Purification Mass Spectrometry (AP-MS). The AP-MS is a large-scale detection approach, and the detected interactions are considered real but need a validation with different approaches, in particular *in vivo*. *In vivo* methods have been and still are used to visualize the interaction in living models, to confirm a specific interaction that occur within a biological system. One of the most used techniques is Bimolecular Fluorescent Complementation (BiFC), becoming fundamental in this field to visualize PPIs in a variety of model organisms for its specificity and immediacy. The BiFC assay is based on the reconstitution of an intact fluorescent protein when two proteins are brought together due to their interactions (Kodama and Hu, 2012). These techniques will be discussed jointly to *in vitro* and *in vivo* methods, in the second chapter.

In this PhD project, the mentioned approaches have been used in order to shed more light on molecular mechanism underpinning the Prosys dependent tomato defense responses, in particular in this chapter the *in silico* one.
For long time Sys was considered as the only part of the precursor harbouring biological activity. However, recent evidence demonstrated that Prosys devoid of the Sys sequence contributes to defense responses (Corrado *et al*., 2016). This observation prompted the investigation of the biochemical and structural characteristics of Prosys which revealed that the protein is intrinsically disordered (IDP) (Buonanno *et al*., 2018). IDPs do not have a well-defined structure under physiological conditions, although they have key roles in cell signaling and regulation of transcription, and translation. Protein unfolded regions are involved in PP or other biomolecular interactions, interplaying with different partners in many-to-one and one-to-many binding equilibria (i.e., acting as “hubs”) (Uversky *et al*., 2008). This Prosys structural characteristic suggested novel ideas for a better understanding of the multiple resistances observed in transgenic plants (as described in chapter 1). Therefore, we focused the work on the identification of Prosys protein partners, using both *in silico* and *in vivo* approaches. In this chapter, results from bioinformatics and database resources to obtain a Prosys sub network are described.

2.2 RESULTS

2.2.1 PROTEIN-PROTEIN INTERACTIONS OF TOMATO DEFENCE RELATED PROTEINS

Starting from the data obtained in a previous work (Coppola *et al*., 2015) in which 695 expressed sequence tags (ESTs) were identified by microarray analysis of transgenic tomato plants constitutively expressing Prosys (RSYS), interactions among defence-related proteins and specifically Prosys-related proteins were investigated. According to the available reference tomato genome, the 695 ESTs correspond to 503 differentially expressed genes (DEGs). These DEGs were used to query two databases of plant PPIs: Predicted Tomato Interactome Resource (PTIR; http://bdg.hfut.edu.cn/ptir/index.html) and Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; https://string-db.org/), trying to import PPIs from tomato and Arabidopsis. To this purpose, DEGs were firstly converted in their Arabidopsis correspondent proteins to query the Arabidopsis interactome that is very abundant since a big effort has been carried out on the model species. From the 503 tomato DEGs, a list of 309 Arabidopsis proteins was obtained. Similarly, DEGs were used to query tomato interactome (PTIR) that was smaller and less informative than the Arabidopsis one. All the interactions captured during this database query phase were integrated. The predicted interactions are direct interactions among proteins. Both databases consulted, classified as prediction databases, used the incorporation of data present in the scientific literature and obtained based on orthologies with other organisms. The orthologous interactors were mapped to reference species interactomes and the interacting proteins were noted. The prediction of each interaction contained in the databases considered was assessed based on sharing Gene Ontology terms (GO), co-expression, co-localization and the availability of interacting protein domains. In addition, as reported in Szklarczyk *et al*., 2016 and Yue *et al*., 2016, a level of reliability was assessed for each interaction by evaluating the evidence supporting direct contact. Dissimilar to PTIR, STRING is a huge interaction database, in fact it currently covers

2031 organisms, a much larger number than other databases freely available online. The procedure performed for the analysis of the RSYS tomato PPIs and RSYS network (figure 2.1) are reported in attachment materials.

2.2.2 PROSYSTEMIN SUB-NETWORK

Due to the high complexity of the network showed a Prosys sub-network was extrapolated. Figure 2.1 shows the flowchart used to generate the Prosys sub-network which consists of 99 nodes and 98 interactions (figure 2.2) obtained extrapolating from RSYS network all Prosys direct interactors. Eleven nodes represent proteins encoded by DEGs (triangle and square shape in figure 2.2) while the other putative interactors, indicated with circular shape, come from the queried interactomes. The size of the nodes, compared to the previous network, is uniform because only the interactions in which Prosys is involved are showed. For this reason, all nodes have a degree equal to 1, except for Prosys for which they are 98. Prosys′ 98 interactors (Table A in attachment) were classified based on their role in the regulation and participation in defensive mechanisms by means of published research. Therefore, clusters with a specific role in defense were highlighted. All the identified interactions come from the STRING database, which associates a specific score, or confidence level, between 0 and 1 with each predicted interaction. This value corresponds to the probability that the interaction is correctly identified and is assigned based on the evidence supporting each interaction (the scores relating to the Prosys interactors are indicated in Table A in attachment). The interactors were divided and classified in Gene Ontology (GO) categories (elliptic colored shape), highlighting several classes of defense-related proteins. The groups related to oxidative burst (light blue group) and calcium signalling (red group), the first line of defense activated in attached plants, including four and three interactors, respectively. Several MAPKs appear to be putative Prosys interactors, (fluorescent green). Two groups highlighted the Prosys interactions with genes related to Salycilic acid (SA) and Jasmonic acid (JA) pathways, two important phytohormone playing key roles in plant defense. The former (in red) is known to mediate host responses

upon pathogen infection (Lefevere *et al*., 2020), includes six subtilisin-like proteases, osmotine-like protease and NPR1 protein, known to be a SA receptor (Wu *et al*, 2012). The latter (in blue) plays a critical role in inducing systemic responses to herbivory (Zhang *et al*., 2020; Royo *et al.*, 1999; Laudert *et al*., 2000; Stenzel *et al*., 2003), and includes four lipoxygenases and protease inhibitors. Another group of defense-related putative interactors are associated with and cell wall (green group), whose reorganization is temporally consequent to the attack of a parasite, with the aim of preventing / reducing its penetration into the leaf. Prosys putative interplays with ethylene pathway were also found (dark blue group) and include an ethylene receptor and an ethylene-responsive transcription factors both also associated with plant defence. Interestingly the group of transcription factors (pink group), includes 10 proteins likely involved in the activation of defence-related genes. Other putative interactors are associated with abiotic stress (fuchsia group), other plant hormones (grey group) and pigment metabolic pathways (orange group). Figure 2.2 also shows a list of coloured squares that indicate the different sub-cellular localization of the proteins.

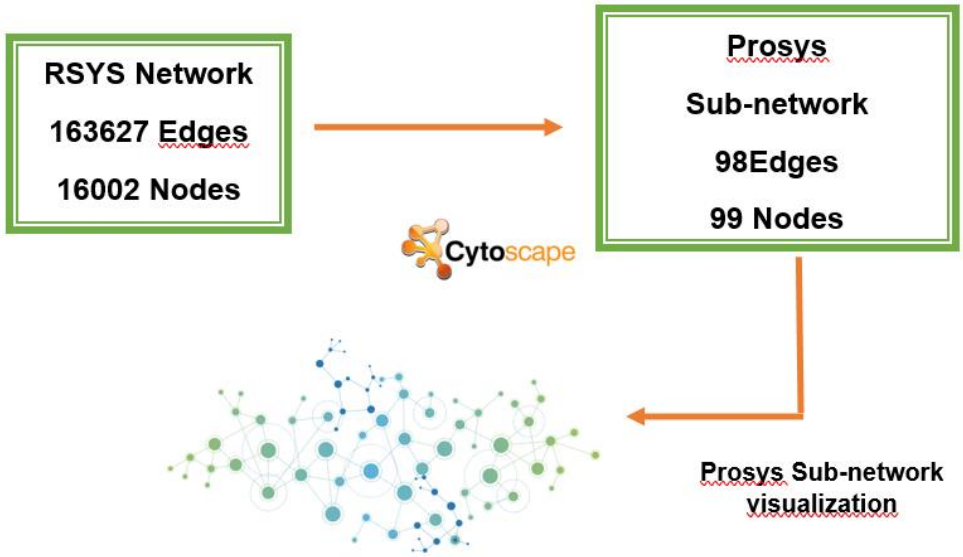


Figure 2.1. Schematic representation of the procedure for Prosys sub-network production and visualization. The RSYS interactome dataset was

analysed focusing on Prosys sub-network, composed by 99 proteins and 98 interactions, then visualized through Cytoscape.

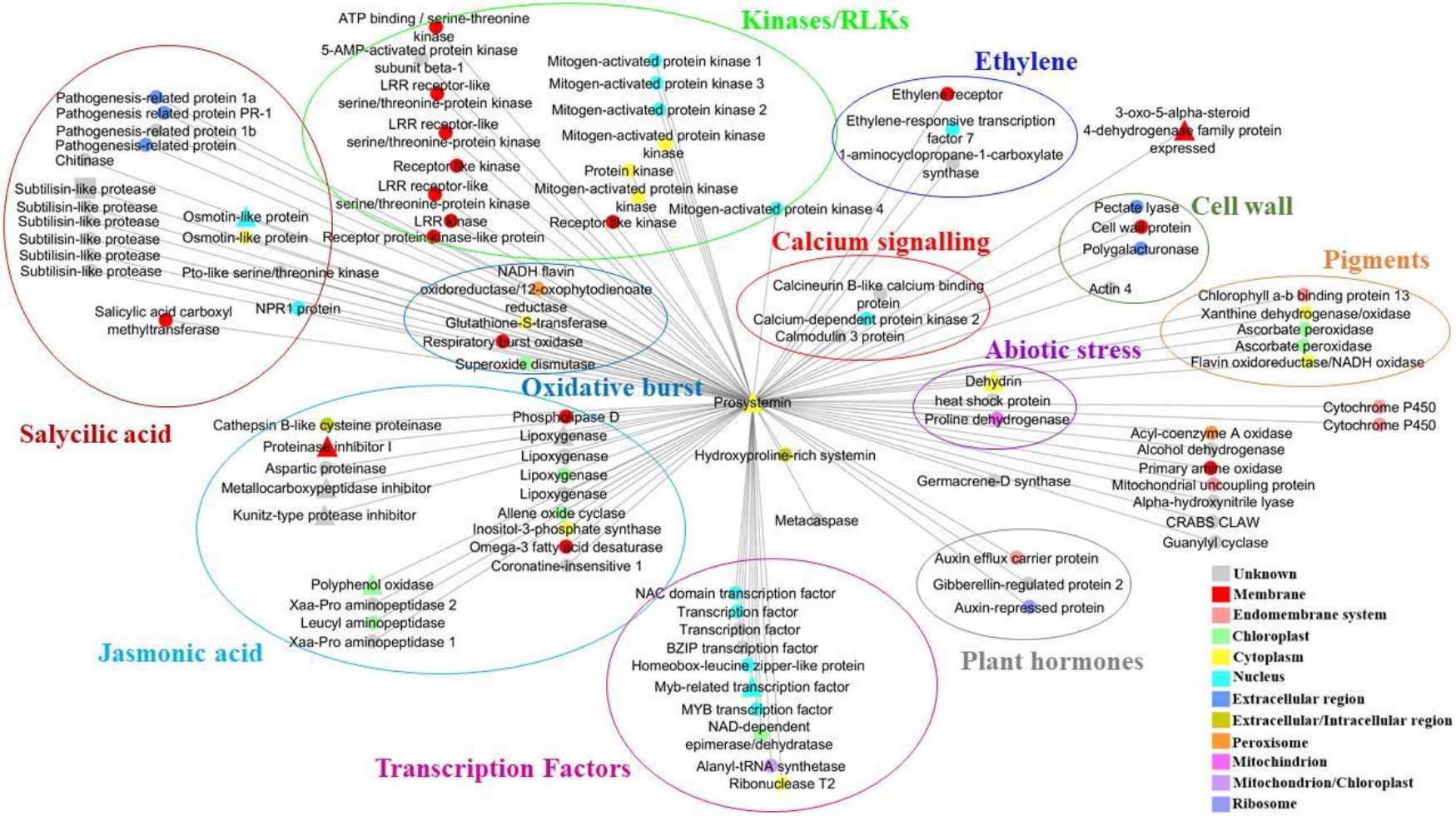


Figure 2.2. Prosys sub-network. The nodes were coloured based on the cellular localization of the protein. The up-regulated genes are showed with triangular shape; the square shapes are reserved for the genes down-regulated by *Prosys* over-expression.

2.3 **DISCUSSION**

The decision to study Prosys interactors instead of Sys interactors was dictated by the fact that the pro-hormone, classified as IDP (Buonanno *et al*., 2018), tends to interact with different molecular partners. In addition, the results obtained by Corrado and collaborators in 2016 demonstrated that the expression in tobacco plant of a mutated Prosys gene lacking the Sys coding region altered the proteomic profile of tobacco leaves and increased plant resistance against *B. cinerea*. There results suggested that the N-terminal part of the precursor is biologically active, or at least contain aminoacid stretches with biological activity.

Prosys over-expression in RSYS plants determined a transcriptomic reprogramming which caused a strong response to biotic stress, as showed through different bioassays (Coppola *et al.*, 2015). In wild-type plants, upon mechanical or insect damages, the pro-hormone is processed, Sys peptide released and bind the membrane receptor to trigger several rapid signaling events, as a Ca^{2+} burst, H^{+} influx, and K^{+} efflux, which leads to extracellular alkalinization, depolarization of the plasma membrane, and the rapid activation of MAP kinases (MAPKs) (Zhang *et al*., 2020). The MAPK cascade determines the activation of the plastid and peroxisomal enzymes that biosynthesize jasmonic acid (JA), converted in the cytosol to jasmonoyl-L-isoleucine (JA-Ile), the bioactive form of JA that promote, locally and systemically, the expression of defense-related genes (Sato *et al*., 2011). In transgenic plants, all those events presumably occur constitutively, likely partially controlled by some feedback regulation events. Generally, the occurring interactions rarely are persistent over time, as they are the results of specific bio-molecular events that occur within the cells and did not happen accidentally (De Las Rivas and Fontanillo, 2010). In our case the interactions were likely more stable than usual over time as the modified plant physiological state lasted over time.

The RSYS network parameters (figure in attachment) showed the centrality of the nodes, useful for understanding the role of different proteins within the network and varies between 0 and 1. This

observation is supported by the betweenness centrality that is a specification of the betweenness concept. It refers to the amount of control that a node can exert on the interactions of other nodes in the network, and it is defined by a ratio that can assume a value between 0 and 1. The nodes with betweenness centrality of 1 are very important in the network and perfect candidate to be “hubs”. A “hub” is a central node and removing that from the network could change its shape, in fact the removal of more than one hub could fall apart the network. Figure 2.3 b (attachment) shows the distribution of betwenness centrality referred to the number of neighbours. The comparison of nodes with the same number of neighbours in RSYS network evidenced a few nodes with betweenness centrality of 1, several nodes of an intermediate value and many of 0 value. In addition, the average of clustering coefficient distribution (figure 2.3 c in attachment) was consistent with these two previous parameters. The distribution of the clustering coefficients shows the tendency of each node to form clusters: while some proteins do not form clusters, others have a medium-high ability to cluster. In other words, proteins that act in a concerted way will tend to fall close to the network because they are functionally connected. In fact, the comparison of nodes with the same number of neighbours resulted in the identification of some with a high attitude to be part of a cluster, showing a high average of clustering coefficient, and many other for which this value is very low. All together, these parameters indicated that the RSYS network is a scale-free network, with some nodes that can be defined as “hub” with central position and role in the shape of the network. Several criteria have been used to define hubs in protein network, all generally referring to the node degree. Therefore, in biological terms, having a high centrality for a protein means to be fundamental for the correct functioning of the network and for the interconnection of the different proteins. This network is the first Prosys sub-network and it shows the central role played by Prosys during defense responses, starting from the oxidative burst until the modulation of phytohormone pathway and defense related genes through the activation of transcription factors. First Prosys is processed, and Sys peptide binds the membrane receptor, which activates the intracellular cascade. Sys receptor has been widely

discussed over the years, and Wang *et al.*, in 2018, demonstrated that perception of Sys depends on a pair of distinct Kinases (LRR-RKs) termed SYR1 and a homologue SYR2. SYR1 acts as a genuine Sys receptor that binds this small peptide with high affinity and specificity, but neither of them was found in the predicted network. These receptors probably have a high affinity only for Sys peptide after its release, and the Prosys three-dimensional folding may hides the Sys peptide located in the C-term region of the pro-hormone, that show a high disorder index (Buonanno *et al.*, 2018).

Prosys over-expression determined a cytoplasmic variation in the calcium ions concentration, which induces the activation of calcium sensitive proteins (Luan *et al.*, 2002). This observation is in good agreement with the interactions occurring between Prosys and proteins included in the red oval that includes calcineurin B-like (CBL, Solyc03g083320.2), calmodulin (CaM, Solyc03g098050.2) and the calcium-dependent protein kinase 2 (CDPK2, Solyc04g009800.2). CBL and CaM are small proteins containing multiple binding domains for Ca^{2+}, that upon Ca^{2+} binding transduce the Ca^{2+}signal binding target proteins (Luan *et al.*, 2002; Zhang *et al.*, 1995; Kuboniwa *et al.*, 1995). CaMs and CBLs interact with target proteins and regulate their activity, CaM target proteins have been identified in higher plants and include protein kinases, metabolic enzymes, cytoskeleton-associated proteins; CBL proteins interact with a family of SNF1-like protein kinases (Reddy *et al*., 1996 and 2002; Snedden *et al*.,1996; Zielinski, 1998; Snedden and Fromm, 2001). CBL and CaM need to interact with target proteins such as CDPK (Luan *et al*., 2002) in order to transmit the signal. CDPK, in addition to an EF-hand domain (helix-loop-helix) to bind a cation, have a Ser / Thr kinase domain that acts as calcium signal receptors (Wang *et al*., 2016). These genes are differentially expressed in the compatible interaction between tomato and the actinomycete *Clavibacter michiganensis*, together with several genes involved in basal defense, as reinforcement of cell wall, oxidative burst, hormone-mediated defense, and transcription factors involved in the activation of PR proteins (Balaji *et al*., 2008). Going on with the activation ofthe defence signalling cascade,the involvement of MAPK complex is observed: several MAPKs (Solyc12g019460.1, Solyc08g014420.2, Solyc06g005170.2, Solyc05g049970.2) and

MAPKK (Solyc03g097920.1, Solyc03g123800.1, Solyc12g009020.1) have been found in the network, grouped in green (figure 2.2). The substrates of protein kinases can be MAPK (Mitogen Activate Protein Kinase), which, through phosphorylation and dephosphorylation processes, regulate the transduction of the stress signal. Leu-rich Repeat Receptor Kinase (LRR) Ser / Thr kinase (RLK) receptors (green group in figure 2.2) are located on the cell membrane and play an essential role in signalling during the pathogen recognition (PAMPs) and in the subsequent defense mechanisms activation (Afzal *et al*., 2008). These receptors determine the rapid activation of the MAPK chain reaction and the entry of calcium, as well as the production of ROS. The production of ROS, whose associated genes are in the blue group in figure 2.2, is the response of plants that occurs as a result of various stresses. ROS represent one of the primary signals of the defense signalling, and are released few seconds after perception of damage, for example, inflicted by the attack of phytophagous insect. The superoxide anion (O^{2-}), for example, is released locally in the damaged tissue while hydrogen peroxide (H_2O_2) is produced both locally, to the wound, and systemically throughout the plant (Kessler and Baldwin, 2002). *Prosys* interacts with the 12-oxyphitodienoate reductase 3 (OPR3, Solyc07g007870.2), enzyme involved in the biosynthesis of the JA (Breithaupt *et al*., 2006). The enzyme NADPH oxidase (RBOH1, Solyc08g081690.2) is associated to oxidative burst and belongs to family of transmembrane proteins that guarantee the transport of electrons from a cytosolic donor to the extracellular oxygen acceptor, generating the superoxide radical O^{2-} (Lambeth, 2004). The latter is short-lived, therefore, either spontaneously or through superoxide-dismutase (SOD, Solyc06g048410.2), it is converted into hydrogen peroxide (H_2O_2) (Bowler *et al*., 1994). Glutathione-S-Transferase (GST, Solyc01g099590.2) is a cytosolic enzyme that counteracts the damage caused to the cell by oxidative stress. It is an enzyme that catalyses the conjugation of toxic and hydrophobic chemicals to glutathione, increasing its solubility and promoting its sequestration in the vacuole or its transfer to apoplast (Coleman *et al*., 1997). Prosys involvement in the response to abiotic stress has already been discussed in relation to salinity stress. Orsini and collaborators (2010)

observed a lower stomatal conductance and a higher plant biomass in plants expressing constitutively the Prosys cDNA in response to moderate saline stress. Prosys interacts with abiotic stress factors (purple grouping in figure 2.2) such as dehydrin (Solyc02g084850.2), Proline Dehydrogenase (ProDH, Solyc02g089620.2) and heat shock protein 70 (HSP70, Solyc06g076020.2). The gene dehydrin, as shown in transcriptomic data, is up-regulated in RSYS plants. Dehydrin belongs to a group of proteins induced in plant tissues following drought or salts excess, with the aim of increasing the cellular content of abscisic acid (ABA) (Hanin *et al*., 2011). This hormone induces closure of the stomata by decreasing the turgor pressure in the guard cells, due to an increase in intracellular calcium and activation of the potassium K^+ output channels. In the same way, Prosys appears to interact with another protein associated with the response to water stress, namely ProDH. Following abiotic stress, prolines are accumulated in the cell to stabilize sub-cellular structures and reduce free radicals (Claussen, 2005). For example, in Arabidopsis, in response to high salt stress concentration (200 nM), the proline level increases more than fifty times in fresh weight (Peng *et al*., 1996; Savouré *et al*., 1995). The ProDH enzyme is involved in the catabolism of proline, but even in the transfer of electrons directly to chain transport to produce the anion superoxide (Zhang and Becker, 2015). Cecchini and collaborators (2011) assessed its role in biotic stress by silencing the ProDH gene in Arabidopsis infested with *Pseudomonas syringae*. In fact, they demonstrated the implication of the protein in inducing the hypertensive response and resistance through the enhancement of ROS accumulation. Heat Shock Protein 70 (HSP), typical response to stress due to high temperatures, also interacts with Prosys. Under stress conditions, the accumulation of proteins not correctly folded into the cells occurs. The rapid induction of HSP was fundamental for the regulation of correct proteins folding in order to guarantee their correct functionality in stressful conditions. Cell localization was not well clarified in this case, although generally their activity is associated with cytoplasm and nucleus (Usman *et al*., 2017). The identification of this interaction was very interesting because it suggests a possible involvement of the peptide also in response to thermal shock. All phenomena described contribute to

determine the defense response in the plant through the release of transcription factors that, by binding the DNA, regulate the transcription of the genes involved in plant defense. A transcription factor induced by the constitutive expression of the *Prosys* gene is MYB (Myeloblastosis related proteins, Solyc06g053610.2), which is involved in the activation of the abiotic and biotic stress response genes (Baldoni *et al.*, 2015). Furthermore, tomato exogenous treatment with SA and Me-JA showed a significant change in MYB expression (Li *et al.*, 2016), suggesting an indirect involvement of Prosys in the JA / SA crosstalk. Furthermore, MYB interact with another family of transcription factors named WRKY, implicated in plant defense and response to various environmental stresses (Yang *et al*., 1999; Du and Chen, 2000; Robatzek and Somssich, 2001; Yu *et al*, 2001). The WRKY are involved in plant defense, some of these factors have been shown to confer disease resistance (Deslandes *et al*., 2002), triggering expression of defense-related genes (Eulgem *et al*., 1999; Robatzek and Somssich, 2002) and a common component in SA- and JA- mediated signal pathway (Li *et al*., 2004). In addition, WRKY transcription factors recognize the promoter region of the *NPR1* gene (Yu *et al*., 2001), present in the network (Solyc07g040690.2). *NPR1* gene is a positive regulator of inducible plant disease resistance. Expression of *NPR1* is induced by pathogen infection or treatment with defense-inducing compounds such as SA (Yu *et al.*, 2001). Spoel and collaborators, in 2003, proposed this possible scenario: the NPR1 gene is activated by SA accumulation after pathogen infection. Activated NPR1 then is localized to the nucleus, where it interacts with TGA transcription factors, ultimately leading to the activation of SA-responsive PR genes. In the cytosol, activated NPR1 negatively regulates JA-responsive gene expression, possibly by inhibiting positive regulators of JA-responsive genes or by facilitating the delivery of negative regulators of JA-responsive genes to the nucleus. The suppression of JA-responsive genes that encode enzymes from the octadecanoid pathways, such as LOX2, ultimately results in the inhibition of JA formation. This probably is a small part of a more complex scenario regarding SA/JA crosstalk, where Prosysmay play a key role interacting with different partners as

showed in the figure 2.2, that includes proteins involved in the pathway regulated by SA.

The Pto-like serine/threonine kinase activity gene (Solyc05g013320.1) has been associated with the salicylic acid pathway, experiments conducted on plants overexpressing the Pto gene have shown spontaneous cell death, accumulation of salicylic acid, high expression of genes related to the pathogenesis and increased resistance to a wide range of pathogens (Li *et al*., 2002). Mysore and collaborators (2003) have shown that the constitutive expression of the Pto gene in tomatoes confers resistance to *Pseudomonas syringae*, expressing the AvrPto protein. The shown overexpression of this gene, even in the absence of AvrPto, is consistent with the activation of various defense responses and could confer resistance to bacterial and fungal phytopathogens. Osmotine is a protein rich in cysteine residues and is involved in osmo-regulation (Ullah *et al*., 2018). It belongs to the PR family protein and has been used to produce transgenic plants resistant to fungi and tolerant to osmotic stress (Hakim *et al*., 2017). The Osmotin-like protein (Solyc08g080620.1) identified in the network is classified as PR-5 protein, which has been found to be up-regulated in RSYS plants. In tobacco, Xu *et al*., (1994) demonstrated that not all PR proteins are involved in the same metabolic pathways; in fact, PR-5 was induced by both SA and ET / JA, while other proteins of this family are SA-specific (Niki *et al*., 1998). The up-regulation of PR-5 in relation to Prosys can be explained by the need to balance the different hormone and to counter the attack of necrotrophic micro-organisms, since overexpression of osmotine causes cell death phenomena (Hakim *et al*., 2017). On the other hand, the Subtilisin-like protease (Solyc04g078110.1), which is a serine-protease characterized by a catalytic triad formed by aspartate, histidine, and serine (Dodson and Wlodawer, 1998) is encoded by a gene that is down-regulated in transgenic plants. Experimental evidence indicated their secretion, after glycosidation, in the extracellular space (Figueiredo *et al*., 2014). These enzymes exhibit various biological functions, both related to the life cycle of the plant and to the response to abiotic stress, in particular in the resistance to drought and saline stress (Budic *et al*., 2013; Liu *et al*., 2007), and to biotic stresses (Granell *et al*., 1987). Experiments

conducted in SA-treated tomatoes revealed the ability of the subtilisins P69B (Solyc08g079870.1) and P69C (Solyc08g079880.1), identified in the network, to be induced (Jordà *et al*. 1999; Tornero *et al*., 1997); for example, subtilisin P69C has been shown to process the LRP protein of the LRR protein family, mediating pathogen recognition (Tornero *et al*., 1996). The gene encoding a methyl-transferase (Solyc09g091550.2) is also very important, as it is related to the synthesis of the volatile Me-SA. The link identified with Prosys reinforces the involvement of the molecule in indirect defense of plants. The blue grouping of figure 2.2 includes proteins involved in the ethylene-regulated pathway that are listed in table A (attachment).

Prosys involvement in the octadecanoid pathway explains its correlation even with the ET pathway. The ACS (1-Aminocyclopropane-1-Carboxylate Synthase, Solyc01g095080.2) interactor regulates the synthesis of ET, ensuring the formation of the precursor called 1-aminocyclopropan-1-carboxylic acid (ACC). The ERF (Ethylene-responsive transcription factor 7, Solyc06g065820.2) gene codes transcription factors that regulate the expression of genes activated in response to ET following abiotic and biotic attack (Müller and Munné-Bosch, 2015). Five ERFs have been found in tomato *Solanum pimpinellifolium* overexpressed in conditions of saline stress and the production of S.p. ERF-B7 overexpressing transgenic plants has shown greater tolerance (Yang *et al*., 2018). The putative interaction of *Prosys* with the ACC enzyme, with the ERFs elements and with the ethylene receptor (Solyc09g075440.2) is very interesting because it would imply a direct regulation by the peptide of the signaling pathway mediated by ET, which acts synergistically with the JA. The JA pathway (celestial group in figure 2.6) includes several proteins (listed in table) that interact with Prosys, many of which encoded by overexpressed genes. In Arabidopsis, the interactor identified in the phospholipase D network (PLD, Solyc06g068090.2) is an enzyme accumulates in response to wounding, to activate the production downstream of JA and lipoxygenase 2 (LOX2) (Wang *et al*., 2000). PLD is a catalyst for the membrane phospholipids hydrolysis that allows the production, together with other phospholipases, of phosphatidic acid (PA, Phosphatidic Acid), or a

secondary messenger that modulates the activity of kinase, phosphatase, phospholipase, and proteins involved in crossing the membrane, in signaling mediated by Ca^{2+} and in oxidative stress (Munnik, 2001). In plan, the PLD / PA complex plays a fundamental role in the response to bacterial and fungal pathogens following PAMPs-mediated activation (Zhao, 2015). The lipoxygenases identified in the network, one of which (Solyc03g122340.2) is encoded by an overexpressed gene, are enzymes involved in the biosynthesis of oxylipins, in particular of JA. The allene oxidase interactor (Solyc02g085730.2) is essential for the biosynthesis of JA and the constitutive expression of its gene in tomato plants has resulted in a quantitative increase in jasmonate family in response to stress (Stenzel *et al*., 2003).

The Inositol-3-Phosphate Synthase (Solyc04g054740.2) is involved in the conversion of D-glucose 6-phosphate to myo-inositol 3-phosphate and has been associated with an increase in tolerance to abiotic and biotic stress in plants (Zhai *et al*, 2015). Protease inhibitors and polyphenol oxidase are induced by the constitutive expression of the Prosys cDNA. In the network enzymes, in relation to *Prosys*, which act at the cell wall level, have been identified such as pectate lyase (PL, Solyc02g093580.2) and polygalacturonase (PG, Solyc08g060970.2). The latter are involved in the hydrolysis of pectins, followed by the release of oligogalacturonides (OGAs) involved in the induction of the plant's defenses (Walling, 2000). In addition, Prosys also interacts with the node corresponding to the Hydroxyproline-rich Systemins (HypSys), responsible for the production of other small peptides (18-20 amino acids) belonging to the Sys family and involved in the induction of protease inhibitors and other insect defense genes (Pearce, 2011). Studies conducted in tobacco plants have led to the identification of two peptide forms, TobHypSys I and II, without sequence similarities with the Sys and rich in hydroxyproline, proline, threonine, and serine. Given the absence of a Sys orthologous gene in tobacco, these peptides are thought to be involved in wound-induced JA release, to amplify the signal from the leaves to the roots (Zhang and Baldwin, 1997). Similar peptides have also been identified in tomatoes and have been named TomHypSys I, II and III,

respectively of 18, 20 and 15 amino acid residues (Pearce and Ryan, 2003). While in tobacco these peptides play a key role in the defense response, in tomato Prosys predominates. However, studies conducted on plants constitutively transformed with an antisense construct for TomHypSys peptides have highlighted their concerted action with Prosys, activating the response to mechanical damage (Narvaez-Vasquez *et al*., 2007). The interaction found in the network supports the involvement of both plant peptides in the regulation of long-distance defense response. Prosys interact with a metacaspases, known for promoting the induction of programmed cell death during biotic and abiotic stress (Liu *et al*., 2016). Metacaspases are a family of cysteine-proteases and eight were identified in tomatoes (SIMC1 to SIMC8). The network identified the direct interaction of Prosys with the metacaspase SlMC6 (Solyc01g105310.2), which belongs to the proteins from the tomato interactome, but currently there is no evidence in the literature. Prosys involvement in indirect defense is supported by the presence in the network of the germacrene-D-synthase node (Solyc12g006570.1). This enzyme is involved in the biosynthesis processes of volatile terpenoids released by the plant following the attack of herbivores (Colby *et al*., 1998). Corrado and collaborators (2007) highlighted the role of Prosys in increasing the attractiveness of the females of parasitoid attributable to the induction of germacrene-C-synthase, responsible for the production of sesquiterpenoids. Coppola and collaborators (2017) have shown that Sys promotes plant-to-plant communication, probably through the alteration of the volatile mixture emitted. The communication promotes the expression of genes and signals associated with the defense (priming of defense), that alert the receiving plants about a possible attack by (micro) invading organisms. The putative direct interaction of Prosys with germacrene synthase is very interesting and reinforces the experimental observations already discussed in the literature, so that transgenic plants overexpressing *Prosys* show the ability to fortify both the direct and indirect defenses. It is therefore a valid tool potentially applicable in crop protection, given its ability to modulate multiple defense pathways simultaneously. Recently, in 2019, Coppola and collaborators shown how tomato plants treated with *Sys* peptide,

showed increased expression of defense-related genes, with enhanced levels of direct and indirect defense.

In summary, the *in silico* prediction of Prosys molecular interactions in tomato defense reflect the broad spectrum modulating activity of the molecule that is likely the consequence of a large number of molecular interactors. The results obtained showed that PPIs prediction could address studies opening new orizons, giving at the same time new input to research. The use of this information could help to shed light on complex mechanisms as in defense response.

2.4 **CONCLUSION**

PPIs affect almost all metabolic processes and pathway, and currently a high number of methods are used for their identification. Each method has its own positive and negative aspects, including costs, time, and reliability of results and, forthis, *in silico* predictions of PPIs have been becoming, over the years, increasingly important, offering new solutions and perspectives. The high amount of data provide a large number of potential interacting pairs, but they unfortunately often have a higher error rates than other approaches. Therefore, computational methods for PPIs prediction need to complement with experimental methods; in fact, they can efficiently integrate data from numerous sources in order to make predictions reliable (McDowall *et al*., 2009). The 98 Prosys direct interactors,confirmed its implication in tomato defense response. Over the years, researchers focused on Sys membrane receptors, after proteolytic cleavage of the prohormone. Beside some proposed SYS receptors that proved to be a wrong identification (Scheer and Ryan, 2002; Scheer *et al*., 2003), the real SYR1 and SYR2, were recently identified (Wang *et al*., 2018). Our hypothesis and the evidence obtained over the years, have also led us to speculate the direct involvement of Prosys, in the activation of defense mechanisms, binding specifics partner. This proof-of-concept appear to be correct as shown by the numerous interactions that appear to link Prosys with several other proteins.

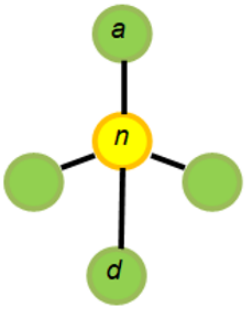
2.5 **MATERIALS AND METHODS**

2.5.1 IN SILICO PREDICTION OF PROTEIN INTERACTIONS

The 695 differentially expressed tomato ESTs were used to query several plant PPIs databases, available for the plant model species Arabidopsis and tomato (Yue *et al*., 2016; Szklarczyk *et al*., 2019). To query Arabidopsis interactome, tomato DEGs were converted in their Arabidopsis correspondent proteins through blastx analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the Arabidopsis RefSeq database as reference, applying an e-value filter (Exp Max=10^{-5}) and collecting only first hit for each query. To get the TAIR identifiers for these proteins, the identifier converter available at Babelomics 4.2 website (www.babelomics.org) was used. The same procedure was performed to obtain the tomato corresponding protein. The protein list obtained in this way was ready to be used for the PPIs analysis. The 309 TAIR identifiers were subjected to PPIs analysis using their corresponding database. The database Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was used for the Arabidopsis corresponding proteins. Then, the database: Predicted Tomato Interactome Resource (PTIR) was used to extrapolate the PPIs predicted in tomato. Both are freely accessible online: PTIR (http://bdg.hfut.edu.cn/ptir/index.html) and STRING (https://string-db.org). Then, we decided to focus on Prosys sub-network; thus, a network contained only Prosys direct interactors was extrapolated. The networks acquired were imported in the Cytoscape 3.8.2 software (www.cytoscape.org), where all the information was integrated and unified. The network and the attribute file were imported in Cytoscape software in order to paint, analyse and integrate the interactions. The parameters that describe the network were studied using the graph theory. The attribute file obtained from Ensembl Plant (http://plants.ensembl.org/index.html) was uploaded and then it was possible to associate the localization, the function and the metabolic process involved in each protein-node. Based on this information, stylistic changes have been made to make the network visualization clearer. After removing any duplicates and self-loops, Cytoscape's Network Analyzer Tool function enabled the analysis of the graph

through the automatic calculation of a series of parameters. The main parameters analysed were:

• **Betweenness centrality** evaluates the centrality of a protein in the network, it is defining by the number of shortest paths passing through a node, where “shortest path” stands for the minimum path (sequence of edges) connecting two nodes (figure 2.3).



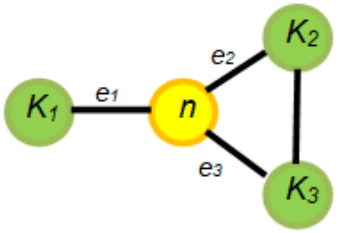
$$B_n = \sum_{ad} \frac{P_{ad}(n)}{P_{ad}}$$

P_{ad}= number of shortest paths between *a* and *d*

$P_{ad}(n)$= number of shortest paths between *a* and *d* through *n*

Figure 2.3. The figure shows the Betweenness centrality and the formula to calculate its value.

• **Clustering coefficient** evaluates how many connections exist between a node *n* and all its neighbours *k* and is described by the ratio of the number of interactions between neighbours of n and the maximum number of interactions that can possibly exist between them (figure 2.4).



$$C_n = \frac{2e_n}{k_n(k_n - 1)}$$

Figure 2.4. The Clustering coefficient indicates the number of interaction (*e*) between a node (*n*) and its neighbours (*k*).

• **Connections degree:** refers to the number of edges (interactions) connected to a node (protein).

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CHAPTER 3

IDENTIFICATION OF PROSYSTEMIN INTERACTORS THROUGHT *IN VITRO* AND *IN VIVO* STUDIES

In collaboration with Y. Zhang and A.R. Fernie and R. Rao

ABSTRACT

In silico methods offered us the possibility to investigate the putative interactors of tomato Prosys resulting in expected and unexpected results, that required further investigations and confirmations. In this chapter the results obtained from the analysis of the Prosys interactors through methods based on laboratory analysis are reported: Affinity Purification Mass Spectrometry (AP-MS) and Bimolecular Fluorescent Complementation (BiFC). AP-MS, an *in vitro* method used to identify protein-protein interactions (PPIs), allowed to carry out a large-scale screening detecting more than 300 proteins that physically interact with Prosys which included some molecular partners previously identified with the *in silico* approach. For example, the heat shock protein 70 (Sl-HSP70-1), which plays a key role in stress responses, and NAD-dependent epimerase\dehydratase (NaDED), possibly associated with both sugar and hormonal plant defense signaling. The obtained results were validated through BiFC, an *in vivo* approach, that allowed to visualize the interactions in plant tissue. The BiFC system evidenced the interaction of Prosys with an ATP-dependent clp protease previously detected with the AP-MS, and confirmed the interaction of Prosys with the NaDED, detected both *in silico* and *in vitro.* In addition, two Prosys interactors located in the *in silico* network, MYB Transcription Factor and a MAP-Kinase were validated with BiFC.

3.1 INTRODUCTION

The term “Interactome” was introduced in 1999 by a group of French scientists led by Bernard Jacq and it and is used to describe the whole set of possible interactions, occurring inside a cell (Lu and Zhang, 2013). Despite the fact that interactions can occur within molecules belonging to different biochemical families such as protein-nucleic

acids (Davidson, 2010), proteins-lipids (Fantini and Yahi, 2015) or within the same family, most commonly the interactome refers to PPIs and protein-DNA interaction networks, also defined gene regulatory networks (Davidson, 2010). Identifying key players and their interactions are fundamental for understanding biochemical mechanisms at the molecular level. The study of the interactions among proteins within cells and organisms may lead to the identification of the functions of proteins and protein complexes, one of the main goals of proteomic studies (Park, 2004). In fact, more than 80% of proteins work in protein complexes (Berggård *et al*., 2007) and PPIs affect a wide range of biological processes, including cell-to-cell interactions (Danese *et al*., 2000), metabolic processes (Zhang *et al*., 2017), developmental control (Yanagida, 2002; Sukenik *et al*., 2017), control of DNA replication and progression of the cell cycle (Droit *et al*., 2005), as well as a myriad of other minor but important functions. Studying PPIs and understanding their function may help in resolving the mechanisms of action of single proteins and protein complexes involved in biological processes (Morris *et al*., 2014). For example, PPIs might explain the speed of some metabolic reaction probably due to the proximity of proteins (Laursen *et al.*, 2014). Starting from the last decade, the number of PPIs identified has increased significantly and, consequently, numerous databases were created to catalogue and annotate these interactions (www.ptir.atcgn.com; www.string-db.org).

Following the definition of the Prosys PPIs established through *in silico* approaches (Chapter 2) an obvious extension of the study included the experimental validation of a number of interactions. We were also encouraged to direct us towards this goal by the recently observed characteristics of Prosys amino acid sequence that confers to the precursor an intrinsic disorder (Buonanno *et al*., 2018). In other words, the pro-hormone is an Intrinsically Disordered Protein (IDP), a class of proteins showing high level of structural instability, characterized by the ability to interact with many different partners (Sun *et al.*, 2012; Sun *et al.*, 2013). This behaviour likely reflects the broad spectrum of action observed in plant over-expressing Prosys in

which several defense-associated pathways were activated (Coppola *et al*., 2015).

The PPIs study may proceed via different approaches that, beside the *in silico* one (described in Chapter 2), include *in vitro* and *in vivo* procedures, both based on recombinant and cloning technologies. *In vitro* methodologies, allow the identification of proteins that physically interact and include affinity chromatography, co-immunoprecipitation, and protein chip arrays. The interactions detected by these strategies need a validation with a different technique, generally *in vivo*, to verify them in a living organism such as yeast two-hybrid.

The *in vitro* method used in this work was AP-MS. AP-MS is a large-scale screening approach to study PPIs and one of the most used technique to isolate and identify protein-binding partners of a target protein. AP-MS experiments have been widely used to generate protein-protein interaction networks and information-rich data (Bürckstümmer *et al*., 2006; Morris *et al*., 2014; Puig *et al*., 2001; Zhang *et al*., 2017). This technique was developed with different detection methods, but the basic principle is based on the well-known protein complex that occurs following the interaction between an antibody and a bait protein or through tag fused to the bait protein via recombinant DNA technologies. These complexes could be precipitated using magnetic beads, on which the ligand is coupled, and later submitted to affinity purification (AP), followed by mass spectrometry (Zhang *et al*., 2019). The success of AP-MS depends on the efficiency of trypsin digestion of the protein interacting complex and the recovery of tryptic peptides for MS analysis. The protocol used provides a proteomic-based method to directly digest complexes on the beads for the successive mass spectrometry. AP-MS can be performed in many plant species, with the main prerequisite being the availability of a sequenced reference genome. Generally, this technique is the first approach in protein interaction studies, coupled to *in vivo* methods, as BiFC or yeast two-hybrid, to validate the interaction detected. Presently, these features have been successfully applied in *Arabidopsis thaliana* (Zhang *et al.*, 2017; Zhang *et al.*, 2019).

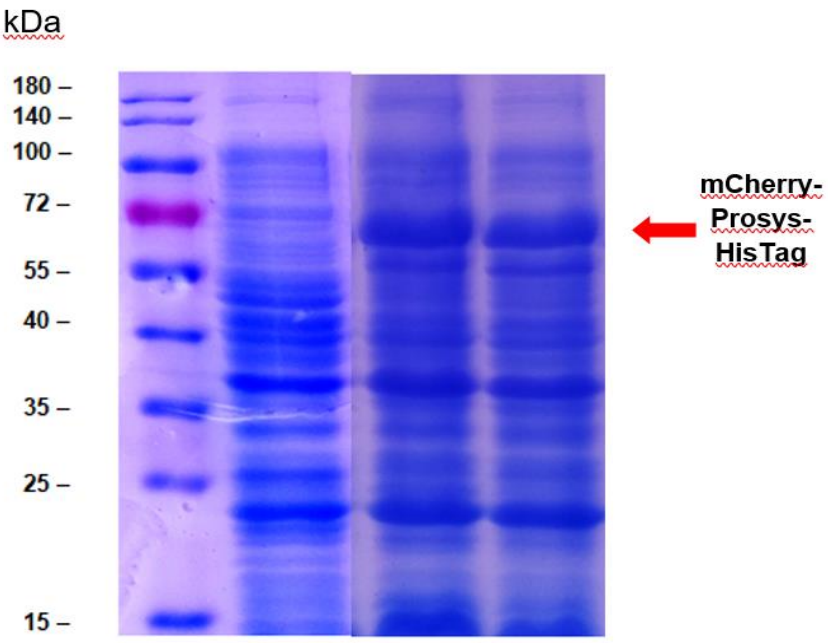
The study of protein interactions *in vivo* is the best way to confirm the veracity of an observed PPIs because the method realizes the natural and complex conditions that occur within a biological system. BiFC is an *in vivo* technique becoming fundamental in this field to visualize PPIs in a variety of model organisms. The technique is based on the fusion of unfolded complementary fragments of a fluorescent reporter protein to the putative interacting proteins. The interaction of these proteins will bring the fluorescent fragments within proximity, allowing the reconstitution of the reporter protein in its native three-dimensional structure and emission of the fluorescent signal (Kodama and Hu 2012). The BiFC assay was originally developed using the yellow spectral variant (YFP) of the green fluorescent protein (GFP), but considering the self-florescence of plants, Yellow Fluorescent Protein (YFP) and Red Fluorescent Protein (RFP) instead of Green Fluorescent Protein (GFP) are often used for plant BiFC studies (Jach *et al*., 2006). The fluorescent signal emitted, can be detected using an inverted fluorescence microscope equipped with an argon laser which, exciting the fluorescent marker, allows the sub-cellular localization of the interaction within the cell. In addition, the intensity of the fluorescence emitted allows the visualization of the fluorescence distribution inside the cells. This method based its success on *Agrobacterium tumefaciens* infiltration, thanks to which it is possible to express genes, for a rapid evaluation of protein-protein interaction (Zhang *et al*., 2020). All these features render BiFC system a key technique to visualize protein-protein interactions *in vivo*. The aim of the work presented in this chapter was to experimentally confirm the Prosys interactions predicted *in silico,* through AP-MS (*in vitro*) and BiFC methods to visualize these interactions in a plant cell.

3.2 RESULTS

3.2.1 *IN VITRO* RESULTS: AP-MS

To study Prosys interactors with AP-MS, the cDNA was amplified (fig. showed in materials and methods) from pMZ vector containing Prosys cDNA and cloned in different destination vectors with protein tag indifferent positions. The vectors generated were pET301 containing the expression cassettes: mCherry-Prosys-HisTag and pET300 HisTag-Prosys-mCherry. Both vectors were expressed in the *E.coli* Rosetta® strain, a specific host which enhances the expression of eukaryotic proteins because contains tRNA codons rarely used in prokaryotic. The vectors contain a strong polymerase promoter, from bacteriophage T7, for the chemical induction of cloned sequences by Isopropyl-β-D-1-thiogalactopyranoside (IPTG) determing different concentrations of the recombinant protein (as shown in materials and methods). The SDS-PAGE of total proteins extracted from bacteria showed, as expected, several protein bands (figure 3.1). The Prosysprotein has a predicted mass is 23 kDa, as has a previously reported (Delano *et al*.,1999), however the mobility of the protein in a standard SDS-PAGE is higher than expected because of the high percentage of charged amino acids (44%). Previous studies showed that Prosys recombinant protein produced in *E.coli* or expressed in tobacco was detected as a 40 kDa protein (Rocco *et al*., 2008; Zhang and Hu, 2017). The mCherry molecular weight is 28.8 kDa while the (His)6-tag is 1.267 kDa therefore, the fusions proteins are expected to be around 70 kDa, as indicated by the arrow in figure 3.4.

a)


kDa
180 –
140 –
100 –
72 –
55 –
40 –
35 –
25 –
15 –
mCherry-
Prosys-
HisTag

b)

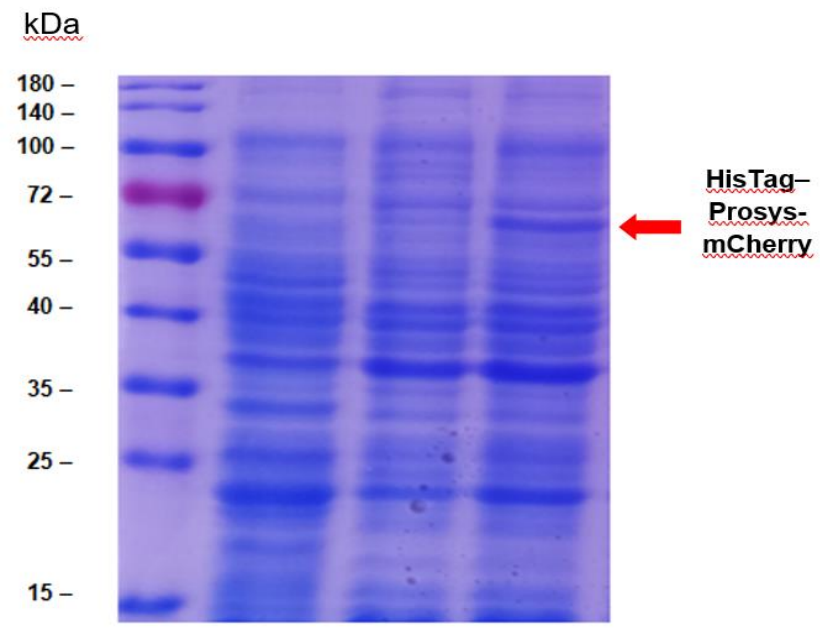

kDa
180 –
140 –
100 –
72 –
55 –
40 –
35 –
25 –
15 –
HisTag–
Prosys-
mCherry

Figure 3.1 a-b. The pictures show the SDS-PAGE of total protein expressed and extracted from *E. coli* transformed with (a) pET301-mCherry-Prosys-HisTag and (b) pET300-HisTag-Prosys-mCherry. (a) Lane 1: protein ladder; lane 2: protein extracted before adding IPTG; lane 3: protein extracted after 8 h from the addition of IPTG; lane 4: protein extracted after 24 h from the addition of IPTG. (b) Lane 1: protein ladder; lane 2: protein extracted before adding IPTG; lane 3: protein extracted after 8 h from the addition of IPTG; lane 4: protein extracted after 24 h from the addition of IPTG.

Thanks to the presence of the (His) 6-tag (Ht), the two different recombinant proteins (with Ht and mCherry [mC] located at N-terminal or C-terminal, mC-PS-Ht and Ht-PS-mC respectively) were submitted to AP by adding them to metal ion beads and mixing with total protein extracts from tomato wounded leaves. Following AP, the protein complexes formed were digested with LysC/Trypsin and analysed by mass spectrometry. A single replicate for an AP experiment constitutes a single sample for liquid chromatography–mass spectrometry (LC-MS) measurement. Three replicas for each vector were analysed by MS. Proteins were identified from spectra, using Mascot (Matrix Science, London, UK), and quantified with the Progenesis IQ software (Nonlinear Dynamics, Newcastle, UK) (Zhang *et al*., 2019). A mass/charge ratio (m/z) for each replica was originated and then used to calculate the average of the m/z obtained from all the replicas. The detected m/z were normalized using the mCherry m/z. The normalized signal intensities were then processed to calculate the Fold-Change Abundance (FC-A) score by using the SAINT algorithm embedded within the CRAPome software (Mellacheruvu *et al*., 2013). FC-A is a confidence score computed for each bait-prey interaction pair. This score value allowed to establish the significance of an interaction and to detect false positive and contaminants. In a simpler way, FC-A score is computed through the ratio between the average of the value of replicas of every single protein detected by MS and the mCherry average. In order to verify any possible impact of the location of mC and Ht on the Prosys interactions with proteins, the replicas of the two groups (mC-PS-Ht and Ht-PS-mC recombinant proteins) were compared by T-test (table B in attachment). Only for three proteins the P value was significant (<0,05) indicating a relative effect of the positions of mC and Ht.

More than three hundred proteins showing an apparent affinity with Prosys were identified from spectra using Mascot (Matrix Science, London, UK) (table B). Unfortunately, most of these proteins are uncharacterized or only scarce information are available. The ribosomal subunit, the translation-related proteins and the proteins not detected in one or more replicas were deleted. Putative interactors showing an FC-A value higher than four were considered effective interactors (Zhang *et al.*, 2019). The proteins were assigned in term of cellular localization, functions, role in defense response querying online accessible database as UniProt (www.uniprot.org), KEGG (www.genome.jp) and Ensemble Plants (www.plants.ensembl.org). Proteins with significant scores are listed in table B (attachments) and represent possible Prosys interactors. On top of the raw data, as result that confirm the success of the experiment, we obtained Prosys protein (Solyc05g051750), expressed at high level with an FC-A score of 3835.44; this occurred because the protein was present in all the protein complexes detected by MS.

Four interactors previously predicted with the *in silico* analysis were also detected by AP-MS although with different scores. Two of them were characterized by high score values, a NaDED (Solyc09g065180) with a FC-A score of 36.64 and a HSP (Solyc06g076020) with a FC-A score of 10.28. The other two interactors had a FC-A score lower than the fixed threshold value, respectively, 1.38 for the Inositol-3-phosphate synthase (Solyc04g054740) and 1.79 for Alanine-tRNA ligase synthetase (Solyc01g111990). The attention was then focused on cytoplasmic proteins, as Prosysis located in the cytosol (Narváez-Vásquez and Ryan, 2004). Several cytoplasmic interactors showed a high score for example: transcription factor S-II (Solyc07g007840), phosphogluconate dehydrogenase 2, PGD2 (Solyc05g010260), SNAP receptor activity (Solyc12g089150), heat shock proteins (HSP) (Solyc07g065840), transcription elongation factor (Solyc07g007840) and several enzymes involved in different processes such as two different oxidoreductases (Solyc05g010260; Sloyc11g010960) and a calcium ion binding protein (Solyc01g099770). Intriguingly one interactor is related to the ethylene biosynthetic process (Solyc02g036350).

3.2.2 *IN VIVO* RESULTS: BiFC SYSTEM

The interactors detected with AP-MS and with *in silico* analyses were subjected to further confirmation with BiFC system. Four interactors were selected for BiFC confirmation the NaDED, detected both *in silico* and *in vitro*; the ATP dependent clp protease detected *in vitro*, the MYB transcription factor and the mitogen-activated protein kinase 6 (MAPK6), both detected *in silico*. The negative control was performed cloning in the same vectors Prosys and the tomato elongation factor 1α (Solyc06g009970). The recombinant vectors were analysed by PCR and sequenced, and then used to the transient expression in *N. benthamiana* via *A. tumefaciens* mediated protocol. The fluorescence emission was visualized by confocal microscopy.
The results of the infiltration of young leaves of *N. benthamiana* with the recombinant vectors are shown in figures 3.2, 3.3, 3.4 and 3.5 and listed in table 3.1. For all the protein pairs tested, BiFC signals were detected in cytoplasm and nucleus. Figures 3.2 shows the interaction between Prosys protein and the ATP-dependent clp protease binding protein detected with AP-MS. The figures 3.3 shows the interaction between Prosys protein and NaDED, previously detected *in silico* and *in vitro*. The figures 3.4 and 3.5 show Prosys interaction with MYB transcription factor and MAPK respectively, both detected in the *in silico* network. Figure 3.6 shows the absence of interaction between Prosys and the tomato Elongation Factor 1α was used as a negative control.

Table 3.1. List of Prosys interactors detected with BiFC, *in silico* network and AP-MS system.

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

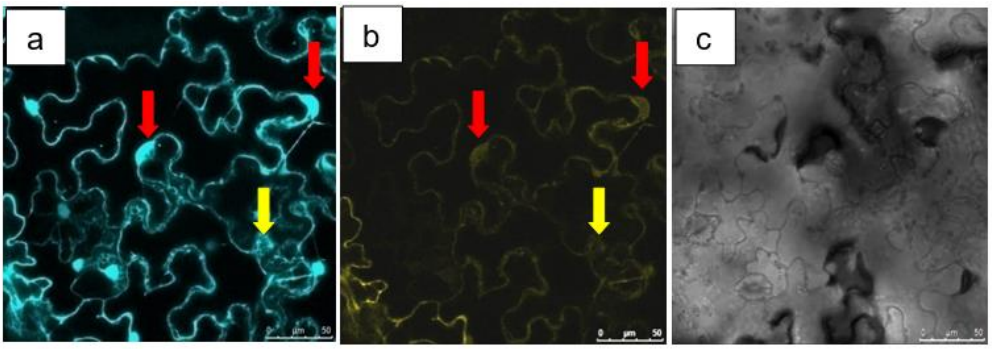


Figure 3.2 a-b-c. Confocal microscope images showing the interactions between Prosys protein and ATP-dependent clp protease ATP-binding subunit. Young tobacco leaves were infiltrated with *A. tumefaciens* transformed by the vector pBiFCt-2in1-NC-Prosys-ATP-dependent clp protease. The red arrows indicate the interactions inside the nucleus; the yellow one inside the cytosol. The interaction between the proteins gives a yellow fluorescence signal due to the fusion of two YFP non-fluorescent fragments (b). The reconstitution of YFP from its' fragments (YFPN, N-terminal fragment [amino acids 1–155]; YFPC, C-terminal fragment [amino acids 156–239]) is the result of the interaction between the proteins. Furthermore, the vectors also contain a red fluorescent protein (RFP), used as control to verify the expression of the protein inside the cell; the exposure to different wavelengths determined different color emission: a) excitation of YFP (490–515 nm) and RFP (555 nm) (green light emission); b) excitation of YFP (yellow light emission); c) blank: same focal plane without laser excitation.

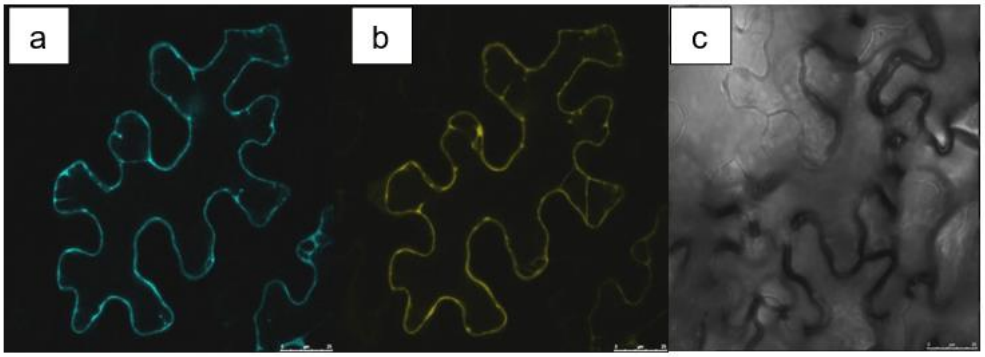


Figure. 3.3 a-b-c. Confocal microscope images showing the fluorescence emitted due to the interactions between Prosys protein and NaDED protein. Young tobacco leaves were infiltrated with *A. tumefaciens* transformed by the vector pBiFCt-2in1-NN-Prosys-NaDED. a) excitation of YFP (490–515

nm) and RFP (555 nm); b) excitation of YFP; c) blank: same focal plane without laser excitation.

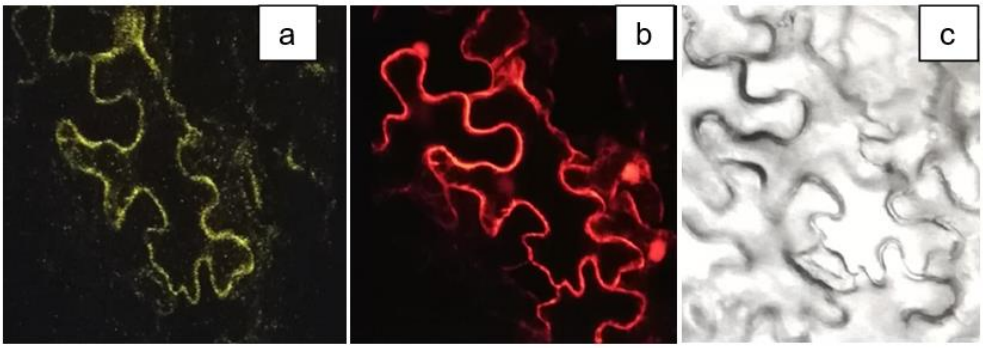


Figure 3.4 a-b-c. Confocal microscope images showing the interactions between Prosys protein and MYB transcription factor using the vector pBiFCt-2in1-NN-Prosys-MYB. In this and in next visualization the merge function between red and yellow laser was not used. a) excitation of YFP (490–515 nm); b) RFP (555 nm) excitation laser; c) blank: same focal plane without laser excitation.

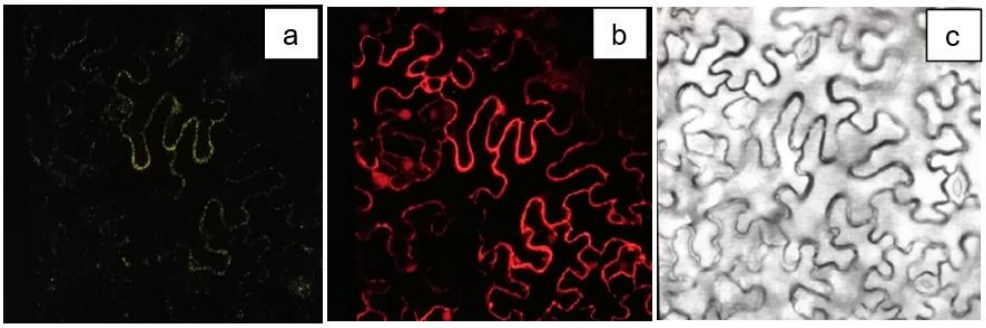


Figure 3.5 a-b-c. Confocal microscope showing the interactions between *Prosys* protein and MAPK6. a) excitation of YFP (490–515 nm); b) excitation of RFP (555 nm); c) blank: same focal plane without laser excitation.

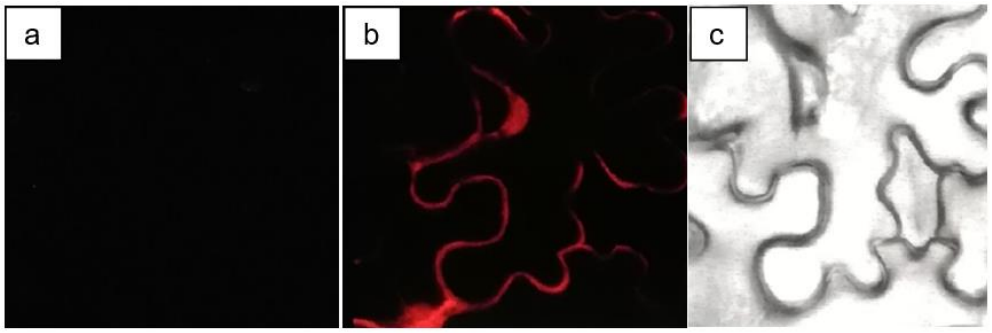


Figure 3.6 a-b-c. Confocal microscope images showing the negative control. The vector used, pBiFC-2in1-NC, contained Prosys and the Elongation Factor 1α. a) excitation of YFP (490–515 nm), and no yellow light emission was observed as absence of PPI. b) excitation of RFP (555 nm); c) blank: same focal plane without laser excitation.

3.3 DISCUSSION

The interactions detected via the AP-MS approach confirmed that Prosys is involved in a complex scenario as previously drawn by the in silico network as it appears to physically interact with several proteins, at least in the condition used in this study. Since the majority of the interactions (80%) found were independent from the steric effect determined by the different position of mC and Ht (N- and C-terminal), we can conclude that the position of the two tags had a negligible impact on the bond formation. Only in three cases (transcription factor S-II, phosphogluconate dehydrogenase II and alcohol dehydrogenase) the interaction with Prosys resulted affected by mC and Ht position. Interestingly, Prosys interaction with NaDED was confirmed with the three different approaches used. NaDED is a member of a family protein with catalytic activity, localized into the cytosol and involved in different biological processes such as rRNA processing, and positive regulation of translation and transcription (https://www.uniprot.org/uniprot/A0A3Q7I5A7). In addition, different homologs are localized in chloroplast suggesting the implication in different cellular mechanism (https://diurnal.sbs.ntu.edu.sg/sequence/view/25434). The NaDED family protein is involved in carbohydrate metabolic biological processes, which includes the formation of carbohydrate derivatives

by the addition of a carbohydrate residue to another molecule (Cao *et al*., 2013). Sugars can stimulate plant immunity and up-regulate defense genes expression (Bolouri-Moghaddam and Van Den Ende, 2012). For example, a high level of sugars in plant tissues enhances plant resistance against pathogenic fungi (Morkunas and Ratajczak, 2014). This mechanism was defined “high-sugar resistance”. It is important to note that sugars constitute the primary substrate providing energy and structural material for defense responses in plants. Sugars trigger an oxidative burst at early stages of infection, inducing certain pathogenesis-related proteins (PR). Moreover, some sugars act as priming agents inducing higher plant resistance to pathogens. Sugars may also act as intermediates, interacting with the hormonal signaling network regulating the plant immune system (Morkunas and Ratajczak, 2014); for example, different phytohormones including ethylene and jasmonate, interact with the sucrose signaling pathway (Tauzin and Giardina, 2014). For all these reasons, the Prosys-NaDED interaction could be associated with both sugar and hormonal plant defense signaling.

Among the AP-MS results, several HSP were founded. Although the proteins interacting with unfolded peptide like heat shock proteins may be artifact of the AP-MS approach (Zhang *et al*., 2010), the presence of heat shock protein in the network obtained with bioinformatic tools encouraged us in considering this protein a candidate interactor. HSP are small protein expressed in a stressful condition that act as chaperone for other proteins, to permit the correct protein folding (Kiang and Tsokos, 1998). The in silico predicted heat shock protein 70 (Sl-HSP70-1) belongs to HSP70 family that are often expressed in response to stresses such as heat or drought (Zhang *et al*., 2015). The abundant expression of HSP70 in both vegetative and reproductive tissues suggests that the gene family is likely to play roles in tomato growth, development, and fruit ripening (Duck *et al*., 1989; Vu *et al*., 2019). Another interesting HSP found is heat shock protein 90s (Solyc12g015880). This protein is required in the Mi-1 gene mediated resistance against pathogens and pests (Bhattarai *et al*., 2007). Tomato Mi-1 gene encodes a protein with putative coiled-nucleotide-binding site and leucine-rich repeat motifs. Mi-1 confers

resistance to root-knot nematodes (*Meloidogyne spp*.), potato aphids (*Macrosiphum euphorbiae*), and sweet potato whitefly (*Bemisia tabaci*) (Bhattarai *et al*., 2007). The found interaction could also mediate plant resistance against biotic stress agents.

Among the interactors obtained, several have catalytic activity, for example, the gene Solyc05g010260 and Solyc11g010960 with oxidoreductase activity are involved in the oxidation-reduction process of ethylene biosynthetic pathway (solgenomics.net). It was demonstrated that ethylene plays a pivotal role in plant sensitivity against biotic stressors such as bacterial, fungal, and nematode pathogens (Adie *et al*., 2007; Kazan and Manners, 2008; León-Reyes *et al*., 2010; Lin *et al*., 2009) and abiotic stresses such as flooding, salinity, and drought. In addition, ethylene promotes plant growth-rhizobacteria improving plant tolerance to environmental stresses (Haas and Defago, 2005; Lugtenberg and Kamilova, 2009; Barreto-Figueiredo *et al*., 2011; Hol *et al*., 2013). Cross-talk between jasmonate (JA), ethylene (ET), and Salicylic acid (SA) signaling is thought to operate as a mechanism to fine-tune induced defenses that are activated in response to multiple attackers. JA and ET interdependently and synergistically induce the expression of pathogen-responsive genes, such as Plant defensins to support plant tolerance against infections (Dugardeyn and Van Der Straeten, 2008). The found interaction could therefore play a biological role in fine - tuning induced defense.

The interesting interactor, WRKY-43, (Solyc12g042590) (FC-A: 4.23) belongs to the family of WRKY transcription factors (TFs). WRKY TFs are involved in the regulation of various physiological programs in plants, including pathogen defense, senescence, trichome development and the biosynthesis of secondary metabolites (Huang *et al*., 2012). In tomato plants, WRKY genes are involved in different developmental processes and in response to various biotic and abiotic stresses (Huang *et al*., 2012). WRKY genes were shown to be functionally connected forming a transcriptional network, holding central positions in plant defense activation (Eulgem and Somssich, 2007). Huang and collaborators, in 2012, showed that WRKY TFs can

be phosphorylated by MAP-kinases, in response to pathogen-associated molecular patterns (PAMP), activating several defensegenes, among which salicylic and jasmonic acid related defense genes. Although it is known that the plant's genome determined the great degree of phenotypic plasticity required for the adaptation to the multitude of abiotic and biotic stresses that plants have to face in their natural habitat, it is not clear how they integrate the multitude of partly synergistic/partly antagonistic signals that enable them to react properly under specific condition. However, we know that plants are capable of extensive reprogramming their transcriptome in a highly dynamic and temporal manner. This regulation leads to adaptive plasticity of plants being mainly achieved by enforcement of a network of various transcription factors (TFs). In this scenario Prosys-WIRKY interaction might play a role in promoting the TFs enforcement leading to plant defense responses. However, a functional study of Prosys-WRKY TF 43 interaction is needed to shed light on its possible role in tomato defense.

Other experiments are also needed to clarify the proteins that may be molecular partners of Prosys as for most of the PPIs identified *in silico* and *in vitro* no match was found.

BiFC experiments confirmed two Prosys interactors predicted by bioinformatic tools: MYB transcription factor (SlMYB14, Solyc06g053610) and MAP Kinase (MAPK6, Solyc05g049970). MYB genes are widely distributed in higher plants and represent one of the largest transcription factor's family, which are characterized by the presence of a highly conserved MYB domain at their N-termini. MYB proteins are involved in various developmental and physiological processes, including participation in defense responses to biotic and abiotic stresses, hormone synthesis and signal transduction (Dubos *et al*., 2010; Zhao *et al*., 2014). SlMYB14 functions as a JA-responsive TF gene which plays positive roles in flavonoids accumulation and oxidative stress tolerance (Li *et al*., 2021). Flavonoids are secondary metabolites that might act as phytoalexins, compounds released by plants in response to pests and pathogens, to ward off disease and disease-causing agents (Sugiyama and Yazaki, 2014). Oxidative

stress is a component of many stress conditions. During conditions, levels of reactive oxygen species (ROS) increase, potentially resulting in oxidations of DNA, proteins, and lipids. At the same time, ROS have additional signaling roles in plant adaptation to the stress (Voothuluru *et al*., 2013). However, plants are able to reduce ROS accumulation altering the expression of ROS scavenging enzymes such as catalases, Cu-Zn-superoxide dismutase, and peroxidases (Tyburski *et al*., 2009). Therefore, Prosys-MYB interaction might be responsible of the activation of transcription of JA-responsive defense-genes, reduction of ROS accumulation and promotion of flavonoid biosynthesis.

MAP kinases are the component of kinase modules that plays a crucial role in eukaryotic systems often linking perception of external stimuli with changes in cellular organization or gene expression. A surprisingly large number of genes encoding MAPK pathway components have been uncovered in genomes of model plants highlighting their significant role in signal transduction (Hardie, 1999). Recent investigations have confirmed major roles of defined MAPK pathways in development, cell proliferation and hormone physiology, as well as in biotic and abiotic stress signaling (Bigeard and Hirt, 2018). The members of this gene family participate to a complex network for efficient transmission of specific stimuli (Mishra *et al*., 2006). This function directs a cascade of phosphorylations, where MAP kinase (MAPK) is phosphorylated and activated by MAPK kinase (MAPKK), which itself is activated by MAPKK kinase (MAPKKK) (Nakagami *et al*., 2005). In response to stresses, MAPK signaling cascade regulate growth of plants by transcriptional and post-transcriptional regulation such as protein–protein interactions. (Lee *et al*., 2008). MAPK6, localizes to the cytosol and/or nucleus and is associated with intracellular signal transduction and regulation of gene expression (Eulgem and Somssich, 2007). Prosys-MAPK6 interaction could cooperate with other kinases to defense signal transmission as also suggested by the presence of several kinases in the Prosys-subnetwork obtained by bioinformatic approaches. This interaction could be one of the earliest actors in defense signal transmission with the consequent activation of phytohormone

biosynthetic pathways and the successive transcription of hormone-activated defense genes.

The last Prosys interactors detected with BiFC system was the ATP-dependent clp protease ATP-binding subunit (Solyc12g042060). Plant cytoplasmic and intra-plastid proteases have a housekeeping role in plants, releasing amino acids for recycling and eliminating non-functional proteins but have also important roles in plant defense, acting in pathogen and pest recognition and in induction of defense responses (Van der Hoorn and Jones, 2004). Studies involving several plant species described the roles of various proteases in plant defense; for example, in tomatoes, a serine carboxypeptidase is induced by wounding, systemin, and methyl jasmonate treatment (Moura *et al*., 2001), while subtilisin are involved in plant defense against herbivores in tomato and tobacco (Tornero *et al*., 1996; Horn *et al*., 2005). Similarly, non-serine proteases are involved in resistance: Mir1-CP, a cysteine protease identified in maize S. frugiperda-resistant lines, is rapidly induced when plants are injured (Pechan *et al*., 2002; Pechan *et al*., 2000). Moreover, leucine aminopeptidase A, a late wound-response gene of tomato, accumulates after mechanical, insect, and pathogen wounding (Pautot *et al*., 2001; Fowler *et al*., 2009). In this scenario the found interaction between Prosys and the ATP-dependent clp protease ATP-binding has an important value as a candidate enhancer of tomato defense responses.The results obtained push us in the direction of deepening the topic of interactions. The goals in the near future concern the study and validation of new interactions that may give new information on the mechanisms that regulate defense systems. New information also raises many questions, as in the case of interactors identified in different cellular compartments with respect to the protein of interest.

3.4 CONCLUSION

PPIs had and have a strong impact on molecular studies because affect almost all metabolic processes and pathways. *In vitro* and *in vivo* PPIs studies, over the years, offered new solutions and perspectives, in biological mechanisms including plant defense increasing biological knowledge of tools used by living organism such

as plants. Prosys interactors, captured by AP/MS and visualized through BiFC, enlarged the knowledge related to Prosys involvement in tomato defense responses, confirming some proteins predicted *in silico*. The results shown in this chapter clearly demonstrated that Prosys related defense mechanism is very complex, conferming that a very high number of proteins may be involved in defense tools of tomato plants. PPIs identified in this chapetr represent the base for a future fuctional study of candidate interactors.

3.5 MATERIALS AND METHODS

3.5.1 PROSYSTEMIN CLONING FOR AP-MS

The strategy used for the AP-MS sample preparation were published in a protocol by Zhang and collaborators in 2019. The gene cloning protocol used (Gateway® pDONR™ Vectors from Invitrogen) provided a two-step polymerase chain reaction (PCR) to clone the genes of interest and link them to the donor vector using the Gateway® BP reaction enzyme. Prosys gene was amplified, from pMZ vector (Rocco *et al.*, 2008) (fig. 3.7) contains the full length Prosystemin gene, using specific extended primers containing the attB1 and attB2 site (table 3.2).

Table 3.2. List of primers used for Prosys cloning (in *Italic* the attb adapter sequence), the attb extension adapter primer and the sequencing primer (M13 Fw/Rv).

Primer name	Sequence
ProsysFw	*AAAAAAGCAGGCTCCACC*ATGGGAACTCCTTCATATGATATC
ProsysRv	*CAAGAAAGCTGGGTC*ATAGCCGAGTTTATTATTGTCTGTTTGCAT
attB1 adapter	5-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACC-3
attB2 adapter	5-GGGGACCACTTTGTACAAGAAAGCTGGGTCATAGCC-3
M13 Fw	GTAAAACGACGGCCAG
M13 Rv	CAGGAAACAGCTATGAC

Prosys was amplified by PCR in a final volume of 20µl containing 0.02 U/µl of Phusion DNA polymerase (ThermoFisher®), Phusion HF Buffer (ThermoFisher®) in a final concentration 1X, dNTP in a final concentration 200 µM, forward and reverse primers in a final concentration of 0.2 µM. The first-step PCR was run in a thermal cycler with initial denaturation of 30 sec at 98°C, then denaturation 98°C for 15 sec, annealing 30 sec 60°C; extension 1 min/kb 72°C, final extension at 72°C for 5 min.10 µl of the PCR run previously was transferred in a second PCR reaction of 40 µl and used as template. The 40 µl mixture contained 0.1 µM of each the attB1 and attB2 adapter primers, to extend the attB sequences for Gateway® system; Phusion HF buffer in a final concentration 1X, dNTPs in a final concentration of 200 µM, Phusion DNA polymerase 0.02 U/µl. The clone amplified, was first run on 1% agarose gel, and then purified using Kit for Nucleic acid gel extraction and purification from Qiagen.

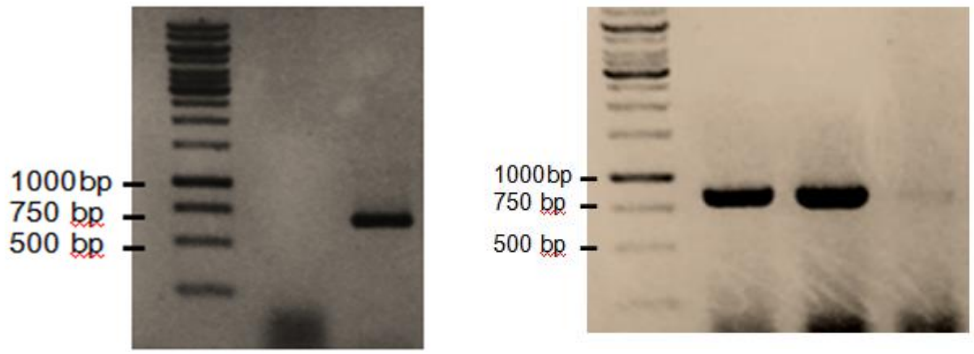


Figure 3.7. Prosys cDNA amplification from pMZ vector. Lane 1 DNA Ladder; Lane 2 negative control; Lane 3 Prosys cDNA.

3.5.2 CREATION OF GATEWAY ENTRY CLONE

The fragment purified was then used for BP reaction between the attB-flanked DNA and attP-containing donor vector (pDONR221) (fig. 3.8), to generate an entry clone. BP reaction were performed in a 1.5 ml tube where was added 15-150 ng of PCR products with attB sites, 150 ng of pDONR™ vector and TE buffer (10mM Tris HCl, 1mM EDTA, pH 8.0) to a final volumeof 8 µl. After mixing, 2µl of Gateway®BP Clonase® were added to the mix. The reaction was

mixed and incubated at 25°C overnight, to increase the efficiency. Subsequently, 1µl of Proteinase K (2 µg/µl) was added and samples incubated at 37°C for 10 min to stop the reaction.

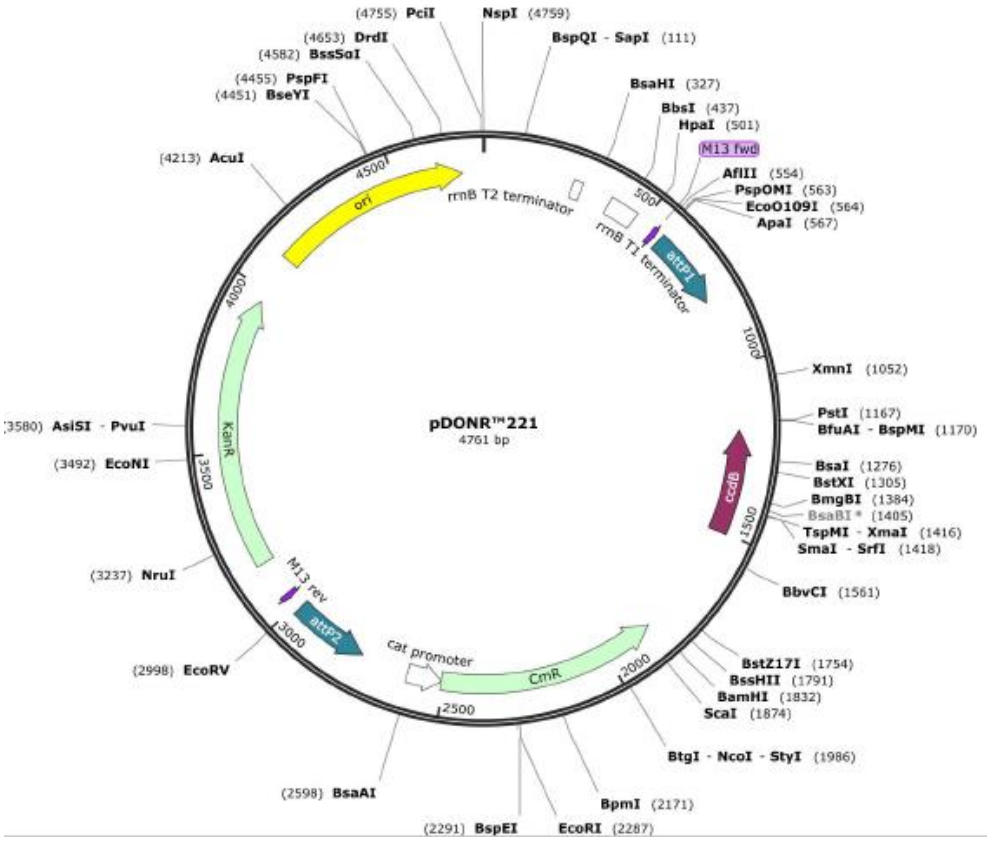


Figure 3.8. Map of the vector pDONR221 used as an entry clone for BP reaction.

3.5.3 *E.COLI* TRANSFORMATION

50 µl of chemically competent cells (DH5α or TOP 10) was thawed, on ice, and mixed gently with 5 µl of BP reaction and incubated on ice for 30 min. The cells have been subjected at heat-shock for 45 sec at 42°C, then again on ice for 2 min; 1 ml of S.O.C. medium was added, and the tube mixed at 850 rpm at 37°C for 1 hour. The cells were then precipitated with ultra-centrifuge at 14000 rpm, suspended and plated on selective plate with kanamycin (50 µg/ml) and incubated overnight at 37°C. The colonies were checked with PCR using the *Prosys* primers. The positive one was growing up overnight in LB broth media, with Kanamycin (50 µg/ml).

3.5.4 PLASMID EXTRACTION AND SEQUENCING

The recombinant plasmid was extracted using Plasmid Extraction Kit by QIAGEN and analysed with NanoDrop™ One (Thermo Scientific™) for the quantification and contaminant identification. 1 µg of the plasmid was sequenced by vector specific primers M13. The entry clone generated, with Prosys full length CDS, was then used for LR reaction to create the destination vector.

3.5.5 LR REACTION: CREATION OF GATEWAY EXPRESSION CLONE

The LR reaction was performed between attL-flanked DNA and attR-containing donor vector, to generate an expression vector. LR reactions were performed using 150 ng of donor vector, 150 ng of destination vector and TE buffer (10mM Tris HCl, 1mM EDTA, pH 8.0) to a final volume of 8µl. After mixing, 2µl of Gateway® LR Clonase® were added to the reaction and incubated overnight at 25°C. To terminate the reaction, 1µl of Proteinase K was added and samples were incubated at 37°C for 10 min. The destination vectors used (150 ng/µl) were pET301-mCherry-HisTag and pET300-HisTag-mCherry. These vectors allowed the expression of the recombinant protein with a six histidine N-terminal tag [(His)6-tag] and mCherry fluorescent protein in a different position as showed in the figure 3.9 and 3.10 (pET301-mCherry-Prosys-HisTag and pET300-HisTag-Prosys-

mCherry). The vectors generated were used for the transformation of *E. coli* competent cells. A single colony, checked by PCR, was grew up overnight in LB broth with Ampicillin (100 µg/ml) and the plasmid extracted with the same protocol showed before).

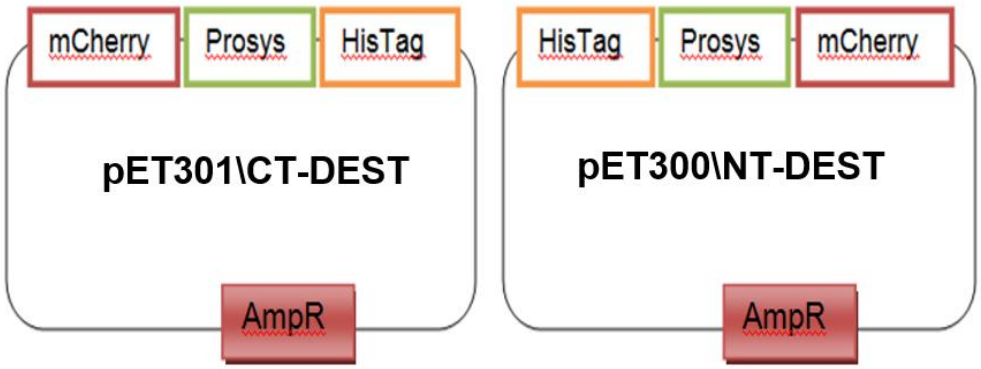


Figure 3.9. Simplified schematic cassette of pET301 and pET300 vectors generated with LR reaction.

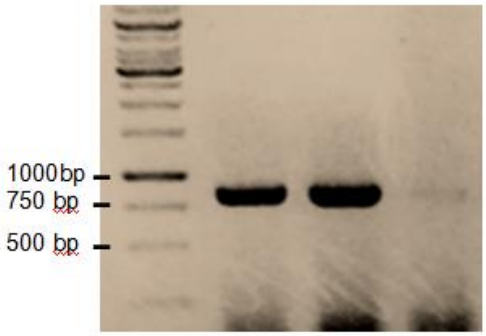


Figure. 3.10. Amplification of pET300/301 vectors with AttL1-2 adapter primer.Lane 1: DNA Ladder; Lane 2: mCherry-Prosys-HisTag (amplified with Prosys Fw-AttL2 Rv); Lane 3: HisTag-Prosys-mCherry (amplified with AttL1 Fw-ProsysRv); Lane 4: negative control.

The plasmid obtained was used to transform Rosetta™ competent cells, a specific strain used to enhance the expression of eukaryotic proteins that contains codons rarely used in *E. coli*. The positive colonies, selected by PCR, were then grown up in LB medium with Ampicillin (100 µg/ml) (Plasmid resistance) and Chloramphenicol (25 µg/mL) (strain resistance). 100 µM of Isopropyl β-d-1-

thiogalactopyranoside (IPTG) has been added to the liquid culture to trigger the transcription of *lac* operone and then the protein of interest (Jobe and Bourgeois, 1972). The colture was monitored at several time-point to obtain the maximum yield of protein for the extraction (fig 3.11).

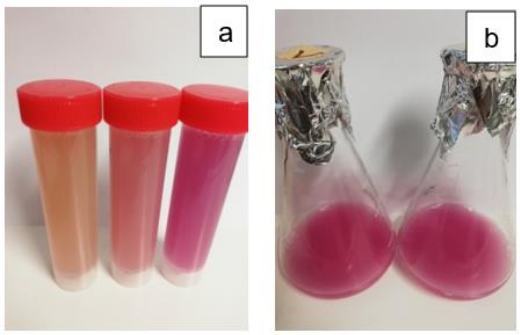


Figure 3.11 a-b. The pictures show the different concentration of the protein: a) 4-8-12 hours after IPTG addition (100nm/l); b) the protein expression in 200 ml flasks before the extraction.

3.5.6 PROTEIN EXTRACTION

The total protein was extracted from cells using Ultra Sonication and a lysis buffer containing Phosphate-Buffered Saline (PBS, containing monobasic potassium phosphate, sodium chloride, and dibasic sodium phosphate), 20 mM pH 7.4, NaCl 20 mM, 5% glycerol and 20 mM Imidazole and Phenylmethylsulfonyl fluoride (PMSF) 0.1M. The lysis buffer was added into the tube to resuspend the pelleted cells and cooling immediately on ice until the solution became homogeneous. The sonication was performed for 30 sec, five times at power 30 KHz. The solution was then centrifuge for 10 min at 4°C to separate the protein from the cellular component; then the supernatant recovered for mCherry Pull-down.

3.5.7 EXTRACTION OF TOTAL PROTEIN FROM WOUNDED LEAVES

To perform the AP-MS and to analyse the protein complexes with recombinant Prosys, generated before, the total proteins from wounded leaves were extracted. Three weeks old tomato plants (Cv

Money-maker) were wounded on the upper side of leaves, to simulate stress condition, and harvested after 9 hours (according with the timing of tomato defense responses published in literature). The materials were immediately frozen in liquid nitrogen, powdered using quartz beads and then stored at -80°C. To extract the total protein 1 g of cell powder and 1 ml of extraction buffer was used, composed by Tris HCl pH 7.5, 25 mM, MgCl2 15mM, EGTA 5mM, DTT 1 mM, PMSF 1 mM, NaCl 150 mM, sterile distilled water up to final volume. The cell powder was then vortexed for 15 sec and immediately cooled on ice until became homogeneous. Then the tubes were centrifuged at 4°C, 3000 g for 10 min, the supernatant separated from the pellet in a new clean tube and the centrifuge repeated at 16000 g, 4°C for 15 min, to eliminate as much as possible leaves material. The supernatant was recovered in a new clean tube for the pull-down.

3.5.8 MCHERRY PULL-DOWN

30 µl of GFP-Trap® (Chromotek) nanobody beads were washed with 500 µl extraction buffer three times in 2 ml tubes and centrifuged at 4500 rpm for 1 min. The total proteins extracted from leaves and the mCherry-Prosys complex were merged in a 1.5 ml tubes with GFP-Trap® and mixed gently at 4°C for 1 hour, to permit the formation of protein complexes. The tubes were centrifuged to precipitate the beads coupled with the protein complexes and the supernatant was eliminated. The beads were recovered using cut-off pipet tip and placed in a spin column, centrifuged 3000 g at 4°C. The spin columns were washed with 500 µl of Wash Buffer I (Tris HCl pH 7.5, 25 mM, MgCl2 15mM, EGTA 5mM, DTT 1 mM, PMSF 1 mM, NaCl 150 mM, sterile H2O up to final volume), Wash buffer II (Tris HCl pH 7.5, 25 mM, MgCl2 15mM, EGTA 5mM, DTT 1 mM, PMSF 1 mM, NaCl 250 mM, sterile H2O up to final volume) and Wash buffer III (Tris HCl pH 7.5, 25 mM, MgCl2 15mM, EGTA 5mM, DTT 1 mM, PMSF 1 mM, NaCl 500 mM, sterile H2O up to final volume) for 3 times each centrifuged at 3000 g for 1 min. The samples were ready for on-beads enzymatic digestion.

3.5.9 ON-BEADS TRYPSIN/LysC IN-SOLUTION DIGESTION AND C18 COLUMN PEPTIDE DESALTING AND CONCENTRATION

The samples were dissolved in a small volume of 6 M urea/2 M thiourea pH 8, then was added 1 µl trypsin/LysC (0.4 µg/µl) and incubated overnight at 37 °C, after the digestion the samples were desalting directly. C18 Stage-SepPak® columns were used for peptide desalting and concentration, coupled with the Visiprep™ 12-Port Vacuum Manifolds and the vacuum pump. The C18 SepPak columns were equilibrated in sequence, with the pump switched on, using 1 ml 100% methanol, 1 ml 80% acetonitrile/0.1% TFA (trifluoroacetic acid) in distilled deionized water, 1 ml of 0.1% TFA in distilled deionized water (two times). The samples were dissolved in 0.1% TFA (add 1/10 volume of 2% TFA to reach pH 2.0).
The samples were loaded onto the SepPak® columns, and the pump switched on; the tube washed with 200 µl of 0.1% TFA that contained the digested sample, centrifuged 1 min at 1000 g, and load this onto the column. The columns were washed with 1 ml of 0.1% TFA two times and then the peptides eluted with 800 µl of 60% acetonitrile and 0.1% TFA into a new 1.5 ml microcentrifuge tube.
The peptides were dried in a SpeedVac™ evaporator. The peptides were resuspended with a final volume of 40 µl of resuspension solution (0.2% TFA/5% acetonitrile) and transferred it to a microtiter plate to perform mass spectrometric analysis. For this step, a Nano LC 1000 liquid chromatograph with a reversed-phase C18 column was used (Acclaim PepMap RSLC, 75 µm × 150 mm, C18, 2 µm, 100 A°).

3.5.10 DATA ANALYSIS AND QUALITY CONTROL

LC-MS/MS analysis was performed on Q Exactive Plus (Thermo Fisher Scientific). Quantitative analysis of MS/MS measurements was performed with the Progenesis QI software (Non-linear Dynamics, Newcastle, UK). Proteins were identified from spectra using Mascot (Matrix Science, London, UK).Mascot search parameters were set as follows: TAIR10 protein annotation, requirement for tryptic ends, one missed cleavage allowed; fixed modification: carbamidomethylation (cysteine); variable modification: oxidation (methionine), peptide mass tolerance=±10 p.p.m., MS/MS tolerance=±0.6 Da, allowed peptide charges of +2 and +3. A decoy database search was used to limit

false discovery rates to 1% on the protein level. Peptide identifications below rank one or with a Mascot ion score below 25 were excluded. Mascot results were imported into Progenesis QI, quantitative peak area information extracted, and the results exported for data plotting and statistical analysis. For each protein, the corresponding identifier Solyc and accession number were obtained consulting Uniprot database (www.uniprot.org), classified in terms of GO categories (www.geneontology.org) and consulting KEGG pathaway (www.genome.jp). The ribosome protein and translation-related protein were deleted at this step. The normalized signal intensities were processed to determine fold-change abundance (FC-A) scores by use of the SAINT algorithm embedded within the CRAPome software (Mellacheruvu *et al*., 2013; Choi *et al.*, 2011; Choi *et al.*, 2012). Compared with the GFP control, the background proteins were deleted at this step by FC-A values of at least four within at least three replicates (Morris *et al*., 2014). Compared with intensity of bait, only the proteins for which the intensity score was more than 2%, corresponding to FC-A values of at least four within at least three replicates, should be regarded as positive interactions. Statistical analysis for this dataset was performed via the use of student T-test.

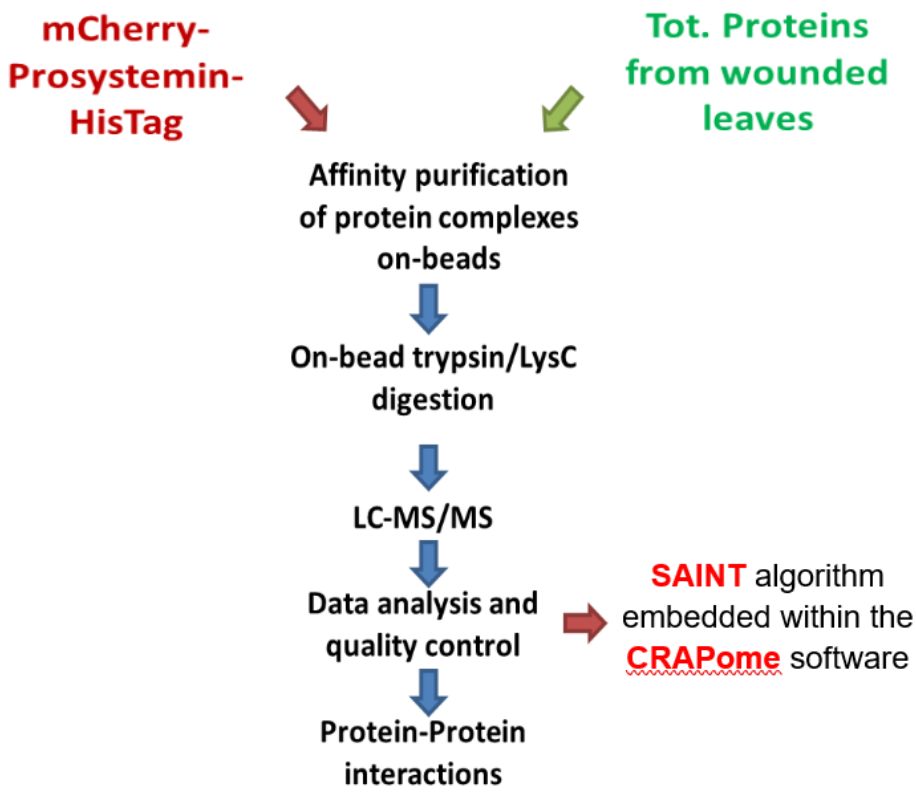


Figure 3.12. Schematic representation of the procedure performed for AP-MS analysis, following the protocol published by Zhang and co-workers in 2019.

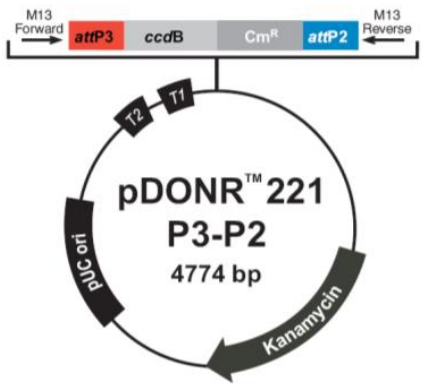
3.5.11 BIFC 2IN1 SYSTEM: CREATION OF GATEWAY ENTRY CLONE AND EXPRESSION CLONE

For this technique was followed the protocol published by Mehlhorn and collaborators in 2018 and the instruction from Multisite Gateway® Pro Manual (Thermo Fisher Life Technologies: www.thermofisher.com). The gene of interest (GOI) were amplified using the specific primer for the full CDS from tomato cDNA, with flanked sequences for the B1 and B4 regions, B3 and B2 (*Italic* style in the table 3.3); the PCR product purified from the agarose gel as showed before (paragraph 3.4.1), then was performed the BP reaction with pDONR B1-B4 and pDONR B3-B2 to create the entry vector. The procedure performed for *E. coli* transformation, vector extraction and sequencing are similar as showed in paragraph 3.5.3 and 3.5.4.

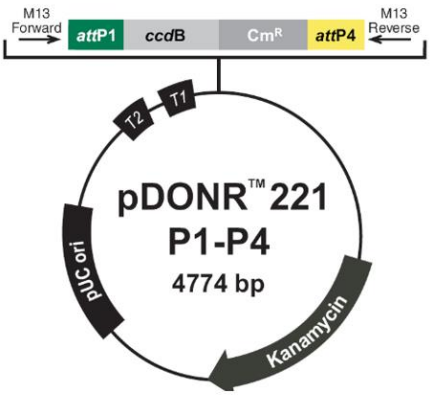
Table 3.3. List of primers used for cloning in BiFC 2in1 system.

Primer names	Sequence
ProsystemiFw B1	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTA*ATGGGAACTCCTTCATATGATATC
ProsystemiRv B4	*GGGGACAACTTTGTATAGAAAAGTTGGGTG*GAGTTTATTATTGTCTGTTTGCAT
EF1αFw B3	*GGGGACAACTTTGTATAATAAAGTTGGA*ATGGGTAAGGAAAAGATTCAC
EF 1αRv B2	*GGGGACCACTTTGTACAAGAAAGCTGGGTG*CTTCCCCTTCTTCTGGGCAGC
NAD-dependent epim\dehydFw B3	*GGGGACAACTTTGTATAATAAAGTTGGA*ATGGCTACTCTTGCTTCTTC
NAD-dependent epim\dehydRv B2	*GGGGACCACTTTGTACAAGAAAGCTGGGTG*GCACTTTCAGGCTTTCCAGA
ATP-dependent clp proteaseFw B3	*GGGGACAACTTTGTATAATAAAGTTGGA*ATGCAGTCAACAAGCATCCCATCG
ATP-dependent clp proteaseRv B2	*GGGGACCACTTTGTACAAGAAAGCTGGGTG*AAAATCCAACTTCCCACAAAAGCA
MAP kinaseFwB3	*GGGGACAACTTTGTATAATAAAGTTGGA*ATGAAGAAAGGATCTTTTGCACC
MAP kinaseRvB2	*GGGGACCACTTTGTACAAGAAAGCTGGGTG*TAGCTCAGTAAGTGTTGCCAATGG
MYB-related proteinFw B3	*GGGGACAACTTTGTATAATAAAGTTGGA*ATGGGTAGAGCTCCTTGTTG
MYB-related proteinRv B2	*GGGGACCACTTTGTACAAGAAAGCTGGGTG*AAATTCTGGTAATTCTGGCA

LR reaction between the pDONR P1-P4 and pDONR P3-P2 (fig. 3.13 a,b) was performed to generate the expression clone with the destination vector pBiFCt-2in1-NN (figure 3.14 a) or pBiFCt-2in1-NC (figure 3.14 b). The difference among the two destination vectors is the position of the splitted fluorescent protein as shown in the figure 3.14 a-b. The vectors were expressed in *E. coli* and the positive colonies (checked by PCR) picked for growing in LB medium with the selective antibiotic and then the plasmid extracted as shown in paragraph 3.4.4. A simplified example of the cassette created is illustrated in figure 3.15. The expression vectors generated were then used to transform *A. tumefaciens* for the transient expression in tobacco leaves by agro-infiltration.

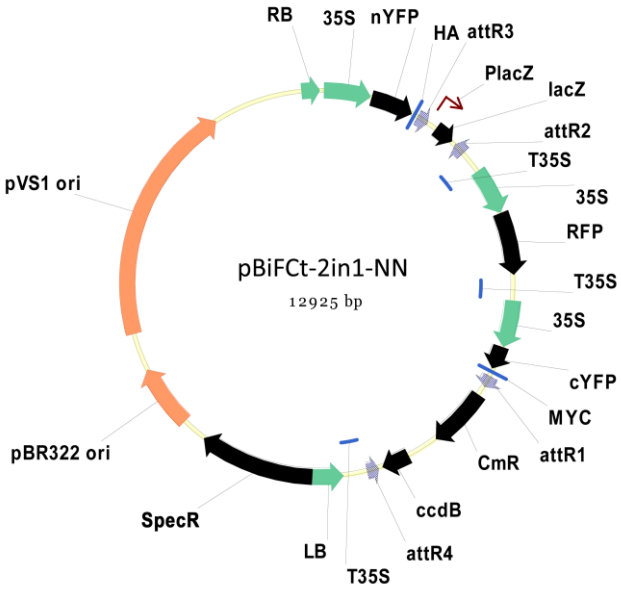


(a)

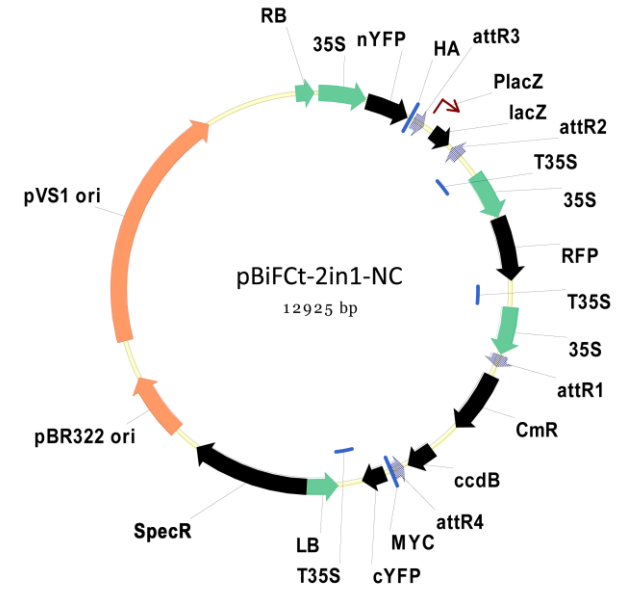


(b)

Figure 3.13 a-b. Schematic representation of pDONR-P1P4(a),and pDONR-P3P2(b).



(a)



(b)

Figure 3.14 a-b. Vector map of pBiFCt-2in1-NN (a) and pBiFCt-2in1-NC (b)

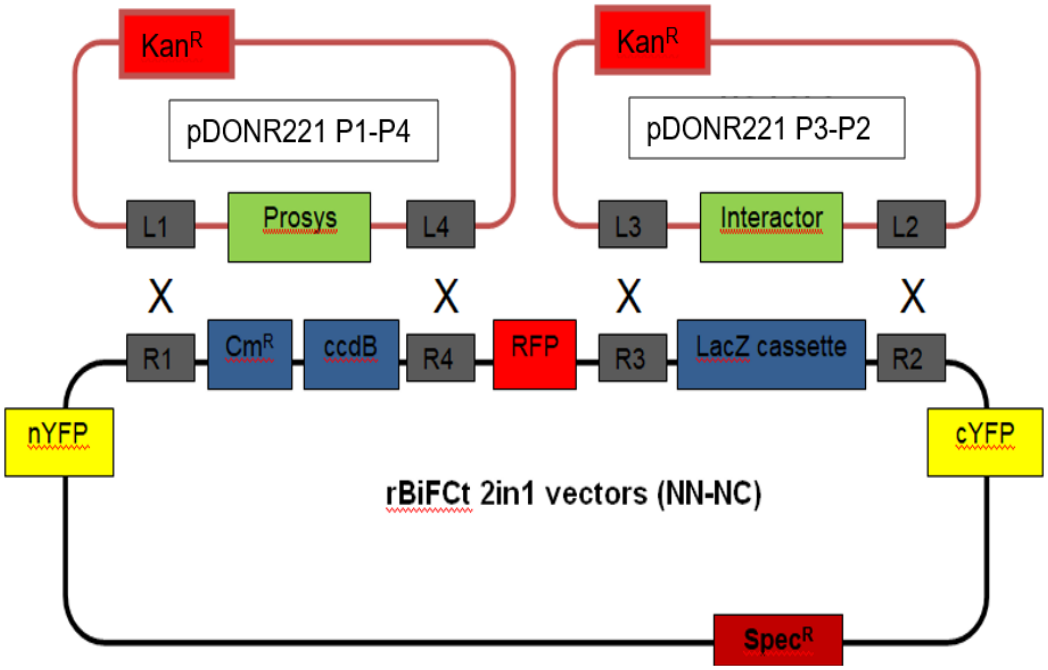


Figure 3.15. Vector maps illustrating the 2in1 concept with its recombination reactions between two entry vectors (pDONR) and an exemplary 2in1 destination vector carrying the two independent cloning cassettes.

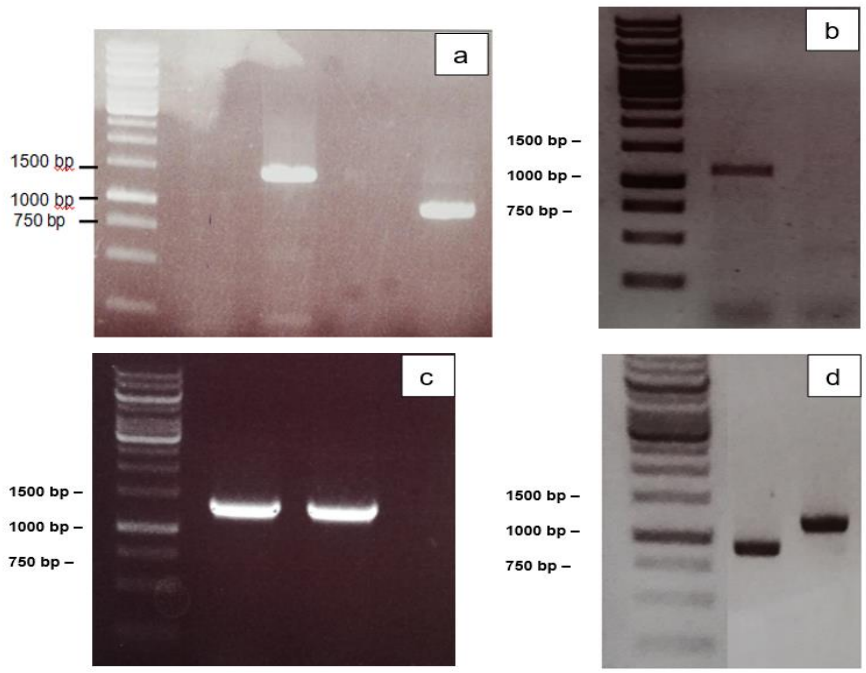


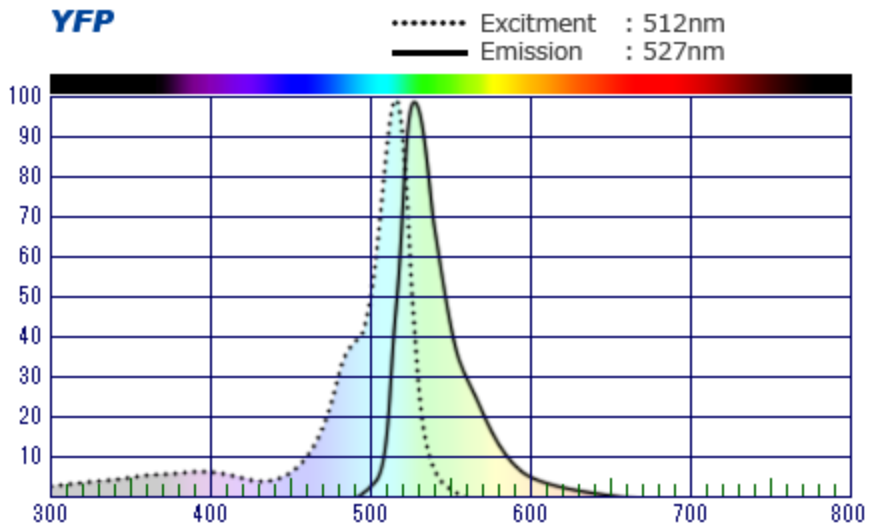
Figure 3.16 a-b-c-d. Genes amplification from tomato cDNA using specific primer for Gateway cloning system; a) lane 1: DNA Ladder, lane 2: negative control, lane 3: ATP-dependent clp protease, lane 4: negative control, lane:5 Prosys gene. b) lane 1: DNA Ladder, lane 2: NaDED, lane 3: negative control. c) lane 1: DNA Ladder, lane 2/3: EF1α, lane 4: negative control. d) lane 1: DNA Ladder, lane 2: MYB transcription factor, lane 3: MAPK6.

3.5.12 AGROBACTERIUM TUMEFACIENS TRANSFORMATION

In 20 ml Yeast Extract Beef (YEB) (1.0 g/l yeast extract, 5.0 g/l beef extract, 5.0 g/l peptone, 5.0 g/l sucrose) medium with carbenicillin (20 µg/ml) and rifampicin (50 µg/ml) were added 200 µl of *A. tumefaciens* AGL1, from the frozen stock, and the cultures incubated overnight with shaking at 28°C. 2 ml of the Agrobacterium overnight culture were added to a 2ml tube and centrifuge for 30 sec at 8000 g at 4°C. The supernatant was discarded and then 2 ml of ice-cold water was added, centrifuged for 30 sec at 14000rpm at 4°C. The supernatant was discarded, and this step repeated with 1 ml, 500 µl and 200 µl of ice-cold water. The last 200 µl were the *A*. *tumefaciens* competent cells. 5 µl of the expression clone DNA sample was added into a 2 ml tube and placed on ice, then 45 µl of Agrobacterium competent cell were added to the tube and incubated on ice 5 min. The solutions were placed into cold electroporation cuvettes and left on ice. The electric shock was performed by the application to the mixture of an electric potential of 1800 V. Following electroporation, 1 ml of YEB medium was added directly to the cuvette, then the solution transferred back into a new 2 ml tube for shaking one to two hours at 28°C. The tubes were microcentrifuge for 1 min at 14000 rpm, discarded the supernatant, and resuspended the pellet by pipetting up and down. The bacteria plated on pre-warmed YEB plates with 20 µg/ml carbenicillin and 50 µg/ml rifampicin, and the appropriate antibiotic for specific selection of vector containing the GOI and incubated at 28°C for two to three days.

3.5.13 *N. BENTHAMIANA* AGRO-INFILTRATION AND IMAGE VISUALIZATION

A single colony of *A. tumefaciens* was scratched and suspended in 500µl washing solution (10mM Magnesium Chloride, 100µM Acetosyringone). The solution was diluted in a final concentration to optical density at 600 nm of 0.5 in 2ml infiltration solution (¼ Murashige and Skoog pH=6.0, 1% Sucrose, 100µM Acetosyringone, 0.05% Silwet L-77 v/v 50µl/l).The additional of Silwet L-77 and keeping the plant at dark 24 hours greatly improve the efficiency of the transient expression (Zhang *et al.*, 2019). Four-week-old plants of *N. benthamiana* were infiltrated by using 1 ml plastic syringe; 300µl of bacterial suspension was infiltrated into young leaves in order to improve the efficiency. Infiltrated plants were left at dark for 24 hour and the left in greenhouse well-watered. Sample collection and observation was conducted after 2-3 days. The plants were checked for protein expression using a DM6000B/SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany), with an Argon (Ar) laser. BiFC fluorescence were imaged with an optimal excitation wavelength for Yellow Fluorescence Protein (YFP) in the range of 490–515nm; the maximal emission intensity is observed in the range of 520–560nm (figure 3.17 a). As for Red Fluorescence Protein, it possesses bright fluorescence with excitation/emission maxima at 555 and 584nm (figure 3.17 b).



(a)

(b)

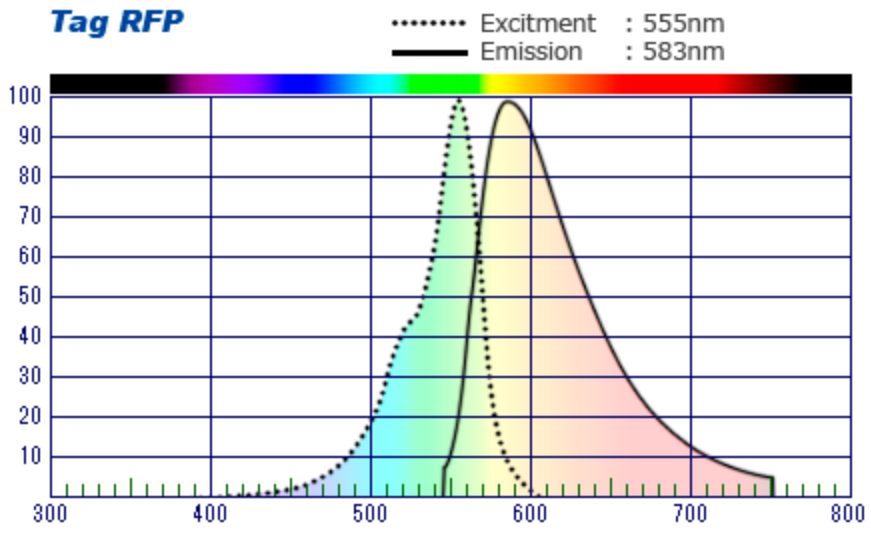


Figure 3.17 a-b. Absorption (dashed line) and emission (continuous line) spectrum of YFP (a) and RFP (b) proteins from Thermo Fischer® Scientific Spectra Viewer.

3.6 REFERENCES

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Final comments

The study of plant responses to biotic stressors has a double finality: to improve the knowledge of the molecular events that leads to defense mechanisms and to discover novel tools for crop protection thus contributing to sustainable agriculture. This is the case of the systemin peptide, discovered long time ago (Pearce *et al*., 1991) and known to be a key actor of tomato defense. Although several studies were performed on this peptide and its role in plant defense against insect herbivores, the only biotechnological approach used was the production of transgenic tomato plants constitutively expressing its precursor, Prosys. In this study we described a novel use of the Sys peptide for tomato crop protection based on its exogenous delivery to the plants. This strategy proved to be very effective in protecting treated plants against insect and fungi pests (Chapter 1). This, in our opinion, is an interesting result that suggest that Sys may be the core of a novel commercial formulate able to reduce the application of chemical pesticide, one of the most important challenge of modern agriculture.

Sys has been considered for long time the only part of Prosys harboring biological activity. However, a study published in 2016 by Corrado and coworkers demonstrated that the expression in tobacco plant of a mutated Prosys gene lacking the systemin coding region altered the proteomic profile of tobacco leaves and increased plant resistance against *B. cinerea*. There results suggested that the N-terminal part of the precursor is biologically active, or at least contain aminoacid stretches with biological activity. Subsequent studies have then demonstrated that Prosys is an intrinsically disordered protein (Buonanno *et al*., 2018) possibly interacting with several different molecular partners as expected by this type of proteins (Dunker *et al*., 2001). Protein-protein interactions have a pivotal role in many biological processes suggesting that targeting macromolecular complexes will open new avenues. The results shown in Chapter 2 and 3 confirm that the precursor may interact with multiple proteins uncovering new molecular events that may play important role in Prosys-dependent tomato defense such as the involvement in

carbohydrate metabolic biological processes, in adaptive plasticity of plants under stress and in the promotion of flavonoid biosynthesis. To improve the coverage of PPIs, a variety of computational methods have been developed to predict PPIs, that used several approaches. Consequently, helpful data resources are now available for plant scientists to better investigate the functional mechanisms of plant proteins (Yang *et al*., 2020 and reference therein). The use of several data resources allowed the construction of the Prosys sub-network shown in Chapter 2. Proteins are usually involved in interactions with an estimated average of 5-10 protein partners (Drews J., 2000) with overlapping or non overlapping binding site(s), displaying the complexity inidentifying, understanding, and predicting protein interaction networks. In addition, different types of protein complexes have been described, like homo- and hetero-complexes (i.e., the interaction between identical or non-identical chains), obligate and non-obligate complexes (transient or permanent) (Jones *et al*., 2000; Nooren and Thornton, 2003; Keskin *et al*., 2005). The results illustrated in Chapter 2 and 3 suggest that Prosys is involved in a much larger number of interactions possibly due to its ID structure and consequent biological function. The understanding of the functional role of the interacting complexes here shown will provide crucial insights into the Prosys-dependent defense mechanism.

Attachment

RSYS NETWORK VISUALIZATION

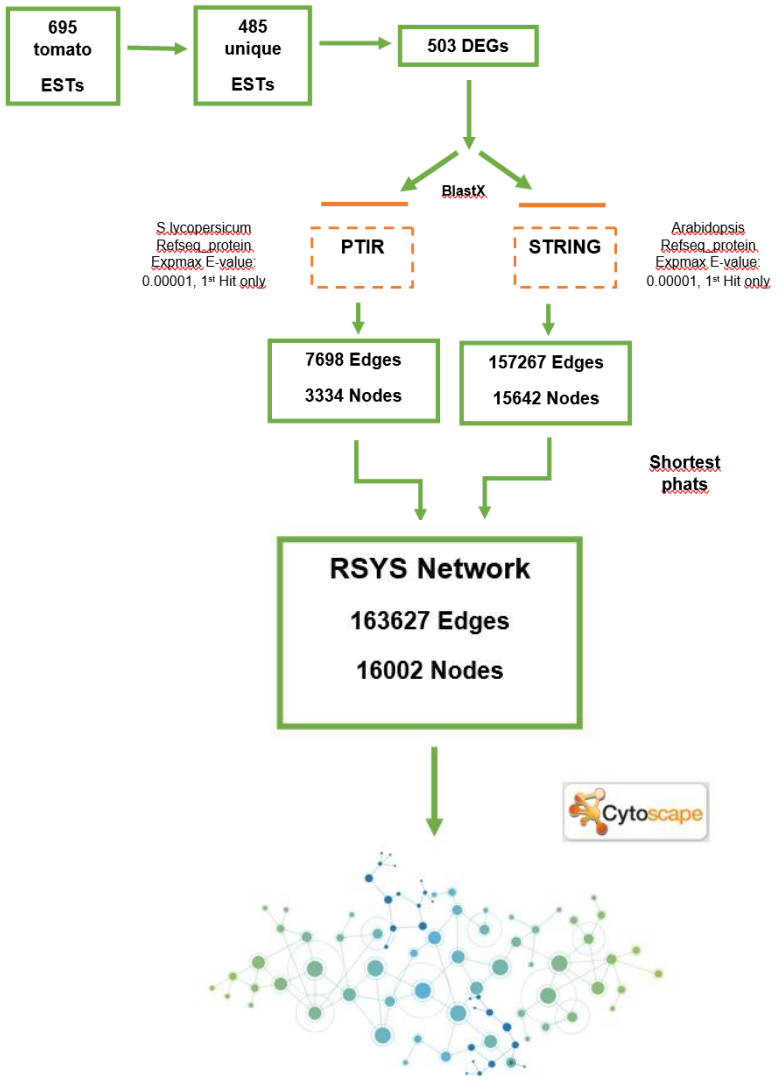


Figure 2.1. Schematic representation of the procedure used to produce the PPIs network in RSYS plants. Tomato EST were first converted in the corresponding DEGs. These DEGs were converted via BlastX and analysed via PTIR and STRING databases obtaining the RSYS network. Nodes: proteins; Edges: interactions.

The interactions obtained querying the available plant databases were imported in Cytoscape 3.8.2 (www.cytoscape.org). The data

interpretation through the two-dimensional visualization of nodes, representing proteins and arcs indicate the interactions. The interactions were loaded in Cytoscape and a one-way interpretation of the network was imposed, in order to ignore the directionality of the arcs. Two distinct networks were obtained, each relating to a database: STRING produced a network of 15.642 nodes while PTIR shows 3.334 nodes. The different result is justified by the size of each database: while STRING reports 34675 tomato proteins, PTIR presents 10626. In order to obtain a single network containing all the PPIs found, the Merge function of the Cytoscape software was used. The network obtained, after removing duplicates and self-loops, is made up of 16.002 proteins and 163.627 interactions. Of these proteins, 306 come from transcriptomic data (RSYS plants), while the rest were found from the tomato interactome. The network obtained includes all the proteins translated *in silico* starting from the DEGs modulated by the constitutive expression of Prosys, in addition to all the tomato proteins for which interactions were predicted. The network was very complex (figure 2.2) and difficult to handle; it was very hard to clearly distinguish the nodes with which Prosys established interactions. Then, a sub-network was extrapolated through the selection of the node corresponding to the Prosys protein (Solyc05g051750.2) and of all the interactions involved with this protein. This was possible using the feature included in the Cytoscape package as showed in the next paragraph.

ANALYSIS AND PROPERTIES OF THE PROTEIN-PROTEIN INTERACTING NETWORK

Cytoscape software allowed to calculate a series of parameters related to the topology of the network obtained, and to perform an evaluation to understand the biological models represented. Nodes and edges, representing proteins and interactions respectively, were represented in two-dimensional way, but a unidirectional interpretation of the network was set (figure 2.2) in order to ignore the directionality of the arcs. The parameters investigated are the connection degree distribution (figure 2.3a) that refers to the distribution of the average of the connection degrees in the network; the betweenness centrality

(figure 2.3b) which indicates the centrality of a node in the network and describes the betweenness distribution of all nodes with neighbours; the average of clustering coefficient distribution (figure 2.3c) that describes the distribution of clustering coefficient of all nodes with neighbours (*k*). The node degree distribution (figure 2.3a) reveals a “scale-free” network assigning a score for each protein (node). This means that there are many nodes with a low score value, with few interactions within the network, and nodes with a high score, so highly connected, in fact the parameter varies between 1 and 4.366. This means that different central nodes are present, and these are very relevant to maintain network structure. The maximum degree value found coincides only with the RNA polymerase enzyme (Solyc02g083350.2), which comes from the transcriptomic data in which it is up regulated by the constitutive expression of Prosys cDNA. The fact this enzyme has 4.366 interactions is likely the consequence of the plant needs to rearrange the transcriptome in response to the constitutive expression of the Prosys cDNA. A minimum value was found, however, for the transcription factor bHLH (Basic Helix-Loop-Helix, Solyc03g118310.2), which appears down-regulated in the transcriptomic data. For a node, having a high centrality value this implies that it is crossed by many short paths and become an obligatory passage between many nodes.

Analyzing the network, an attribute file was obtained from Ensembl Plant(http://plants.ensembl.org/index.html); this file contains for each node the localization, function, and biological process of involvement. Stylistic changes were made, with the Style function of the Cytoscape software, which allowed an easier interpretation of the protein network. In particular, the different colours of the nodes indicate the cellular localization of the proteins, while the size is an indication of their connection degree.

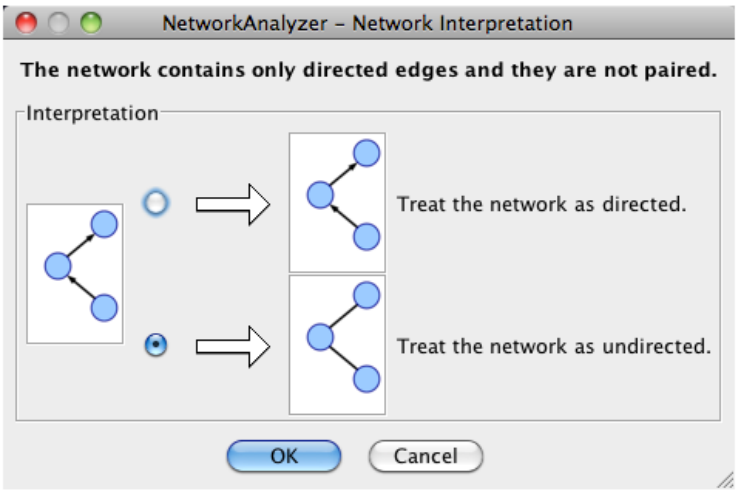
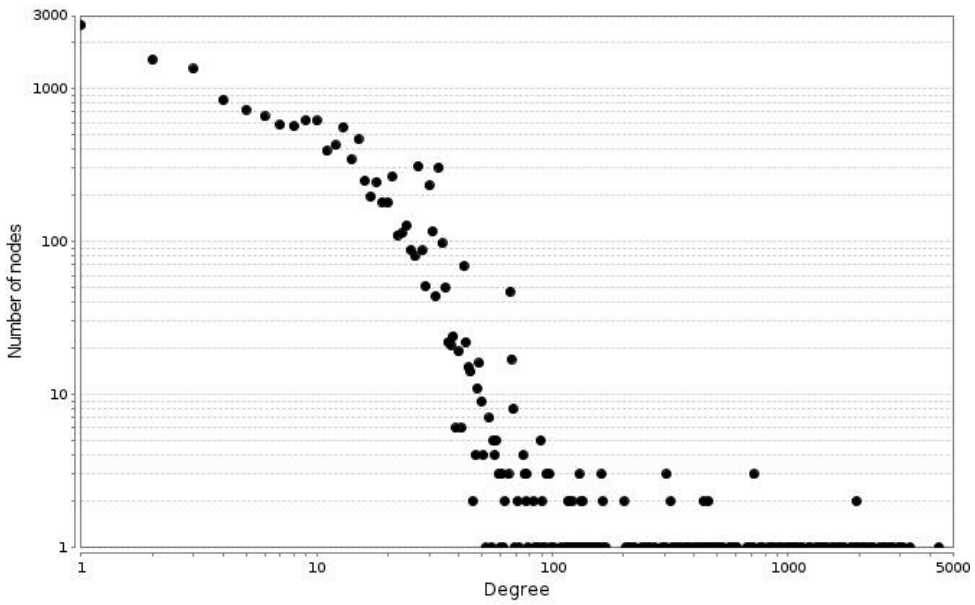
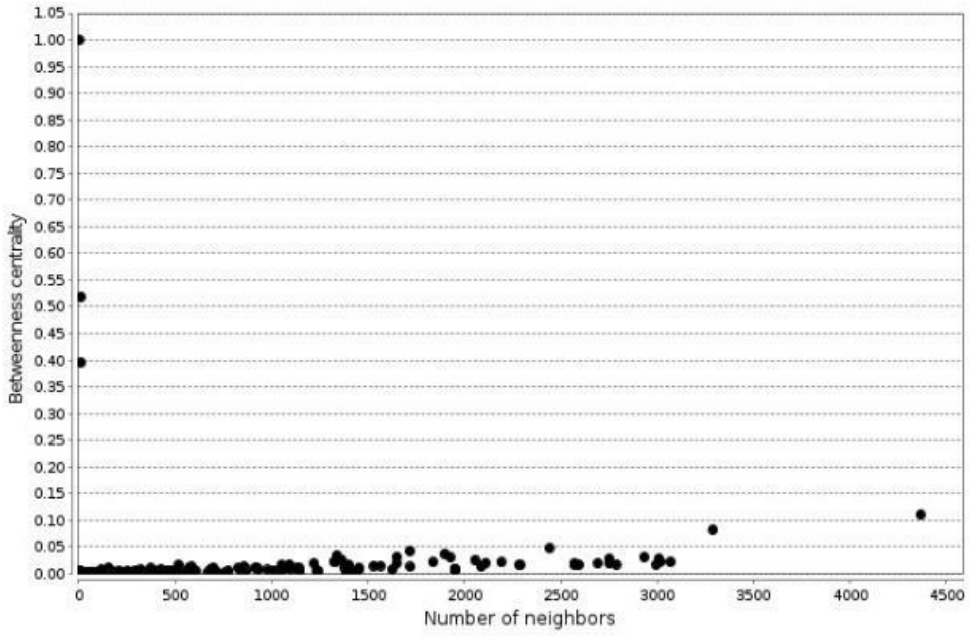


Figure 2.2. Graphical interface of Network Analyzer, bioinformatics tool included in the Cytoscape package, wich allows to set up a one-way interpretation of the network.

a)



b)



c)

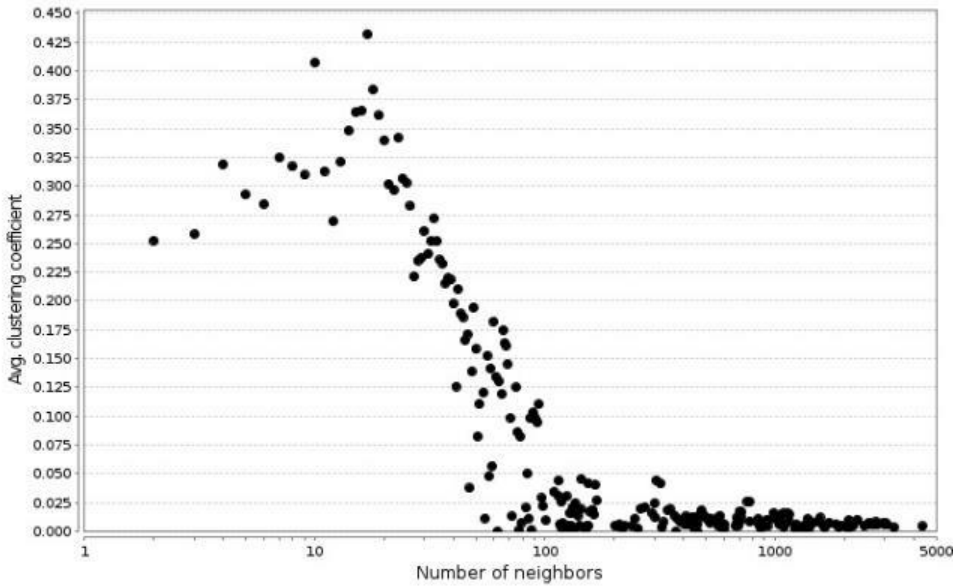


Figure 2.3 a,b,c. The image shows the network parameters analysis carried out in Cytoscape. a) Connection degree distribution; b) Betweenness centrality; c) Average Clustering Coefficient distribution.

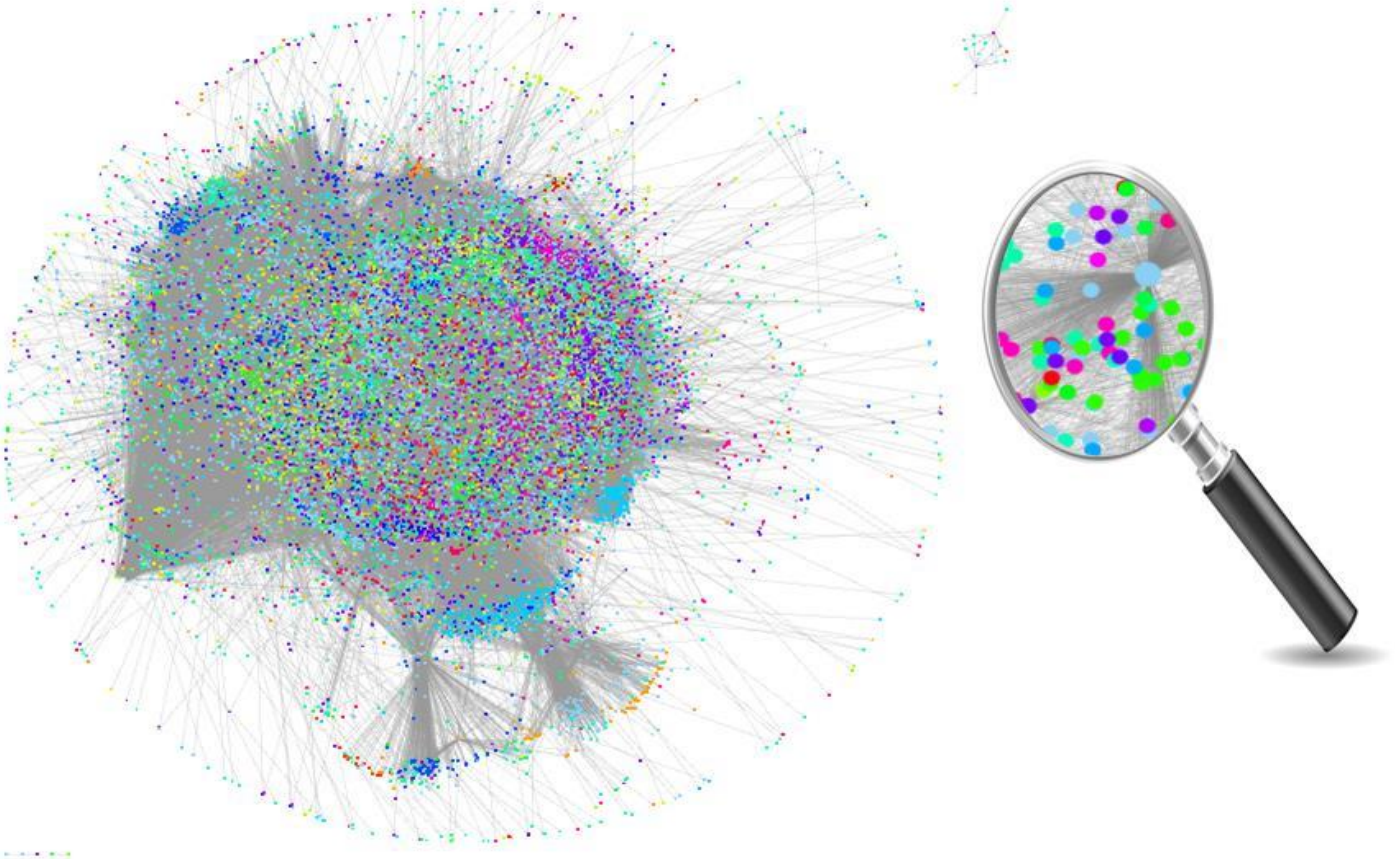


Figure 2.4. Network of PPIs in RSYS plants obtained with Cytoscape 3.6.0 software. The enlargementshows the different size and coloring of the nodes

TABLE A. List of Prosys sub-network interactors from *in silico* prediction. The table show the Solyc identifier for each protein (first column); STRING score* (second column); protein description (third column); function available for each protein (fourth column); sub-cellular localization (fifth column); biological process (sixth column). NA: not available.

*****In STRING, each protein-protein interaction is annotated with one or more 'scores'. These scores do not indicate the strength or the specificity of the interaction. Instead, they are indicators of confidence, i.e., how likely STRING judges an interaction to be true, given the available evidence. All scores rank from 0 to 1, with 1 being the highest possible confidence. A score of 0.5 would indicate that roughly every second interaction might be erroneous (i.e., a false positive).

Identifier	STRINGScore	Description	Localization	Biologicalprocess
Solyc01g099160	**0.412**	Lipoxygenase	NA	oxylipin biosynthetic process, oxidation reduction process, fatty acid biosynthetic process
Solyc01g097270	**0.252**	Chitinase	NA	defense response to fungus and to bacterium
Solyc01g099590	**0.169**	Glutathione-S-transferase	cytoplasm	glutathione metabolic process, toxin catabolic process
Solyc00g174340	**0.694**	Pathogenesis-relatedprotein 1b	NA	NA
Solyc01g009860	**0.165**	NAC domain transcriptionfactor	nucleus	regulation of transcription, DNA-templated
Solyc01g095080	**0.161**	1-aminocyclopropane-1-carboxylatesynthase	NA	ethylene biosynthetic process, fruit ripening, 1-aminocyclopropane-1-carboxylate biosynthetic process
Solyc01g101240	**0.469**	Asparticproteinase	NA	proteolysis, lipidmetabolicprocess

Solyc01g105310	**0.167**	Metacaspase	NA	NA
Solyc01g106620	**0.358**	Pathogenesis-relatedprotein 1a	extracellularregion	NA
Solyc01g111080	**0.250**	Gibberellin-regulatedprotein 2	NA	Metabolicprocess, biosyntheticprocess
Solyc01g111990	**0.163**	Alanyl-tRNAsynthetase	mitochondrion, cytoplasm, chloroplast, plastid	translation, tRNA modification, alanyltRNAaminoacylation
Solyc02g062970	**0.161**	Xaa-Pro aminopeptidase 2	NA	proteolysis
Solyc02g089620	**0.165**	Proline dehydrogenase	mitochondrion	oxidation-reduction process, proline catabolic process
Solyc02g076980	**0.252**	Cathepsin B-like cysteine proteinase	Extracellular space, lysosome	proteolysis
Solyc01g006540	**0.394**	Lipoxygenase	chloroplast	oxylipin biosynthetic process, oxidationreduction process, fatty acid biosynthetic process, green leaf volatile biosynthetic process
Solyc01g006560	**0.159**	Lipoxygenase	NA	oxylipin biosynthetic process, oxidationreduction process, fatty acid biosynthetic process
Solyc01g009230	**0.467**	Xanthinedehydrogenase /oxidase	cytosol	oxidation-reductionprocess
Solyc02g077880	**0.167**	Auxin-repressed protein	ribosome	translation
Solyc02g084850	**0.418**	Dehydrin	cytosol	response to stress, response to abscisic acid, cold acclimation, response to water, response to water deprivation

Solyc02g085640	**0.161**	Xaa-Pro aminopeptidase 1	NA	proteolysis
Solyc02g085730	**0.256**	Allene oxide cyclase	chloroplast	response to wounding, response to insect, response to salt stress, response to ethylene, auxin-activated signaling pathway, response to abscisic acid, response to salicylic acid, response to jasmonic acid, induced systemic resistance, response to hydrogen peroxide, defense response to fungus
Solyc02g093580	**0.701**	Pectatelyase	extracellula rregion	pectin catabolic process, response to nematode
Solyc03g044790	**0.254**	Alpha-hydroxynitrilelyase	NA	NA
Solyc03g079850	**0.256**	Guanylylcyclase	NA	proteolysis
Solyc03g083320	**0.159**	Calcineurin B-like calcium binding protein	NA	
Solyc03g097920	**0.303**	Mitogen-activated protein kinase kinase	cytoplasm	regulation of mitotic cell cycle, signal transduction by protein phosphorylation, stress-activated protein kinase signaling cascade
Solyc03g098050	**0.398**	Calmodulin 3 protein	NA	
Solyc03g098790	**0.700**	Kunitz-typeproteaseinhibitor	NA	negative regulation of endopeptidase activity
Solyc03g122340	**0.693**	Lipoxygenase	NA	oxylipin biosynthetic process, oxidationreduction process, fatty acid biosynthetic process
Solyc03g123800	**0.218**	Mitogen-activated protein kinase kinase	cytoplasm	regulation of mitotic cell cycle, signal transduction by protein phosphorylation, stress-activated

				protein kinase signaling cascade
Solyc04g009800	**0.161**	Calcium-dependentproteinkinase 2	cytoplasm, nucleus	peptidyl-serine phosphorylation, abscisic acid-activated signaling pathway, intracellular signal transduction
Solyc04g011500	**0.161**	Actin 4	NA	
Solyc04g051510	**0.702**	Receptor like kinase	plasma membrane, endosome	defense response, protein phosphorylation, brassinosteroid mediated signaling pathway, brassinosteroid homeostasis, negative regulation of cell death
Solyc04g054320	**0.165**	BZIP transcriptionfactor	NA	regulation of transcription, DNA-templated
Solyc04g054740	**0.159**	Inositol-3-phosphate synthase	cytoplasm	phospholipid biosynthetic process, inositol biosynthetic process
Solyc04g078110	**0.169**	Subtilisin-like protease	NA	proteolysis
Solyc05g007180	**0.256**	Homeobox-leucine zipper-like protein	nucleus	regulation of transcription, DNA-templated
Solyc05g007940	**0.159**	Ribonuclease T2	cytoplasm	nucleic acid phosphodiester bond hydrolysis, RNA phosphodiester bond hydrolysis, endonucleolytic
Solyc05g013320	**0.694**	Pto-like serine/threonine kinase	NA	proteinphosphorylation
Solyc05g049970	**0.161**	Mitogen-activatedproteinkinase 4	cytoplasm, nucleus	regulation of gene expression,MAPK cascade, phosphorylation,protein
Solyc05g051750		Prosystemin	cytoplasm	
Solyc05g052620	**0.511**	Coronatine-insensitive 1		response to wounding, response to insect, response to jasmonicacid, SCF-dependent proteasomal ubiquitin-dependent protein

				catabolic process, negative regulation of defenseresponse,defense, response to bacterium, defense response to fungus
Solyc06g005150	**0.394**	Ascorbateperoxidase	chloroplast	oxidation-reduction process, hydrogen peroxide catabolic process
Solyc06g005160	**0.252**	Ascorbateperoxidase	chloroplast	oxidation-reduction process, hydrogen peroxide catabolic process, response to reactive oxygen species
Solyc06g005170	**0.696**	Mitogen-activatedproteinkinase 3	cytoplasm, nucleus	regulation of gene expression, MAPK cascade, protein phosphorylation, response to cold, response to water deprivation, defense response to bacterium, defense response to fungus, positive regulation of response to salt stress
Solyc06g005500	**0.161**	ATP binding / serine threonine kinase	plasma membrane	transmembrane receptor protein serine/threonine kinase signaling pathway, cell surface receptor signaling pathway, phosphorylation
Solyc06g048410	**0.167**	Superoxide dismutase	plastid	oxidation-reduction process, removal of superoxide radicals, superoxide metabolic process
Solyc06g051400	**0.467**	Omega-3 fatty acid desaturase	integral component of membrane	lipid metabolic process, oxidation-reduction process
Solyc06g053610	**0.309**	Myb-related	nucleus cell	differentiation, regulation of

		transcription factor		transcription from RNA polymerase II promoter
Solyc06g065820	**0.159**	Ethylene-responsive transcription factor 7	nucleus	regulation of transcription, DNA-templated
Solyc06g068090	**0.167**	Phospholipase D	membrane	phosphatidylcholine metabolic process
Solyc06g068520	**0.699**	Hydroxyproline-rich systemin	extracellular region, intracellular	defense response
Solyc06g071810	**0.463**	Receptor like kinase	integral component of membrane	protein phosphorylation
Solyc06g076020	**0.165**	heat shock protein	NA	NA
Solyc06g076350	**0.169**	Transcription factor	nucleus	regulation of transcription, DNA-templated
Solyc07g006900	**0.696**	Auxin efflux carrier protein	endoplasmic reticulum, plasma membrane, auxin efflux carrier complex, lytic vacuole	homeostasis, auxin polar transport, auxin activated signaling pathway, response to ethylene, response to glucose, positive gravitropism
Solyc07g007250	**0.161**	Metallocarboxypeptidase inhibitor	NA	negative regulation of endopeptidase activity, negative regulation of catalytic activity
Solyc07g007870	**0.258**	NADH flavin oxidoreductase/12-oxophytodienoate	intracellular, peroxisome	oxidation-reduction process, oxylipin biosynthetic process, fatty acid metabolic process, jasmonic

		reductase		acid biosynthetic process
Solyc07g040690	**0.161**	NPR1 protein	nucleus, cytoplasm	defense response to bacterium, defense response to fungus, response to herbivore
Solyc07g049690	**0.470**	Cytochrome P450	chloroplast envelope, integral component of membrane, plastid outer membrane	oxylipin biosynthetic process, defense response, sterol metabolic process, oxidation-reduction process
Solyc08g014420	**0.699**	Mitogen-activated protein kinase 2	cytoplasm, nucleus	regulation of gene expression, MAPK cascade, protein phosphorylation
Solyc08g060970	**0.461**	Polygalacturonase	extracellular region	cell wall organization, carbohydrate metabolic process
Solyc08g074620	**0.167**	Polyphenol oxidase	thylakoid, plastid, chloroplast thylakoid lumen	oxidation-reduction process, pigment biosynthetic process
Solyc08g076930	**0.258**	Transcription factor	NA	NA
Solyc08g078390	**0.167**	Acyl-coenzyme A oxidase	peroxisome	oxidation-reduction process, fatty acid metabolic process, fatty acid betaoxidation, fatty acid beta-oxidation using acyl-CoA dehydrogenase

Solyc08g079430	**0.254**	Primary amine oxidase	membrane	secondary metabolite biosynthetic process, oxidation-reduction process
Solyc08g079840	**0.303**	Subtilisin-like protease	NA	proteolysis
Solyc08g079850	**0.161**	Subtilisin-like protease	NA	proteolysis
Solyc08g079860	**0.161**	Subtilisin-like protease	NA	proteolysis
Solyc08g079870	**0.219**	Subtilisin-like protease	NA	proteolysis
Solyc08g079880	**0.303**	Subtilisin-like protease	NA	proteolysis
Solyc08g080620	**0.211**	Osmotin-like protein	nucleus	cell differentiation, regulation of transcription from RNA polymerase II promoter
Solyc08g080640	**0.161**	Osmotin-like protein	cytoplasm	defense response, response to biotic stimulus, killing of cells of other organism, defense response to fungus
Solyc08g081690	**0.252**	Respiratory burst oxidase	integral component of membrane	oxidation-reduction process, cellular oxidant detoxification, response to ethylene, negative regulation of programmed cell death, hydrogen peroxide biosynthetic process, defense response by callose deposition
Solyc09g005080	**0.512**	LRR receptor-like serine/threonine-protein kinase	integral component of membrane	NA
Solyc09g005090	**0.159**	LRR receptor-like serine/threonine-protein kinase	integral component of membrane	NA

Solyc09g007010	**0.161**	Pathogenesis related protein PR-1	extracellular region	response to biotic stimulus, killing of cells of other organism, defense response to fungus
Solyc09g007020	**0.215**	Pathogenesis-related protein	extracellular region	NA
Solyc09g011920	**0.169**	Mitochondrial uncoupling protein	mitochondrialinner membrane, vacuolar membrane, plasmodesma, chloroplast	proton transport, mitochondrial transmembrane transport, photorespiration
Solyc09g065180	**0.252**	NAD-dependent epimerase/dehydratase	chloroplast, plastoglobule, apoplast	rRNA processing, response to cytokinin, plastid translation, positive regulation of translation, positive regulation of transcription, DNA-templated
Solyc09g075440	**0.252**	Ethylene receptor	intracellular, integral component of membrane	signal transduction by protein phosphorylation
Solyc09g084470	**0.691**	Proteinase inhibitor I	integral component of membrane	NA
Solyc09g091550	**0.159**	Salicylic acid carboxyl methyltransferase	integral component of membrane	NA
Solyc09g097770	**0.304**	Cell wall protein	integral	NA

			component of membrane	
Solyc10g047700	**0.161**	Receptor protein kinase-like protein	integral component of membrane	protein phosphorylation
Solyc10g081190	**0.167**	LRR	plasma membrane transmembr ane receptor	protein serine/threonine kinase signaling pathway, cell surface receptor signaling pathway, protein phosphorylation
Solyc10g086220	**0.692**	Flavin oxidoreductase/NADH oxidase	cytoplasm, intracellular	oxidation-reduction process, oxylipin biosynthetic process, fatty acid biosynthetic process
Solyc10g086500	**0.161**	3-oxo-5-alpha-steroid 4-dehydrogenase family protein expressed	cytoplasm, integral component of membrane	lipid metabolic process, brassinosteroid biosynthetic process, oxidation-reduction process, steroid metabolic process
Solyc11g011340	**0.159**	Alcohol dehydrogenase	NA	oxidation-reduction process
Solyc11g012710	**0.159**	5-AMP-activated protein kinase subunit beta-1	NA	cellular response to nitrogen levels, response to sucrose
Solyc11g069800	**0.165**	cytochrome P450	chloroplast, plastid	oxylipin biosynthetic process, defense response, sterol metabolic process, oxidation-reduction process, lipid metabolic process, jasmonic acid biosynthetic process
Solyc11g071810	**0.161**	CRABS CLAW	NA	multicellular organism development
Solyc12g005620	**0.307**	LRR receptor-like serine/threonine-protein	integral component	xylan catabolic process

		kinase	of membrane	
Solyc12g006570	**0.252**	Germacrene-D synthase	NA	metabolic process
Solyc12g009020	**0.459**	Protein kinase	cytoplasm	stress-activated protein kinase signaling cascade
Solyc12g010040	**0.514**	Leucyl aminopeptidase	cytoplasm, intracellular, plastid, chloroplast	proteolysis
Solyc12g011450	**0.169**	Chlorophyll a-b binding protein 13	chloroplast envelope, membrane, photosystem I, photosystem II, plastid, plastoglobule	response to light stimulus, proteinchromophore linkage, light harvesting, photosynthesis, light harvesting in photosystem I
Solyc12g019460	**0.258**	Mitogen-activated protein kinase 1	intracellular, cytoplasm, nucleus	regulation of gene expression, MAPK cascade protein phosphorylation
Solyc12g099120	**0.161**	MYB transcription factor	nucleus cell	differentiation, regulation of transcription from RNA polymerase II promoter

Table B. List of interactors with the highest FC-A score and p-value (<0,05).Inside the table are also listed the raw data referred to every single replicaanalysed by MS. The replicas 1-2-3 are referred to expression

cassette HisTag-Prosys-mCherry; the replicas 4-5-6mCherry-Prosys-HisTag. The T-test has been done comparing replicas 1-2-3 with replicas 4-5-6. Prosys protein is one of the most abundant, confirming the high level of expression by the vector.

Accession	Description	Replicates 1	Replicates 2	Replicates 3	Replicates 4	Replicates 5	Replicates 6	p-value	FC-A
K4C1K6Solyc05g051750	Prosystemin	2,4E+05	9,5E+05	1,9E+05	2,3E+07	3,1E+08	4,2E+08	**0.08**	3835,44
K4CUE5 Solyc09g065180	NAD-dependente pimerase/dehydratase	5,0E+04	2,0E+04	3,0E+07	2,7E+03	1,2E+08	3,8E+07	**0.19**	36,64
K4CBQ4Solyc07g007840	TranscriptionFactor S-II	3,2E+04	5,9E+04	1,3E+05	3,9E+07	3,9E+07	4,6E+07	**0.001**	3926,98
K4BXJ9Solyc05g010260	phosphogluconatedehydrogenase 2, PGD2	2,0E+05	1,1E+05	3,0E+06	7,1E+07	4,3E+07	7,9E+07	**0.01**	405,18
K4DGY6Solyc12g089150	SNAP receptor activity	6,5E+05	5,3E+06	9,2E+05	1,9E+05	6,7E+06	1,9E+08	**0.20**	199,17
K4C9W3Solyc06g076020	Heat Shock Protein	2,4E+06	8,9E+05	5,1E+04	3,9E+06	9,3E+05	6,1E+04	**0.20**	10,28

A4ZYQ6Solyc02g036350	Ethylenebiosyntheticprocess	6,6E+05	1,0E+06	8,0E+05	5,0E+05	6,8E+04	2,4E+06	**0.42**	8,41
K4D5Q4 Solyc11g010960	Alcohol dehydrogenase	6,3E+09	5,9E+09	1,0E+09	2,4E+09	7,4E+09	5,7E+09	**0.03**	483,17
Q6DUX3 Solyc01g099770	Translationallycontrolleddtumour-associated	4,8E+09	1,2E+09	5,3E+09	1,9E+07	2,3E+09	3,3E+09	**0.23**	74,59
K4C1K6 Solyc07g065840	Heat Shock Protein	2,4E+09	1,6E+09	7,9E+09	6,9E+09	9,8E+09	5,6E+09	**0.09**	271,69
K4BVE0 Solyc04g080610	Ornithinecarbamoyltransferase	2,4E+09	2,2E+09	1,2E+09	1,3E+09	4,8E+08	1,7E+09	**0.09**	126,16
K4BFH5 Solyc03g032040	Tonoplastmonosaccharidetransporter	4,1E+07	5,4E+07	7,1E+07	3,6E+07	8,7E+07	6,9E+09	**0.3**	5,18
K4DF00 Solyc12g042060	ATP-binding, Nucleotide-binding	3,5E+08	2,8E+09	1,2E+09	2,5E+07	1,3E+07	2,0E+09	**0.12**	147,18

Table C. List of Prosys interactors detected with AP-MS, with the corresponding FC-A score, predicted interaction and functions.

Bait identifier	Preyidentifier	FoldChange	Predicted Interaction	Function
Solyc05g051750	Solyc05g050970	42,12	no	Transketolase family
Solyc05g051750	Solyc09g065180	36,64	yes	NAD-dependentepimerase\dehydratase
Solyc05g051750	Solyc06g076020	10,28	yes	Heat shock protein
Solyc05g051750	Solyc12g089150	199,17	no	SNAP receptor activity
Solyc05g051750	Solyc05g010260	405,18	no	oxidoreductase activity
Solyc05g051750	Solyc11g008110	15,02	no	transporter activity
Solyc05g051750	Solyc12g042060	147,17	no	ATP binding, nucleotide binding
Solyc05g051750	Solyc12g042920	291,13	no	Cytochrome C complex activity
Solyc05g051750	Solyc01g099770	74,59	no	calciumionbinding
Solyc05g051750	Solyc11g010960	483,16	no	oxidoreductase activity
Solyc05g051750	Solyc10g084050	452,41	no	hydrolase activity
Solyc05g051750	Solyc01g006510	7,77	no	oxidoreductase activity

Solyc05g051750	Solyc01g008120	9,55	no	transcriptioncofactor activity
Solyc05g051750	Solyc01g060280	6,61	no	acetyltransferase activity
Solyc05g051750	Solyc01g080280	4,42	no	catalytic activity
Solyc05g051750	Solyc01g080510	9,36	no	transmembrane transporter activity
Solyc05g051750	Solyc01g095900	7,50	no	methyltransferase activity
Solyc05g051750	Solyc01g108430	11,90	no	nucleic acid binding
Solyc05g051750	Solyc01g109300	16,36	no	Dimethylallyldiphosphatebiosyntheticprocess
Solyc05g051750	Solyc01g111120	6,08	no	triose-phosphateisomerase activity
Solyc05g051750	Solyc07g065840	271,69	no	Heat shock protein
Solyc05g051750	Solyc01g111630	4,57	no	NAD binding, oxidoreductase activity
Solyc05g051750	Solyc01g112290	7,42	no	nucleotide binding
Solyc05g051750	Solyc02g089260	8,04	no	metal ionbinding
Solyc05g051750	Solyc02g091580	7,59	no	peptidase activity
Solyc05g051750	Solyc02g093590	5,91	no	proteinbinding

Solyc05g051750	Solyc03g118410	4,31	no	fatty acid biosyntheticprocess
Solyc05g051750	Solyc03g120670	4,74	no	oxidoreductase activity
Solyc05g051750	Solyc04g008740	27,57	no	catalytic activity, kinase activity
Solyc05g051750	Solyc04g045340	31,26	no	metal ionbindingphosphotransferases
Solyc05g051750	Solyc04g080610	126,16	no	amino acid binding, transferase activity
Solyc05g051750	Solyc04g081100	19,17	no	oxidoreductase activity
Solyc05g051750	Solyc04g081970	16,09	no	proteindisulfideoxidoreductase activity
Solyc05g051750	Solyc04g082630	6,82	no	NAD binding, oxidoreductase activity
Solyc05g051750	Solyc05g009950	280,15	no	Cytochrome B family protein
Solyc05g051750	Solyc06g071910	18,38	no	oxidoreductase activity
Solyc05g051750	Solyc07g053030	3,08	no	ADP binding
Solyc05g051750	Solyc07g054690	175,35	no	Endonuclease activity, RNA binding
Solyc05g051750	Solyc07g062570	24,35	no	ubiquitinproteinligasebinding

Solyc05g051750	Solyc07g065840	271,69	no	ATP binding,nucleotide binding, unfolded protein binding
Solyc05g051750	Solyc07g064160	3,55	no	oxidoreductase activity
Solyc05g051750	Solyc07g066470	7,45	no	hydroxymethylbilan esynthase activity
Solyc05g051750	Solyc07g066600	8,02	no	kinase activity
Solyc05g051750	Solyc08g006780	32,89	no	glycogenmetabolic process
Solyc05g051750	Solyc08g076020	27,59	no	DNA binding
Solyc05g051750	Solyc07g007840	3926.98	no	Transcriptionelonga tionfactor S
Solyc05g051750	Solyc08g080580	5,72	no	transcription regulatory region DNA binding
Solyc05g051750	Solyc09g018750	48,20	no	cell redox homeostasis
Solyc05g051750	Solyc12g009250	21,23	no	chaperonebinding
Solyc05g051750	Solyc12g056120	42,61	no	oxidoreductase activity
Solyc05g051750	Solyc05g008600	29,67	no	catalytic activity
Solyc05g051750	Solyc07g066610	15,39	no	phosphoglycerateki nase activity
Solyc05g051750	Solyc09g014390	77,45	no	Alpha 1 4-galactosyltransfera

				se
Solyc05g051750	Solyc07g066580	14,19	no	transferase activity
Solyc05g051750	Solyc04g009030	8,85	no	oxidoreductase activity
Solyc05g051750	Solyc03g032040	5,18	no	transmembrane transporter activity
Solyc05g051750	Solyc10g018300	10,22	no	catalytic activity
Solyc05g051750	Solyc05g012390	9,07	no	endonuclease activity
Solyc05g051750	Solyc03g096440	9,90	no	Transposase
Solyc05g051750	Solyc11g067290	106,70	no	Acyltransferase-like protein
Solyc05g051750	Solyc11g008110	15,02	no	transporter activity
Solyc05g051750	Solyc07g007840	3926.98	no	Transcriptionelongationfactor S
Solyc05g051750	Solyc08g080580	5,72	no	transcription regulatory region DNA binding
Solyc05g051750	Solyc12g042060	147,18	no	ATP binding, nucleotide binding
Solyc05g051750	Solyc07g006030	31,51	no	response to abscisic acid
Solyc05g051750	Solyc01g057830	456,42	no	nucleic acid binding
Solyc05g051750	Solyc07g066320	12,49	no	DNA-dependentATPase activity

Solyc05g051750	Solyc08g013900	24,62	no	Nitrogen plant regulator
Solyc05g051750	Solyc09g090610	19,33	no	ATP binding, nucleotide binding
Solyc05g051750	Solyc01g099770	74,59	no	Calciumionbinding
Solyc05g051750	Solyc09g072560	780,54	no	isomerase enzyme involved in the synthesis of L-rhamnose
Solyc05g051750	Solyc03g098400	1009,27	no	protein serine/threonine kinase activity
Solyc05g051750	Solyc12g055810	1462,84	no	ATP binding
Solyc05g051750	Solyc04g054740	1,38	yes	inositol-3-phosphate synthase
Solyc05g051750	Solyc01g111990	1,79	yes	alanine-tRNAligasesynthetase

APPENDIX

Foreign working period

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Tutor: Dr. Youjun Zhang

Date and duration:

1st October 2018-1st October 2019 (12 months)

1st September 2020-1stDecember 2020 (3 months)

PUBLICATIONS

Title: FROM AFFINITY TO PROXIMITY TECHNIQUES TO INVESTIGATE PROTEIN COMPLEXES IN PLANTS

Authors: Sandra M. Kerbler, **Roberto Natale**, Alisdair R. Fernie, and Youjun Zhang.

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Title: *BOTRYTIS CINEREA* COLONIZATION OF *SOLANUM MELONGENA*AND *VITIS VINIFERA*PLANTS IS STRONGLY REDUCED BY THE EXOGENOUS APPLICATION OF TOMATO SYSTEMIN

Authors: Donata Molisso, Mariangela Coppola, Anna Maria Aprile, **Roberto Natale**, Pasquale Chiaiese, Rosa Rao.

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Title: TOMATO PLANTS TREATED WITH SYSTEMIN PEPTIDE SHOW ENHANCED LEVELS OF DIRECT AND INDIRECT DEFENSE ASSOCIATED WITH INCREASED EXPRESSION OF DEFENSE-RELATED GENES

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Plants 2019, 8(10), 395; doi:10.3390/plants8100395

Title: RAPID IDENTIFICATION OF PROTEIN-PROTEIN INTERACTIONS IN PLANTS

Authors: Youjun Zhang, **Roberto Natale**, Adilson Pereira Domingues Junior, Mitchell Rey Toleco, Beata Siemiatkowska, Norma Fabregas, and Alisdair R. Fernie

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POSTER COMMUNICATION

Title: THE PROSYSTEMIN PROTEIN NETWORK IN TOMATO PLANT

Authors: **Natale Roberto**, Coppola Mariangela, Albano Manuela, Delano-Frier John Paul, Rao Rosa.

Proceedings of the LXII SIGA Annual Congress Verona, Italy – 25/28 September, 2018. ISBN 978-88-904570-8-1

Abstract – 5.23

Tomato plants release a small defense peptide called Systemin (Sys) from a larger precursor of 200 amino acids called Prosystemin (ProSys) upon the perception of a stress condition. This peptide activates a cascade of events that leads to the production of defense compounds (Ryan, 2000 Biochim. Biophys. Acta 1477, 112-121). Tomato transgenic plants overexpressing ProSys show tolerance towards a wide array of biotic and abiotic stressors (Coppola *et al*., 2015, Plant Mol Biol Rep 33:1270–1285; Orsini *et al*. 2010, Physiol Plant, 138: 10–21). The molecular mechanisms underpinning such a wide array of defense responses are largely unknown. In order to acquire knowledge in this respect we decided to define protein-protein interactions involved in Sys signaling pathway. Starting from transcriptomic profiles imposed by ProSys constitutive expression and by querying interactome databases ('Predicted Tomato Interactome Resource', PTIR, and 'Search Tool for the Retrieval of Interacting Genes/Proteins', STRING), we obtained the *in silico* prediction of a protein network including 16000 nodes (proteins) and about 160000 edges (interactions). We focused our attention on proteins directly interacting with ProSys obtaining a sub-network of 99 nodes and 98 edges. ProSys interactions, coming from STRING database, were divided, and grouped based on Gene Ontology (GO) categories. The network shows a direct interaction of ProSys with enzymes involved in the biosynthesis of the 3 major hormones associated with defense responses against biotic and abiotic stressors, Jasmonic Acid, Salycilic Acid, and Ethylene supporting the role of the protein in the activation of a number of different plant defense responses. In addition, among the ProSys interactors several transcription factors, key players in pest recognition and regulation of immunity, were found. These results may explain the

phenotype observed for transgenic plants. The validation of the predicted interactions is presently in progress.

Title: THE PROSYSTEMIN INTERACTOME IN TOMATO PLANT

Authors: **Natale Roberto**, Zhang Youjun, Siemiatkowska Beata, FernieAlisdair Robert, Rao Rosa.

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Abstract – 1.17

Tomato plants release a small defense peptide called Systemin (Sys) from a larger precursor of 200 amino acids called Prosystemin (ProSys) upon the perception of a stress condition. This peptide activates a cascade of events that leads to the production of defense compounds. Tomato transgenic plants overexpressing ProSys show tolerance towards a wide array of biotic and abiotic stressors. The molecular mechanisms underpinning such a wide array of defense responses are largely unknown. In order to acquire knowledge in this respect we decided to define protein-protein interactions involved in ProSys signaling pathway. The *in silico* prediction shows 99 nodes (proteins), which interact with ProSys; starting from these results we proceed to the experimental evaluation of the ProSys interactors using different approaches. We used the Affinity Purification Mass Spectrum as first method which resulted in more than 500 interactors that include some previously predicted proteins. Here we show some of them such as the NAD-dependent epimerase/dehydratase located into the transcription factors group, the Heat-shock protein (HSP) located into abiotic stress group and a protein related with ethylene biosynthetic process. The stress conditions in plants, caused by drought, salinity, chemicals, cold and hot temperatures, and various pathogen attacks, induce the production of HSP useful to keep proteins in their functional native conformations thus preventing aggregation of non-native proteins. On the other hand, ethylene is known to play an important role in the activation of defense genes. Our results shed lights on the molecular role of ProSys in tomato defense responses: the precursor appear to interact with several proteins that are able to activate defense pathways and to keep cellular homeostasis under stress conditions. Experimentally evidenced interactors will be confirmed at least with a second

approaches (BiFC/FRET/CoIP) and could highlight new perspectives in this field, now largely unknown.

Title: TOMATO SYSTEMIN: A POTENTIAL LINK BETWEEN BIOTIC AND ABIOTIC STRESSES

Authors: Molisso Donata, Lentini Matteo, **Natale Roberto**, Cirillo Valerio, Esposito Marco, Maggio Albino, Rao Rosa.

Proceedings of the SIGA Young Web Meeting 7 July, 2020. ISBN 978-88-944843-0-4

Abstract – SY25

Plant signaling peptides trigger signal transduction of external and internal stimuli that leads to the production of hormones and to the successive activation of genes modulating several physiological events in plants, including defense. Some of these peptides have been defined as plant resistance activators or elicitors that are released upon pest attacks triggering an amplification of the plant's own defense. Tomato Systemin (Sys) is one of the best characterized signaling peptide described in plants. This 18-amino acid peptide is released from a larger cytosolic precursor protein of 200 amino acids called ProSystemin (ProSys). Transgenic plants, constitutively expressing ProSys, have shown a wide transcriptome reprogramming which reflected in novel phenotypes resistant to different pests, salinity, and heat stresses. Most recently, by combining gene expression studies and bioassay with different pests, we have already demonstrated that the exogenous supply of ProSys protein and Sys peptide to tomato plants enhance both direct and indirect defense barriers. However, little is known on the functional link between plant responses to biotic and abiotic stresses. To contribute to this knowledge, we investigated the effect of the application of the Sys peptide, via soil drench, on the regulation of the expression of abiotic stress-related genes, on plant growth characteristics and on metabolic parameters of tomato plants exposed to NaCl (80mM). Our results indicate that the direct delivery of this peptide primed defense genes active incounteracting saline stress (catalase 1 (CAT1), 14-3-3 protein 1 (TFT1), Heat shock transcription factorA2 (HSFA2), Heat shock protein 70 (HSP70), Heat shock protein 90 (HSP90)) and that the subsequent administration of salt stress to the treated plants increased the expression of primed genes. In addition, under salinity conditions, Sys-treated plants exhibited no significant reduction in

shoot biomass accumulation and a higher proline content in the leaf. The present study indicates that Sys peptide represents a link between biotic and abiotic stress resistance in tomato plants. From an applied perspective our data give a significant contribution towards the safe and sustainable strategies for crop protection.

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Review

From Affinity to Proximity Techniques to Investigate Protein Complexes in Plants

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Abstract: The study of protein–protein interactions (PPIs) is fundamental in understanding the unique role of proteins within cells and their contribution to complex biological systems. While the toolkit to study PPIs has grown immensely in mammalian and unicellular eukaryote systems over recent years, application of these techniques in plants remains under-utilized. Affinity purification coupled to mass spectrometry (AP-MS) and proximity labeling coupled to mass spectrometry (PL-MS) are two powerful techniques that have significantly enhanced our understanding of PPIs. Relying on the specific binding properties of a protein to an immobilized ligand, AP is a fast, sensitive and targeted approach used to detect interactions between bait (protein of interest) and prey (interacting partners) under near-physiological conditions. Similarly, PL, which utilizes the close proximity of proteins to identify potential interacting partners, has the ability to detect transient or hydrophobic interactions under native conditions. Combined, these techniques have the potential to reveal an unprecedented spatial and temporal protein interaction network that better understands biological processes relevant to many fields of interest. In this review, we summarize the advantages and disadvantages of two increasingly common PPI determination techniques: AP-MS and PL-MS and discuss their important application to plant systems.

Keywords: affinity purification; proximity labeling; plant protein complex; protein-protein interactions

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1. Introduction

The study of biomolecular complexes is crucial in understanding the molecular mechanisms underpinning biological processes, protein function and subcellular protein localization [1–4]. Biomolecular complexes are principally formed by proteins interacting with other proteins (protein–protein interactions, PPIs), however complexes can also arise through the interaction of proteins with ligands such as nucleic acids, sugars, lipids and hormones [2–4]. As the biological function of a protein is defined by its interactions in the cell, an important step in investigating, disrupting or modulating biological processes lies in understanding how and why PPIs occur [1,4]. Advantages of protein complex formation are myriad, starting from greater proximity between substrate and catalyst to enhanced efficiency of whole biochemical pathways.

The field of proteomics has witnessed the development of many innovative methods for the identification and characterization of PPIs [1,3,4]. As method preferences to study protein complexes have changed over time, so too have the possibilities to obtain annotated or predicted protein complexes and composition. Over recent years, proteome-wide studies and computational approaches both point toward a scenario with an increasing number of heteromeric protein complexes being identified [5,6]. The methodology used to predict or identify protein complexes can be categorized in two ways: experimental

and computational. Computational or in silico approaches are used to predict PPIs via computer simulations and are dependent on the algorithm used [7]. These predictions are based on high throughput proteomics data (binary or mass spectrometry-based methods), primary structure, 3D structure, domain, evolutionary relationship, genomic methods or a combination of these methods [7–10]. Experimental approaches are either performed in vitro or in vivo. While in vitro studies are generally performed on a low throughput scale, in vivo studies can be carried out in a high throughput manner. The most common methods used in the study of PPIs are biochemical protein purification or separation (2D gel electrophoresis, 2-DE [11]; blue native polyacrylamide gel electrophoresis, BN-PAGE; size exclusion chromatography, SEC) followed by mass spectrometry (MS), genetic engineering of cellular systems (yeast two hybrid (Y2H) assays and their variants; phage display), arrays (protein arrays or peptides microarrays), structural studies (NMR spectrometry, X-ray crystallography, cryoelectron microscopy) or fluorescence imaging (fluorescence resonance energy transfer, FRET; bimolecular fluorescence complementation BiFC) [1,3,4,12].

Recent studies highlight significant progress in the use of affinity purification and proximity labeling approaches combined with MS-based quantitative proteomics in studying PPIs [5,13–15]. Affinity purification mass spectrometry (AP-MS) is a fast, sensitive and targeted approach used to detect interactions between bait (protein of interest) and prey (interacting partners) under near-physiological conditions [16]. This method can be applied to large-scale studies and has been demonstrated to have high intra-and inter-laboratory reproducibility [17]. Similarly, proximity-dependent labeling methods are being increasingly used to detect transient PPIs under native conditions in living cells [14]. As the name suggests, proximity labeling (PL) relies on the principle that proteins must be physically close in order for them to interact and is predicted to be more precise in determining interacting partners [18].

Both AP-MS and PL-MS are powerful techniques that have significantly enhanced our understanding of PPIs. While these methods have become increasingly popular in animal systems, application of these techniques in plants remain underutilized. Combined, AP-MS and PL-MS have the potential to reveal an unprecedented spatial and temporal protein interaction network that better understands biological processes relevant to many fields of interest. For example, AP-MS can be theoretically used to detect transient PPIs as well as interactions involving potentially insoluble proteins such as membrane-associated proteins. Furthermore, PL-MS has the potential to detect hydrophobic interactions under native conditions and has been recently used to investigate membrane contact sites between the endoplasmic reticulum and mitochondria in plants [19]. In this review, we summarize two increasingly common PPI determination techniques: AP-MS and PL-MS and discuss their important application to plant systems.

2. Affinity Purification Mass Spectrometry in Plants

Similar to immunopurification or immunoprecipitation (IP), AP utilizes antibodies which can be targeted to the bait, or to a standardized fusion moiety often referred to as an epitope tag [6]. Using protein-specific antibodies, AP-MS has the theoretical advantage of capturing protein complexes under native conditions from plant lysates [5]. However, with limited availability of plant protein antibodies, different bait isoforms that can occlude antibody interaction sites and differing specificities of antibodies, the ability to obtain reliable protein interaction networks remains challenging [4,6]. Therefore, fusion of the bait to various affinity tags has greatly increased the efficacy of this method. Once the bait protein interacts with its respective prey, the resulting complex can be purified from the cell lysate using a matrix that specifically recognizes the affinity tag. Both stable protein complexes and weak PPIs between bait and prey have been detected by AP-MS [20,21]. A critical aspect of this technique lies in protein separation, purification and digestion to reduce the presence of contaminants. Specific protein antibodies can be used to immunoprecipitate the protein of interest under native conditions; however, this approach has only been successfully demonstrated by a few laboratories [5]. While several affinity

tags have been developed to allow co-precipitation of prey and bait proteins under native conditions (Table 1), the use of such tags comes with its challenges. Introduction of an epitope tag can result in non-native folding of the tagged protein or steric hindrance of interactions. As bait fused affinity tags generally need to be overexpressed, such expression can influence the physiological properties of the bait or stoichiometry of the complex. Epitope tags can also result in incorrect localization or alternative localization of the protein of interest. It has been shown that overexpression of the bait may result in false positive interactions [6,22]. For these reasons, it is highly recommended that researchers confirm that the chosen epitope tag does not interfere with the endogenous function, localization, or properties of the bait by complementation of the mutant plant line [3,6]. However, these recommendations are not widely utilized due to the time-consuming nature of producing stable transgenic lines and cannot be followed if wild-type plants are used. The use of clustered regularly interspaced short palindromic repeats (CRISPR) technology could help to improve these limitations. Such technology provides researchers with the ability to directly insert affinity tags into endogenous loci without changing the genomic context of the gene and also maintain the native environment to which protein interactions can then be characterized [3,23].

Table 1. Affinity tags successfully used to investigate plant protein–protein interactions.

Tag	Sequence/Size	Affinity Resin	Elution Conditions	Reference
TAPi tag	45 kDa	Calmodulin binding peptide with two protein A domain	Protein A/low pH	[22,23]
Streptavidin binding peptide (SBP)	WSHPQFEK	Streptavidin	Desthiobiotin	[23,24]
GS^{yellow}	37 kDa	Streptavidin-binding peptide tag with citrine yellow fluorescent protein	Desthiobiotin/pH	[23,25,26]
Fluorescent protein (GFP, YFP)	26.9 kDa	Anti-GFP	pH	[13,23,25,26]
GS^{rhino} tag	21.9 kDa	two IgG-binding domains of protein G and a SBP tag	Streptavidin elution buffer [5]	[5,23,27]
Alternative TAP (TAPa)	26 kDa	2 xIgG-BD with 6 XHis and 9 Xmyc	HR3C cleavage/Imidazole/low pH	[23,28]

Given the increased sensitivity of MS and the application of novel bioinformatic approaches for accurate data analysis, affinity-based methods have improved considerably in recent years [5,29]. While single tag AP-MS is now widely used in large scale studies, selection of the epitope tag and positioning of the tag at either the N- or C-terminus of protein remains critical. In addition to being an efficient purification handle, some affinity tags also provide benefits such as information regarding subcellular localization of the PPI. For example, fluorescent tags (i.e., green fluorescence protein (GFP), yellow fluorescence protein (YFP) and the mFruits family of monomeric red fluorescent proteins (mRFPs)) allow for localization studies to be performed in parallel to AP-MS studies. The ability to simultaneously monitor both protein localization and expression is useful in investigating whether the recombinant protein occurs under native conditions and if the preyed interactions are biologically relevant. For example, differences in the metabolic roles of glycolytic and TCA cycle enzymes fused with C-terminal GFP were observed in the cytosol and mitochondria respectively [30,31]. In addition, one benefit of using epitope tags is that several proteins can be fused with the same epitope and purified with same method. As a result, background contamination should be consistent across all purifications and should enable the use of the same negative controls, including tag-only constructs or wild-type plants. As shown in Table 1, several types of epitope tags have been successfully applied to AP-MS in plants.

The main disadvantage of AP-MS however, remains in the ability to fully characterize affinity matrix/epitope tag interaction properties. The identification of non-specific bound proteins is one of the main disadvantages of a single-step purification approach and contaminant proteins associated with either the solid-phase or the epitope tag are hard to distinguish from positive interactors. Thus, the use of proper negative controls such as protein extracts from wild-type plants, mutant lines, or tag-only expressing plants is critical (Figure 1). In principle, unspecific proteins identified in these controls can be simply subtracted from the list of interactors that are identified by the bait. However, given the limitations of AP enrichment and liquid chromatography–mass spectrometry (LC–MS), false positives are still likely. Alternatively, various algorithms can be applied. For example, the SAINT algorithm [32] allows researchers to determine fold change abundance (FC-A), which can be used to filter out potential false positives. Possible interactions can also be evaluated based on the ratio of spectral counts of the bait versus overexpression of an unrelated protein or tag-only controls [33]. Moreover, a second purification step can be introduced to reduce the amount of non-specific binding proteins [5,21]. In tandem affinity purification (TAP), two types of affinity tags linked by a protease cleavage site are fused to a bait protein and expressed in plants. Two affinity purification steps are then performed to obtain reliable interacting partners (Figure 1b). Interestingly, an *Arabidopsis* plant cell culture system has been developed for TAP technology which allows for the high-throughput identification of protein complexes, even with very low sample volumes (25 mg total protein) [5]. GS tags and their derivatives are the most frequently and successfully used TAP tags in plant research [5,34]. A GS tag consists of two immunoglobulin domains of a streptavidin-binding peptide and protein G linked by a unique cleavage site that is recognized by the etch virus protease from tobacco (*Nicotiana tabacum*). Following an initial affinity purification step with immunoglobulin G agarose beads, protein complexes can be incubated with the tobacco etch virus protease to release the complex from the matrix. In a subsequent purification step, the bait protein complex associates with a streptavidin-conjugated bead trap. Following several washing steps, the protein complex is eluted and determined by LC-MS (Figure 1b; [5,21]). In addition, a multifunctional TAP tag (GS^{yellow}) has been developed that combines the fluorescent properties of citrine YFP with a streptavidin-binding peptide tag. This double affinity tag can not only be used to determine the subcellular localization of proteins in vivo but also the potential function of the protein through AP [26].

The strength of AP-MS is that it can be used to study PPIs in their relevant plant growth and development biological contexts. For example, studies on specific plant organs including leaves [35], flowers [36] and roots [37], have provided improved information on protein complex organization. Furthermore, AP-MS has the potential to provide insight into posttranslational modification of proteins that may regulate the establishment of spatially or temporally dependent protein interactions [38]. For example, interactions between TCA cycle enzymes and phosphatases have been found using AP-MS in *Arabidopsis* plant cell cultures [31,39,40]. Several posttranslational modification candidates have also been found using AP-MS of glycolytic enzymes in our recent research [30]. These modifications can be directly detected using MS/MS; however, only if they are relatively abundant and if such modifications can withstand the numerous processes involved in protein extraction, purification and MS and MS/MS analyses [41]. Furthermore, given that AP-MS is based on the association of stable complexes, the combination of AP-MS with cross linking has been suggested to greatly improve detection of transient and weak PPIs that are normally lost during protein affinity purification steps [12,22,42].

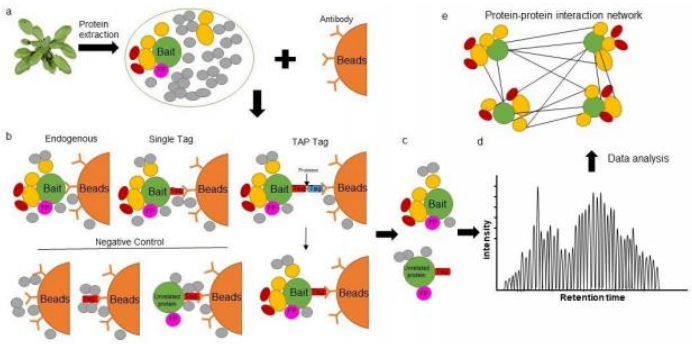

Figure 1. Overview of affinity purification strategies. (**a**) Total protein extraction for affinity purification. (**b**) Bait specific antibodies are linked to beads for protein complex immunoprecipitation under native conditions. Such beads can be used to detect endogenous proteins within a plant, proteins fused displaying a single tag (single affinity purification) or proteins expressing a double (TAP) tag (double affinity purification). Suggested controls used to reduce background contaminants and thus the identification of false positives include using a wild-type plant extract, purification from cells expressing the tag only, or unrelated proteins fused with a tag. (**c**) Several washing steps are used to reduce non-specific interactions. (**d**) Proteins are measured by LC-MS. (**e**) Data analysis to determine a protein–protein interaction network. FP: false positive; UP: unrelated protein.

Chemical cross-linking is a classical approach which is used to freeze PPIs in their native form and has been shown to be especially useful for capturing transient and weak PPIs. For example, membrane protein interactions have been detected in vivo by cross linking with formaldehyde [43]. In the two steps of formaldehyde crosslinking, formaldehyde reacts with a relatively strong nucleophile, most commonly a lysine-amino group from a protein to form a methylol intermediate. Sequentially, the methylol intermediate reacts with another nucleophile, possibly an amino group of a DNA base, to generate a crosslinked product. Thus, formaldehyde could be injected or incubated with plant materials to quickly generate crosslinked protein complexes. Other commonly used cross-linkers include the reversible dithiobis (succinimidyl propionate) during sample extraction to enhance affinity purification of transient and unstable interactions [44]. In addition, a quantitative dimension to AP-MS experiments (q-AP-MS) has been used to overcome issues of non-specific binding of proteins and allows investigation of regulative PPIs under changing physiological conditions [45]. While AP-MS provides a snapshot of the interacting compositions in a multi-subunit complex, it alone, cannot provide insight into the dynamic changes and associations of protein complexes [4].

Two analytical strategies that can be applied to detect dynamic associations of protein complex partners include label-free quantification (LFQ) and stable isotopic labeling. Stable isotope labeling combined with AP-MS has been successfully used to follow the temporal dynamics of PPIs throughout the cell cycle [46] and to investigate protein complexes influenced by different types of cellular perturbation in human and yeast research [47,48]. In *Arabidopsis*, stable isotope labeling combined AP-MS has been used to quantitatively investigate the B-box protein complex, involved in integrating light and hormone signaling pathways during photomorphogenesis from non-specific background proteins [49]. Due to the high cost of labeled substrates and limited labeling efficiency, isotope labeling approaches are restricted in plant research even though it is very sensitive and more accurate than LFQ [50]. In contrast, LFQ technology is easy to perform, cost-effective, and suitable for comparative analyses of large amount samples [51]. LFQ-based technologies use statistical algorithms to analyze relative LC-MS peptide peak abundances based on intensity or counting strategies in multiple replicates [52], so allowing the comparison of samples run

at different times. Given that MaxQuant software is an integrated suite of algorithms for the analysis of high-resolution quantitative MS data, its MaxLFQ module is widely used to calculate highly reliable relative LFQ intensity profiles [53] by first searching against the Araport11 database (www.araport.org, accessed on 28 April 2021). Assuming that the enrichment of most proteins (including non-specific background proteins) is kept constant by the design of the experiment, the algorithm promotes the investigation of proteins that are differentially enriched under the tested conditions [54]. Moreover, AP-MS combined with LFQ has also been suggested to assess PPI dynamics during cellular signaling or after cellular perturbations. Given that both tagged bait samples and negative controls can be purified under different conditions or treatments, comparison of quantitative interaction networks could provide the means to assess dynamic protein complex associations. For example, using quantitative (q) TAP in growing maize (*Zea mays*) leaves, growth-regulating factors have been shown to interact with Angustifolia 3 in the division zone, while this interaction was significantly lower in the expansion zone of the same leaves [55]. Another example is the well-characterized strigolactone-dependent interaction (between the receptor protein Dwarf 14 and Suppressor of More Axillary Growth-Like 7), which displays dynamic changes in protein complex composition in response to the hormone [29].

A high-performance affinity enrichment approach for mass spectrometry (AE-MS)- is a technique that combines AP-MS and LFQ and has become an effective method to determine positive PPIs from false positive interactions [56]. Instead of multiple steps of purifying complexes, AE-MS takes advantage of the specific enrichment of interactors in the context of a large number of unspecific background binders by performing a single-step affinity enrichment of endogenously expressed tagged proteins followed by single-run, intensity-based label-free quantitative LC-MS/MS analysis. Although high amounts of non-specific binding proteins are used in the postprocessing pipeline for more accurate normalization and quality control, bait-interacting proteins are expected to be enriched in extracts when compared to negative controls. Given that similar amounts of contaminants are detected under similar conditions in both samples and negative controls, it is easy to eliminate non-specific binding proteins by observing the ratio of interactors versus noise. False positives can also be removed by background normalization, untagged samples and the intensity profiles across all samples. While AE-MS normally requires a minimum of three replicates, this technique has been widely used for large-scale studies as it provides sufficient amounts of data for statistical analyses [57,58]. Both random sample preparation and negative controls are important to determine reliable PPIs networks. To date, AE-MS has been successfully used to characterize several plant PPIs such as dynamin-related proteins interacting with PIN-Formed auxin efflux carriers [59], the protein interaction network of the plant TCA cycle [31,40], MADS domain transcription factor complexes during *Arabidopsis* flower development [57], vascular development-regulating basic helix–loop–helix transcription factor dimers [60] and a glycolysis interaction network [30].

3. The Proximity Labeling Method

PL-MS is a high-throughput approach for the systematic analysis of PPIs in vivo. While PL-MS is already firmly established in mammalian and unicellular eukaryote systems, application of this technique in planta remains challenging. PL utilizes enzymes that produce reactive molecules that covalently interact with proteins in close proximity. Labeled proteins can be isolated using conventional affinity purification methods and identified via immunoblot analysis or by protein mass spectrometry, Proximity labeling overcomes some of the limitations of AP-MS and Y2H, as abundant soluble proteins as well as insoluble membrane proteins can be effectively enriched under stringent denaturing conditions, which in turn, facilitates their identification. PL can detect weak, transient or hydrophobic PPIs in their native state and provides an unedited spatial and temporal protein interaction network for better understanding of a specific biological process. In addition, fusion of PL enzymes to a minimal targeting motif that restricts proteins to a particular subcellular location or structure, can be used to map the protein population

therein [61]. While application of PL-MS to plant systems remains in its infancy, we summarize the recent development of this technology and highlight its potential in studying plant PPIs (Figure 2).

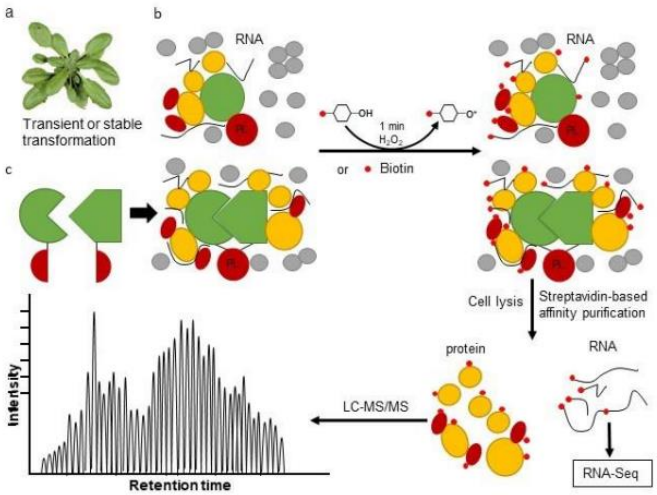

Figure 2. Overview of proximity labeling system. (**a**) Transient and stable protein with proximity-labeling (PL) enzyme transformation. (**b**) PL assay based on the tagged PL enzyme. A biotin ligase or APEX PL enzyme is fused to the target protein and expressed in plants. Upon the addition of a substrate, such as biotin or biotin-phenol and hydrogen peroxide (H_2O_2), proteins or RNAs are tagged by biotin. (**c**) Interacting pairs are fused to the PL enzyme at either the N- and C-terminus to investigate the composition of protein complexes. As two proteins interact in cells, the two halves of a split-PL are reorganized as a full PL enzyme and initiate the labeling of proximal partners of the protein complex. After protein extraction and incubating with streptavidin beads, biotin-labeled proteins or RNAs can be enriched for subsequent LC-MS/MS or high-throughput sequencing analysis.

PL-MS has emerged as a powerful tool to characterize PPIs. Over recent years, this technology has grown immensely with the development of new PL enzymes and the application of PL in studying protein interaction networks (including protein-DNA and protein-RNA interactions). Currently, enzyme-mediated activation of radical sources (EMARS), engineered ascorbate peroxidase (APEX) and proximity-dependent biotin identification (BioID) are three commonly used PL technologies [62]. As BioID and its derivatives are highly specific, non-cytotoxic and reproducible, these approaches are increasingly becoming the PL method of choice. Proximity biotinylation is based on the *Escherichia coli* enzyme, BirA. First reported in 2014, BioID relies on the promiscuous activity of a modified BirA protein (mutation of R118G) that releases highly reactive and short-lived biotinoyl-5′-AMP and can modify proteins within a distance of 10 nm [18]. Due to the covalent biotinylation of prey, biotin-labeled targets are stable following stringent cell lysis treatments associated with protein extraction and affinity purification (for example streptavidin beads) with multiple washing steps. This method can also be combined with mass spectrometry measurements to screen for PPIs or detect biotin-labeled proteins with high spatial resolution in living cells. This method has been successfully used to evaluate physiologically relevant PPI networks [14], especially in the detection of transient asso-

ciations or low-affinity interactions that arise through posttranslational modifications of proteins and signal transduction. An improved smaller enzyme, BioID2, isolated from *Aquifex aeolicus*, has also been produced with the advantage of maintaining the correct localization of a fused protein and requires less biotin for labeling compared to BioID [63].

Similar to BioID, APEX is a 27 kDa monomeric protein which biotinylates prey near the target protein when supplied with ATP, H_2O_2 and biotin-phenol at 37 °C [64]. The advantage of using APEX-fused bait proteins is that the time needed to biotinylate all neighboring proteins rich in tyrosine residues is just 1 min, which is significantly faster than the 18–24 h required for BioID. An improved variant of APEX, named "APEX2", was also developed with increased catalytic activity to reduce the toxic effects of using H_2O_2 and biotin-phenol on living cells [65]. Compared to APEX, BioID has the advantage of using non-toxic biotin as a substrate and so avoids the introduction of oxidative stress conditions to cells or tissues. However, as APEX uses quick labeling times, this method has been shown to have greater success in studying dynamic processes such as cell signaling and transient PPIs. Recently, a new PL enzyme and its truncated mutant, termed TurboID (35 kDa) and miniTurboID (28 kDa) respectively, were developed as directed evolution of BirA [66]. The two new versions of BirA combine the advantages of both BioID and APEX and are able to identify interactions involved in fast, dynamic processes without causing damage to living cells [15,67]. Furthermore, several PL enzymes including HRP, APEX2, BioID and TurboID can be split into two parts, similar to the BiFC system, that then can be reconstituted into a functional entity when brought into close proximity. This system is particularly useful for studying membrane contact sites and additional interacting factors of spatio-temporally defined protein complexes [14].

PL technology overcomes several limitations of traditional interaction detection approaches and has been widely used in different biological contexts to highlight different molecular interactions. Furthermore, PL methods demonstrate its potential in detecting interactions with rapid kinetics [68,69]. Several studies underscore the great potential of the PL technique [61,70–72], particularly in the detection of weak or transient interactions as well enzyme-substrate interactions that are often difficult to detect by conventional methods. For example, the mitogen-activated protein kinase (MAPK) signaling pathway is often dynamically involved in various physiological processes under different stress responses. While traditional methods have been limited to simultaneously capturing the substrates of MAPK in different states, Dumont and colleagues used APEX2-based PL to map the interactome of p38α and p38γ MAPKs under both steady and activated conditions and revealed novel substrates of p38 [73]. Regarding the proteomic composition of specific regions of an organelle or membrane-associated proteins, information remains scarce due to a lack of techniques to purify these sub-organellar regions. However, PL methods have been successfully applied to study the composition of several large membrane-associated protein complexes, such as the nuclear pore complex (NPC) [18], G-protein-coupled receptors [68,69] and CaV1.2 voltage-gated calcium channels [74]. Indeed, different labs have independently established TurboID-based PL techniques in plant systems including *Arabidopsis*, tomato root cultures and *N. benthamiana* [15,75–77]. Comparing the activity of BioID, BioID2, TurboID and miniTurboID in different plant systems, studies have shown that TurboID displays higher efficiency in biotinylating proximal proteins twhen compared to BioID and BioID2 in planta [75,77]. MiniTurboID has the advantage of minimizing the deleterious effect of the tag fusion on the function of target proteins but still shows reduced labeling efficiency compared to TurboID [66].

The use of PL methods is being increasingly applied to different fields of research. This is due to their accessibility, simplicity, and most importantly, potency in probing transient or weak PPIs as well as membrane bound proteins or proteins of low abundance. When performing PL, the first and perhaps most critical step is to choose the enzyme appropriate for ones needs. Secondly, researchers should make sure that the fusion of the PL enzyme to a bait protein does not interfere with its localization or its functions. Lastly, it is important to include appropriate controls to minimize false positives or false negatives.

The emerging developments in PL technology provide an incredible opportunity to profile dynamic interaction networks under different conditions, thus offering a global vision of the entire cellular functions, which will greatly advance our understanding of various biological processes.

4. Combining Proximity Labeling and Affinity Purification-Mass Spectrometry

While AP-MS results in the identification of proteins that form stable complexes, PL enables the identification of proteins that are in close proximity to the bait, which results in overlapping yet distinct protein identifications. By integrating AP- and PL-MS data, one has the ability to comprehensively characterize a protein's molecular context and so several combined AP and PL experiments have been trialed. Enzyme combinations allow for both AP-MS and BioID analysis within a single construct and with almost identical protein purification and mass spectrometry (MS) identification procedures such as FLAG-BirA* tag [78,79], Multiple Approaches Combined (MAC)-tag [16] and Strep-Tactin [27] have now been developed. However, there are limitations in combining these two approaches due to the large size of BirA* and the small affinity purification peptide of a Flag or His tag. This strategy of combining AP and PL has not been used in plants to date; however, the generation of specific antibodies for PL tags may facilitate the combination of these two methods in the future.

5. Perspectives and Conclusions

The study of PPIs is a rapidly evolving field. AP-MS and PL-MS possess different specificities that can be used according to the type of interactions studied. Moreover, every method has its own strengths and weaknesses. In the future, it is likely that new enzymes will be developed and current systems such as BioID and PL will be further optimized to enhance the applicability of such methods. For AP based methods, the most relevant improvements will be in the reduction of contaminants through new digestion/purification procedures. Another goal will be the extension of their use to different subcellular environments such as vacuoles or peroxisomes, as well as application of these methods in plant species other than in model species [75,77,80]. Another important aspect lies in regard to data analysis, there are a large number of computational tools available to analyze interaction proteomics data. For example, SAINT (Significance Analysis of INTeractome) is an approach based on spectral counting of protein–protein interactions from label-free quantitative proteomics data in AP-MS experiments [81]. Several bioinformatics methods for MS-based proteomics data analysis are well summarized at Chen et al. [82]. In conclusion, the two methods considered in this review offer a broad possibility to study the different interactions that occur in various organisms, shedding light on the complex mechanisms that underlie all biological processes.

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Colonization of *Solanum melongena* and *Vitis vinifera* Plants by *Botrytis cinerea* Is Strongly Reduced by the Exogenous Application of Tomato Systemin

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Abstract: Plant defense peptides are able to control immune barriers and represent a potential novel resource for crop protection. One of the best-characterized plant peptides is tomato Systemin (Sys) an octadecapeptide synthesized as part of a larger precursor protein. Upon pest attack, Sys interacts with a leucine-rich repeat receptor kinase, systemin receptor SYR, activating a complex intracellular signaling pathway that leads to the wound response. Here, we demonstrated, for the first time, that the direct delivery of the peptide to *Solanum melongena* and *Vitis vinifera* plants protects from the agent of Grey mould (*Botrytis cinerea*). The observed disease tolerance is associated with the increase of total soluble phenolic content, the activation of antioxidant enzymes, and the up-regulation of defense-related genes in plants treated with the peptide. Our results suggest that in treated plants, the biotic defense system is triggered by the Sys signaling pathway as a consequence of Sys interaction with a SYR-like receptor recently found in several plant species, including those under investigation. We propose that this biotechnological use of Sys, promoting defense responses against invaders, represents a useful tool to integrate into pest management programs for the development of novel strategies of crop protection.

Keywords: crop protection; signaling peptide; plant defense; foliar application; hydroponics; antioxidant activity

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1. Introduction

The success of modern agriculture relies in part on discovery and adoption of pesticides for pest control [1]. However, the onset of different concerns on the impact of pesticides on the environment, biodiversity, as well as on human health, pressed the introduction of more stringent pesticide registration procedures. Furthermore, the tendency in European Union policy is to encourage the development of eco-friendly and sustainable control strategies to protect crops reducing chemical inputs [2]. One of the main challenges facing the agricultural sector is to reduce the negative impact on soil, water, and the atmosphere.

Sustainable strategies for pest control have been applied to agricultural practices, such as biological control. This approach includes the use of beneficial microorganisms

or bioactive compounds that bio-stimulate plant performance against pathogens by competing or by directly antagonizing them [3–6]. Some other alternative control strategies of plant diseases are based on the use of plant resistance inducers (PRIs, also called elicitors or plant defense/resistance activators), which offer the prospect of durable, broad-spectrum disease control [7]. PRIs can be chemical compounds [8] as well as biological stimulators [9] able to activate and/or prime plant defense responses by their exogenous application [10]. Depending on their nature, they either mimic plant downstream signaling molecules, such as phytohormone or derivates, or act as non-self molecules, classified as microbe/pathogen/herbivore-associated molecular patterns (MAMPs/PAMPs/HAMPs), or signals from damaged cells, generally referred to danger- or damage-associated molecular patterns (DAMPs) [11–14] or phytocytochines [15]. PRIs are recognized by plasma-membrane localized pattern recognition receptors (PRRs) to initiate signal transduction pathway [7]. One of the best characterized DAMP is systemin (Sys), an octadecapeptide synthesized as a part of a larger precursor protein, prosystemin (ProSys) [16,17]. Sys was isolated from tomato leaves and proved to be able to activate the octadecanoid pathway, which leads to the production of the plant hormone jasmonic acid (JA) and its derivatives, powerful activators of plant defense genes [18,19]. Transgenic tomato plants constitutively expressing *ProSys* proved to be resistant to insect herbivores and phytopathogenic fungi [20–22] and tolerant to moderate salt stress [23]. Homologs of the tomato *ProSys* gene have been identified only in some economically important species of Solanoideae subfamily, but other genetically distinct families of plant defense signal peptides have been described in several species [24–30].

Upon either pests or other environmental challenges cues, Sys interacts with a leucine-rich repeat receptor like-kinase (LRR-RLK), RLK SYSTEMIN RECEPTOR 1 (SYR1) and with lower affinity its homologous SYR2, triggering a complex intracellular signaling pathway that leads to the generation of early and late defense responses [31]. It was recently observed that although both SYR1 and SYR2 receptors are restricted to the species of Solanoideae subfamily (e.g., tomato, potato, eggplant, and pepper), other SYR-like genes are present in other plants species, including *Vitis vinifera* [31].

Sys perception at the cellular surface induces depolarization of the plasma membrane, mitogen-activated protein kinases (MAPKs), the opening of ion channels, with the consequent increase of intracellular Ca^{2+} concentration, and accumulation of reactive oxygen species (ROS) [32].

Since ROS participate in signaling events, they are highly reactive but also toxic to the cells. To control the level of ROS and protect cells under stress conditions, plants have developed a sophisticated ROS scavenging system that includes the activity of several enzymes such as catalase (CAT) and ascorbate peroxidase (APX) as well as non-enzymatic low molecular compounds such as phenolics compounds [33–36].

Eggplants (*Solanum melongena* L.) and grapevine (*Vitis vinifera* L.) are particularly susceptible to important fungal pathogens, among them *Botrytis cinerea*, the agent causing grey mold which diminish yield and depreciate quality throughout their entire biological cycle [37,38]. Phytochemicals are commonly used to prevent and reduce the damages of this pathogen infection, but pathogen strains with pesticide-resistance have been reported [39,40]. In an effort to protect crops from such a dangerous enemy and yet reduce the impact of chemicals on the environment, considerable interest has been focused on the identification of novel biotechnological tools that use elicitors to strengthen the endogenous defenses of plants. In this work, we demonstrated that the direct delivery of Sys to *Vitis vinifera* and *Solanum melongena* plants strongly reduces *B. cinerea* plant colonization.

2. Materials and Methods

2.1. Peptides

Two different purified peptides were assayed: Systemin (Sys) and its scrambled form (Scp) that does not activate the plant defense response in tomato. Peptides synthesis, purification, and stability are reported elsewhere [41].

2.2. Plant Materials and Growth Conditions

The eggplant variety used was "Violetta Lunga". For this crop, two different growth systems were carried out: In soil and in hydroponic culture.

Seeds were germinated in Petri dishes on wet sterile paper and kept in the dark for three days in a growth chamber at 24 ± 1 °C and 60% relative humidity (RH). Upon roots emergence, for soil culture, eighteen plantlets were transferred to a polystyrene plateau with inert substrate S-type (Floragard, Oldenburg, Germany) in a growth chamber at 26 ± 1 °C and 60% RH with a photoperiod of 18/6 h light/dark. After two weeks, plants were transplanted in pots of 9 cm diameter with sterile soil mixture using the same growth conditions. For hydroponic culture, eighteen plantlets of 2 cm were transferred to hydroponic system and divided into three different plastic containers (5 L) supplemented with $Mg(NO_3)_2 \cdot 6H_2O$ (384 mg/L), $Ca(NO_3)_2 \cdot 4H_2O$ (812.9 mg/L), KNO_3 (101.5 mg/L), K_2SO_4 (319.3 mg/L), KH_2PO_4 (204.8 mg/L), Hydromix (14.0 mg/L). Four weeks-old plants were used for biological and molecular investigations unless otherwise indicated.

Grapevine, cultivar "Cabernet Sauvignon" cuttings (rootstock genotype 101.14 CL. 759), were grown in a greenhouse in pots of 20 cm diameter until they developed six to eight leaves. The second and third youngest adult leaves from each cutting were used for biological and molecular investigations.

2.3. Plant Treatments with Peptides and Botrytis cinerea Assay

Intact leaves of eggplant and grapevine plants grown in soil were treated with 100 pM of Sys or Scp peptides in PBS buffer (phosphate buffer saline, 10 mM phosphates, 140 mM NaCl, 2.7 mM KCl, pH 7.4, Sigma-Aldrich, Milan, Italy) while to eggplants growing in hydroponics, peptides were added into a nutrient solution at the same final concentration. Control plants were similarly treated with PBS buffer.

Four weeks-old plants, leaf-treated or grown in hydroponics enriched with the Sys or Scp, were tested for resistance to the necrotrophic airborne pathogen, *B. cinerea*, as already reported [42]. The assay used five leaves per treatment 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 ± 1 °C under 16/8 h light/dark photoperiod and 90% RH as also described by [43,44].

Necrosis areas were measured at 1, 3, 5, and 7 days post inoculum (pi) with a digital caliber (Neiko 01407A, Neiko Tools, Taiwan, China).

2.4. In Vitro Antifungal Assay

The antifungal assay was carried out as already reported [45]. Briefly, a sterile 12-well plate was filled with potato dextrose broth (PDB 1/2) medium containing Sys and Scp peptides at the final concentration of 100 pM. A solution with *B. cinerea* spores was added to each well in order to reach a final concentration of 10^4 spores/mL in each well, the plate was placed in a shaker and incubated for 24 h at 25 ± 1 °C. To assess the fungal growth, the value of optical density (OD) at a wavelength of 600 nm was measured in triplicate on a BioPhotometer Spectrophotometer UV/VIS (Eppendorf, Hamburg, Germany).

2.5. Gene Expression Analyses

Total RNA extraction, single-strand cDNA synthesis, and quantitative reverse transcription (RT)-PCR were performed as already reported [46]. Expression analysis of selected defense-related genes was monitored 3 h and 6 h after Sys foliar and hydroponic application, respectively. Gene expression analysis was carried out using two technical replicates for each of the three biological replicates. Relative quantification of gene expression was carried out using the comparative method with the $2^{-\Delta\Delta Ct}$ formula [47] where ΔCt = Ct target gene—Ct endogenous control and ΔΔCt = ΔCt sample—ΔCt calibrator. The housekeeping *APRT* (*adenine phosphoribosyl transferase*) and the *EF-1α* (*elongation factor-1α*) genes were the endogenous reference genes, respectively, for eggplant and grapevine plants,

2.6. Biochemical Analyses

Total phenolic content (TPC) and antioxidant enzyme activities were assessed spectrophotometrically in treated leaves of eggplant and grapevine plants collected at various time intervals: 1, 3, 6, and 24 h after peptides treatment using three technical replicates for each of the three biological replicates. Untreated leaves were used as control.

For the extraction of total soluble proteins, frozen leaf sample (0.1 g) was ground with 1 mL ice-cold 50 mM $KHPO_4$ (pH 7.8) containing 0.1 mM EDTA. Homogenates were centrifuged at 14,000 rpm for 20 min at 4 °C.

Protein concentration was measured by the Bradford method using bovine serum albumin as a standard protein [48]. TPC was evaluated by using Folin–Ciocalteu colorimetric method as described before [49].

The catalase (CAT) activity was measured following the previously described protocols [50,51], monitoring the decrease in absorbance at 240 nm. Ascorbate peroxidase (APX) activity was analyzed by measuring the decrease in absorbance at 290 nm monitored according to the method previously described [52].

2.7. Statistical Analyses

For the evaluation of Sys effect on *B. cinerea* growth and infection, necrosis area differences between controls and Sys-treated or Scp-treated sample were compared and analyzed by one-way Analysis Of Variance (ANOVA) coupled with Tukey–Kramer Honestly Significant Difference (HSD) test. Differences in relative quantities of defense transcripts were analyzed by comparing ΔCt values for all the replicates of tests and controls using a two-tailed Student's t-test. Moreover, the quantification of the amount of total phenolic content and the evaluation of the activities of antioxidant enzymes were analyzed by one-way ANOVA coupled with Tukey–Kramer multiple comparisons test. Error bars referring to standard error have been displayed.

3. Results

3.1. Systemin Exogenous Supply Reduces B. cinerea Colonization of Eggplant and Grapevine Leaves

The performance of Sys-treated eggplants and grapevine against *B. cinerea* was evaluated at 1, 3, 5, and 7 days post inoculum (pi). The assay was carried out using detached leaves harvested 6 h after peptides, Sys or Scp, application to intact plants [41,42]. Disease severity was quantified by measuring the necrotic leaf areas caused by fungal colonization. In eggplants, as shown in Figure 1, Sys significantly reduced the lesions since five days pi (Figure 1A), whereas in leaves deriving from hydroponic cultures, a reduction of the lesions was evident already 24 h pi (Figure 1B). No differences were observed for eggplants treated with buffer and Scp-peptide. Similarly, grapevine Sys-treated leaves displayed a marked reduction of *B. cinerea* induced lesions after seven days pi compared with the control ones (Figure 1C). Likewise to the previous experiment, no effect was detected in Scp or buffer treated leaves. These results demonstrate that the exogenous supply of Sys peptide to healthy plants reduced disease severity.

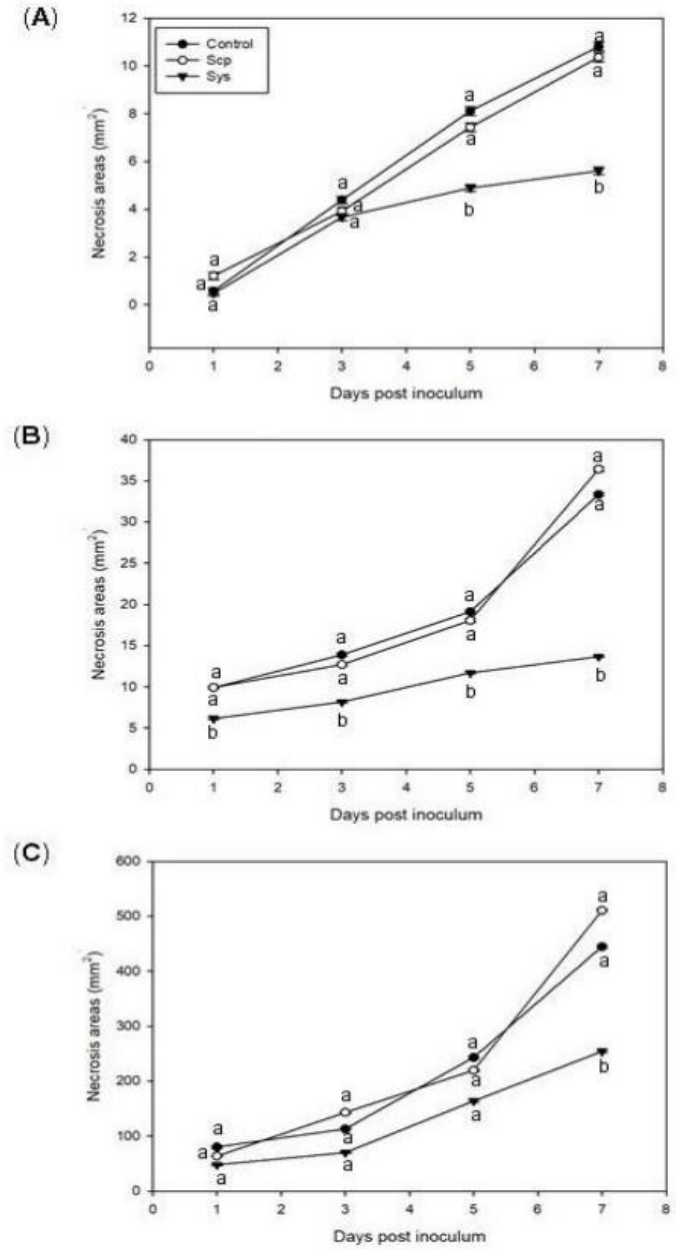

Figure 1. *Botrytis cinerea* necrosis area assay. Sys was applied to eggplant leaves (**A**) or added to hydroponic solution (**B**), while for the grapevine plants, only leaves were treated (**C**). Response to *B. cinerea* infection on leaves from plants treated with 100 pM Sys or Scp or Control (PBS 1X). The graph displays the average (±S. E., standard error) of the lesion size at 1, 3, 5, and 7 days post-inoculation. Letters indicate statistically significant differences (one-way Analysis of Variance, ANOVA, Tukey–Kramer Honestly Significant Differences (HSD) test with $p < 0.05$). Error bars indicate standard error.

Moreover, in order to evaluate whether the reduction of *B. cinerea* necrosis area was due to a direct antimicrobial effect of the Sys peptide on the fungus, an in vitro assay to measure fungal growth in the presence of Sys and Scp peptides was carried out.

As shown in Figure 2, Sys peptide did not directly impact fungus vitality. The growth of *B. cinerea*, monitored by measuring the absorbance at 600 nm, was similar in all three treatments. This result indicates that the observed reduction of *B. cinerea* plant colonization is determined by the induction of plant endogenous defenses upon Sys treatment.

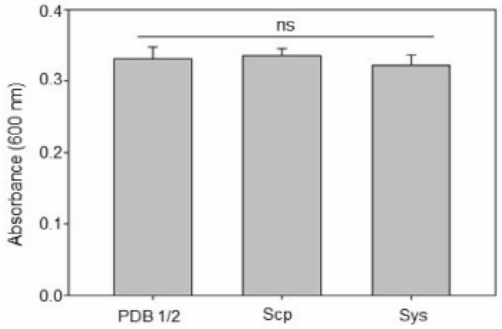

Figure 2. In vitro antifungal vitality assay. Each 12-well sterile plate was filled with 1 mL of PDB 1/2 medium containing the peptides at the final concentration of 100 pM, except for the broth sterility control wells. Thereafter, spores of *B. cinerea* were added to each well, and fungal growth was assessed 24 h after pathogen inoculation by evaluating the optical density (OD) of the medium at 600 nm. Letters indicate statistically significant differences (one-way ANOVA, Tukey–Kramer Honestly Significant Differences (HSD) test with $p < 0.05$; ns, not significant). Error bars indicate standard error.

3.2. Systemin Exogenous Supply Activated the Expression of S. melongena and V. vinifera Defense-Related Genes

In order to verify the ability of Sys-treatments to induce the expression of defense-related genes, we performed a qRT-PCR of selected genes for the two plant species. The genes analyzed were: *Allene Oxide Synthase* (*AOS*), *Wound-induced proteinase inhibitor I* and *II* (*Pin I* and *Pin II*), *Pathogenesis-related protein 4* (*PR4*), *Dihydroflavonol 4-reductase* (*DFR*) and *Polyphenol oxidase* (*PPO*) for eggplants, the *basic-helix-loop-helix* (*bHLH*) *transcription factor* (*TF*) (*MYC2*), *AOS*, *Pin I*, *Pin II*, *PR4*, *Phenylalanine ammonia-lyase* (*PAL*) and *Flavonol synthase 5* (*FLS5*) for grapevine plants. The expression of the target genes was analyzed at two time intervals after treatment. Relative quantification of treated samples was referred to the mock-treated control (relative quantification, RQ = 1).

As shown in Figure 3A, in eggplants, a strong increase of *AOS* transcript was recorded 3 h after Sys application followed by a reduction of the transcript after 6 h from peptide application. Conversely, the expression profile of *Pin I* and *II* showed a gradual increase in their transcripts that reached the highest expression level 6 h after Sys treatment. Moreover, *PR4*, *DRF*, and *PPO* transcripts resulted significantly up-regulated (Figure 3A). We also monitored the expression of the same genes in leaves of eggplants grown in hydroponics enriched with the peptide. As shown in Figure 3B, *Pin I*, *Pin II*, and *PR4*, transcripts resulted significantly up-regulated after 6 h and no significant variation in transcript level was recorded for the other three genes.

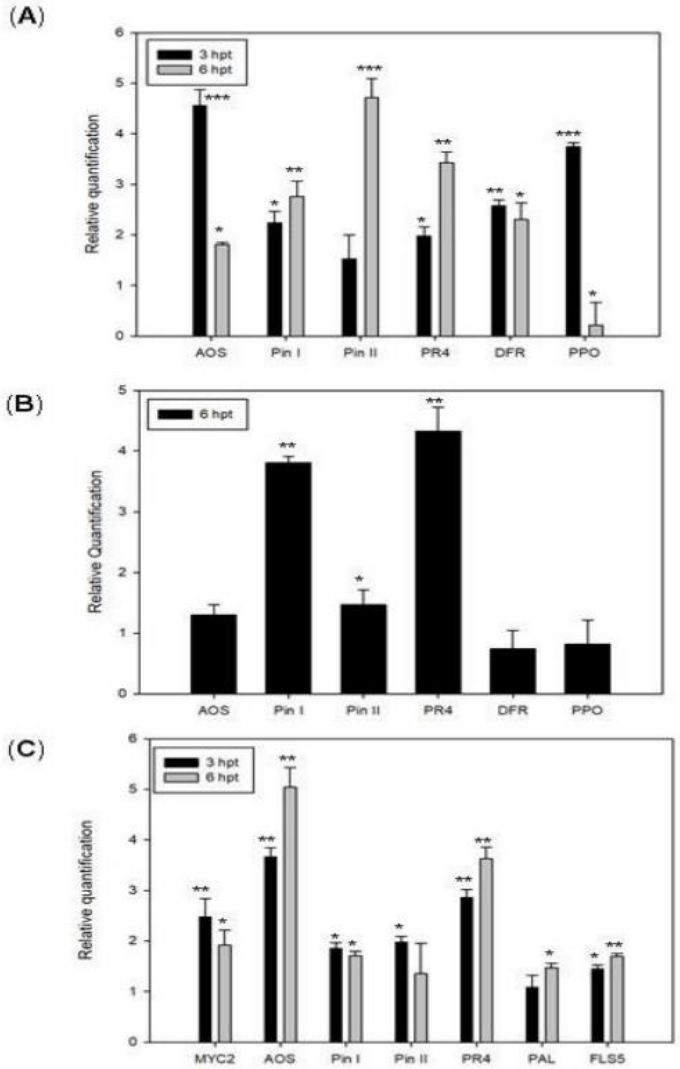

Figure 3. Expression analysis of defense-related genes following Sys treatments (100 pM) on eggplants and grapevine plants. Relative gene expression of defense-related genes by qRT-PCR in eggplants-treated leaves (**A**), in leaves of eggplants grown in a hydroponic system (**B**) and in grapevine-treated leaves (**C**). Quantities are relative to the calibrator control condition, mock-treated plants. Asterisks indicate data statistical significance (Student's *t*-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Error bars indicate standard error.

Figure 3C shows the results of the gene expression analyses in treated leaves of grapevine plants. All the target transcripts resulted significantly up-regulated. Taken together, the results demonstrate that Sys, under two different delivery systems, is able to induce the transcription of defense-related genes in both plant species.

3.3. Systemin Increases the Production of Total Soluble Phenolic Content and Antioxidant Capacity in Treated Eggplant and Grapevine

We quantified the amount of total phenolic content (TPC) and analyzed the activities of some key antioxidant enzymes that are responsible for rapid scavenging of ROS. Sys induced in treated plants a rapid antioxidant response, the TPC pool increased significantly by about 70% in eggplants (Figure 4A) 3 h after Sys application while the response of grapevine plants was more rapid with the increase of TPC after 1 h of roughly 16%. In addition, the TPC content in the treated plant species reached the highest content 3 h after Sys treatment (Figure 4, Table S2). On the contrary, as expected, the application of Scp peptide to the plants did not induce any TPC content variation (Figure 4, Table S2).

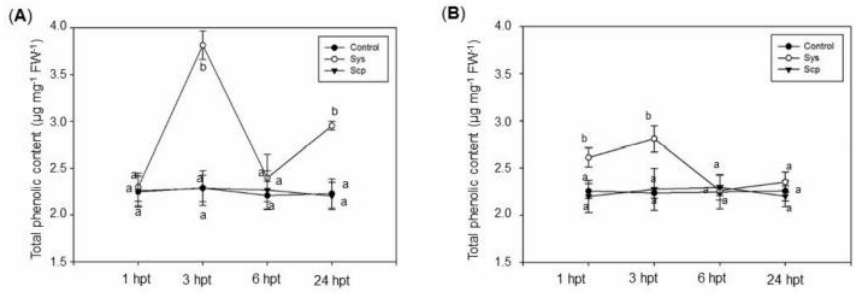

Figure 4. Total phenolic content (TPC) in eggplant (**A**) and grapevine (**B**) leaves treated with Sys. TPC was measured in control (PBS1X) and in treated leaves at 1, 3, 6, and 24 h after 100 pM Scp or Sys application. Letters indicate statistically significant differences (one-way ANOVA, Tukey test with $p < 0.05$). Error bars indicate standard error.

In addition to the investigation on the non-enzymatic components that regulate redox status, we monitored two enzymes that are included in the other arm of the antioxidant defense machinery. A significant increase in the activities of CAT and APX enzymes was observed in eggplant-treated leaves, respectively, of about four times and 100 times higher than control, 1 h and 6 h following Sys application, respectively (Figure 5A,B, Table S3). A different profile of CAT activity was observed in grapevine-treated leaves, which showed a steady increase after 3 h up to 40 times higher the control value 24 h post-treatment (Figure 6A, Table S4). In the same species, a significant increase in APX, about 11 times control value, was observed 24 h post-treatment (Figure 6B, Table S4). No significant variation in the activity of those enzymes was registered in leaves treated with Scp (Figures 5 and 6, Tables S3 and S4).

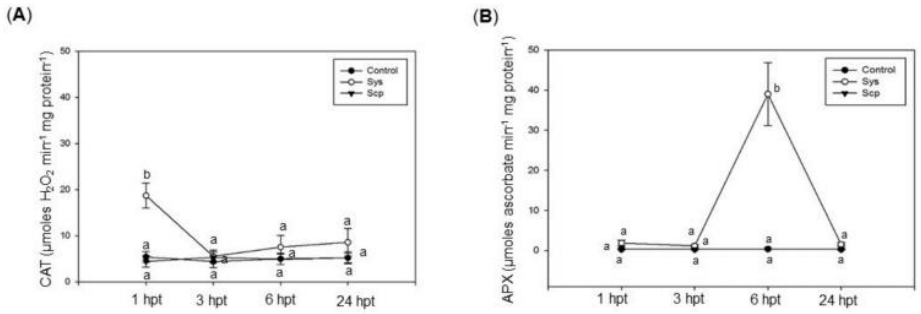

Figure 5. Catalase (CAT) (**A**) and ascorbate peroxidase (APX) (**B**) activity at various time intervals in eggplant leaves treated with Sys. CAT and APX activity was assessed in control leaves (PBS1X) and in treated leaves at 1, 3, 6, and 24 h after 100 pM Sys and Scp application. Letters indicate statistically significant differences (one-way ANOVA, Tukey test with $p < 0.05$). Error bars indicate standard error.

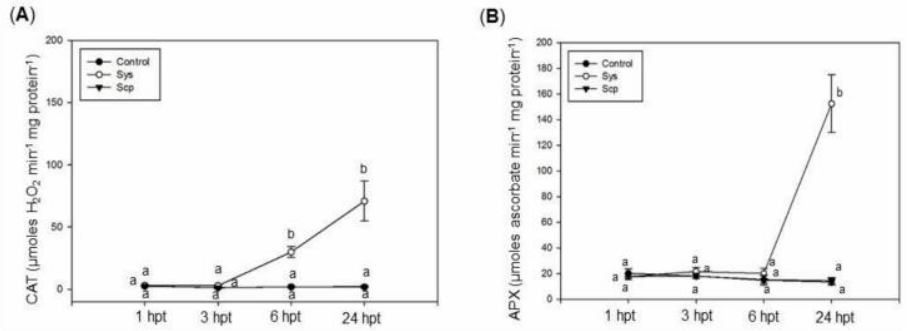

Figure 6. Catalase (CAT) (**A**) and ascorbate peroxidase (APX) (**B**) activity at various time intervals and grapevine leaves treated with Sys. CAT and APX activity was assessed in control leaves (PBS1X) and in treated leaves at 1, 3, 6, and 24 h after 100 pM Sys and Scp application. Letters indicate statistically significant differences (one-way ANOVA, Tukey test with $p < 0.05$). Error bars indicate standard error.

4. Discussion

The development of safe and sustainable crop protection strategies is a challenging goal facing our society. This is increasingly pursued through bio-inspired research efforts, aiming to mimic natural mechanisms of pest suppression by exploiting biotechnological applications of biomolecules active in plant defense [53]. A promising control strategy is based on the application of elicitors to the plant that stimulate and/or potentiate plant defense responses affecting the fitness and behavior of herbivores and pathogens [42,54].

Among pathogenic plant agents, the necrotrophic fungus *B. cinerea* is a very dangerous fungus that infects many economically important crops, such as grapevine, strawberry, tomato, and eggplant. Grapevine is one of the major fruit crops in the world based on hectares cultivated with this crop and its economic value [55]. The species is particularly sensitive to various pathogenic fungi, including *B. cinerea* that causes significant losses in terms of production and quality. This pathogen is controlled by fungicide treatments, but pathogen strains with fungicide resistance have been reported [39]. Eggplant is one of the most important vegetable crops, especially for the Mediterranean basin, after potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) [56]. The plants are very susceptible to important fungal pathogens, including *B. cinerea*, throughout their entire biological cycle and the fungal control has been adversely affected by the development of fungicide resistance [40]. Therefore, the identification of novel biotechnological tools able to protect these crops from such a dangerous enemy is of great importance.

In this paper, we investigated the ability of tomato Sys to protect *S. melongena* and *V. vinifera* plants from *B. cinerea*, demonstrating, for the first time, that the exogenous supply of the peptide to intact healthy plants severely counteracted fungal growth. This is likely the consequence of the induction of plants defense-related genes that promote the accumulation of compounds active in plant defense [10,57]. Consequently, Sys-treated plants respond more effectively than controls when exposed to biotic stress. Both peptide delivery systems (leaf application or hydroponics uptake) proved to be very effective in conferring measurable protection against the necrotrophic fungus. The absence of inhibition of mycelium growth in the presence of Sys fully excluded that the peptide has a direct effect on the fungus. Therefore, the observed reduction of plant colonization is likely the consequence of the activation of plant endogenous defenses following Sys treatment. As a matter of fact, we observed the induction of a set of defense-related genes. *AOS*, a gene of the octadecanoid pathways, leads to the biosynthesis of JA that subsequently activate the late defense genes *PPO*, *Pin I*, and *Pin II*. Tomato *PPO* is induced by Sys and jasmonate, and it is involved in defense against pests [58,59]. In addition, *PPO* and *protease inhibitors* (*PIs*) are up-regulated by tobacco Sys as well as by the endogenous supply of a JA derived compound, the methyl jasmonate (MeJA) [60,61]. It has been demonstrated that PIs are very effective against *B. cinerea* both in vitro and in vivo: PIs isolated from

young cabbage leaves were able to inhibit *B. cinerea* spore germination and germ tube elongation in vitro [62], whereas a strong inhibitory activity of a PIs mixture purified from tuber sprouts was observed against *B. cinerea* spore germination, germ tube elongation, and necrotic symptom development in vivo [63]. We also observed that the exogenous supply of Sys, under two different delivery systems, is able to induce the transcription of *PR4* genes in the two species. Pathogenesis-related proteins are a group of proteins involved in higher-plant responses to biotic stresses, whose expression is triggered by several pathogens, including fungi, bacteria, and viruses [64]. Many in vitro studies revealed that over-expression in various crops of *PR* genes (*PR2*, *PR3*, *PR4*, *PR5*, *PR12*), alone or in combination, leads to enhanced disease resistance against biotrophic and necrotrophic fungal phytopathogens [65]. Therefore, the disease reduction observed in our experimental plants is likely due, at least in part, to the increased level of protease inhibitors, polyphenol oxidase and PR4. Sys-treated eggplants showed an increased level of *DFR* transcript. *DFR*, together with *PAL*, *CHS*, *CHI* represents an essential component of the anthocyanin biosynthetic pathway. Developmental stages, diverse stresses, such as drought, temperature, wounding, and pathogen attack, are known to regulate anthocyanin biosynthesis. Previous studies showed that MeJA significantly induces anthocyanin accumulation through the up-regulation of genes encoding for anthocyanin biosynthetic enzymes, such as *DFR*, *LOX*, and *UF3GT* [66,67]. Sys-treated eggplants likely increase the MeJA production that may modulate the anthocyanin biosynthetic pathway [68].

Moreover, in grapevine, we observed that Sys application activated the phenylpropanoid pathway, as shown by the increased level of *PAL* transcript, and the induction of *MYC2* and *FLS5* genes. PAL, the first enzyme of the phenylpropanoid pathway, is involved in the biosynthesis of secondary metabolites, especially the production of phytoalexins and salicylic acid (SA) which were proposed to reduce the incidence of plant disease through antifungal activity and to stimulate plant defense responses, respectively [69,70]. It has also been shown that priming of *PAL1* is associated with responses to pathogen infection and wounding [71]. Interestingly, it was recently demonstrated that the exogenous application of MeJA in grapevine raises *PAL* gene expression and the consequent accumulation of several bioactive compounds (e.g., total phenolic and anthocyanin concentration) [72,73]. Therefore, in grapevine like in eggplant, Sys may induce an increase of MeJA that likely contribute to the accumulation of defense compounds. In addition, the up-regulation of *MYC2*, in Sys-treated grapevine plants, linked to the observed disease reduction, confirmed that this transcription factor is required for JA-mediated defense responses against the necrotrophic fungus *B. cinerea* [74].

Flavonols are the most abundant component of flavonoids, important secondary metabolites with a myriad of functions, including plant defense following pathogen attack, thanks to their antioxidant properties [75]. The increased level of *FLS5* transcripts registered in Sys-treated grapevine plants may favor the accumulation of these compounds that reduce disease severity following fungal infection.

Taken together, the most likely explanation of these results is the ability of Sys to bind SYR-like receptors or closely related genes recently identified in eggplants and grapevine plants, besides other plant species [31]. Following Sys-SYR interaction, the initiated signaling pathway leads to the systemic defense responses by the induction of JA synthesis that triggers the plant defense machine able to reduce the growth of with *B cinerea*.

It was previously shown that in tomato Sys causes very rapid changes in cellular redox homeostasis with the generation of excessive ROS [76,77], which may damage cell organelles. Since our data show that Sys is perceived by both eggplants and grapevine plants, in Sys treated plants, ROS likely increased and the plants reacted by activating the antioxidant defense machinery that boosted the TPC and the activity of CAT and APX enzymes, two key actors of the enzymatic H_2O_2 scavenging mechanism in plants [78].

Generally, in plants, the metabolism of H_2O_2 is controlled by several antioxidant scavenging enzymes, such as SOD, APX, and CAT [79,80]. The increased level of CAT and APX activities observed in treated plants of both species is likely functionally related to the cell requirement of a reduction of redox potential caused by Sys treatment. Similarly, the increased level of phenolic compounds may be linked to this function. In fact, they participate as antioxidants in the prevention of the plant from suffering molecular damage caused by microorganisms, insects, and herbivores [81]. In addition, it is worth noting that phenolic compounds play an important role in plant disease resistance responses representing an early defense plant reply to several biotic stresses [82]. As they are toxic to pathogens, their accumulation at the infection site can restrict pathogen development and the successive plant colonization or contrast infections by increasing the mechanical strength of the host cell wall [83]. Jasmonates (JAs), or their derivates, enhance the accumulation of phenolic compounds in different plant species contributing to the resistance against *B. cinerea* [84,85] and have a pivotal role in the reduction of H_2O_2 level by the enhancement of antioxidant enzymes activity in plant cells [86,87]. Sys-treated plants likely increase the JAs production that may modulate the activity of CAT and APX antioxidant enzymes in both plant species. Previous studies showed that the application of MeJA to in vitro cultures induced not only the expression of defense-related genes but also the antioxidant enzyme activity and the over-production of secondary metabolites [86]. Our results demonstrate the increase of both phenolic content and the antioxidative activity of CAT and APX enzymes likely determined by the activation of the JA pathways triggered by Sys treatment. In our experimental conditions, the increased level of TPC likely contributed to the observed reduction of damages on Sys treated leaves [84,88].

In conclusion, tomato systemin induces resistance against *B. cinerea*, indicating that the two species perceive the non-self-peptide and activate the defense and the antioxidant machineries. These results open a novel perspective on the use of plant peptides in crop protection. From an applied perspective, the exogenous delivery of plant signaling peptides integrated into pest management programs may offer a useful contribution to the reduction of chemical pesticide both in greenhouses and in the field.

Supplementary Materials: The following are available online at https://www.mdpi.com/2309-608X/7/1/15/s1. Table S1: Oligonucleotide sequence, gene symbol, accession number and plant species; Table S2: Effect of systemin peptide on total phenolic content at different times of leaf treatment in eggplant and grapevine plants; Table S3: Effect of systemin peptide on catalase (CAT) and ascorbate peroxidase (APX) activity at different times in eggplant treated leaves; Table S4: Effect of systemin peptide on catalase (CAT) and ascorbate peroxidase (APX) activity at different times in grapevine treated leaves.

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Ethical Statement: The plant species and variety used in this experiment is a widely cultivated variety and we have followed all proper ethical standard. All of the reagent and fertilizers used are properly recommended by the authority.

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Tomato Plants Treated with Systemin Peptide Show Enhanced Levels of Direct and Indirect Defense Associated with Increased Expression of Defense-Related Genes

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Abstract: Plant defense peptides represent an important class of compounds active against pathogens and insects. These molecules controlling immune barriers can potentially be used as novel tools for plant protection, which mimic natural defense mechanisms against invaders. The constitutive expression in tomato plants of the precursor of the defense peptide systemin was previously demonstrated to increase tolerance against moth larvae and aphids and to hamper the colonization by phytopathogenic fungi, through the expression of a wealth of defense-related genes. In this work we studied the impact of the exogenous supply of systemin to tomato plants on pests to evaluate the use of the peptide as a tool for crop protection in non-transgenic approaches. By combining gene expression studies and bioassays with different pests we demonstrate that the exogenous supply of systemin to tomato plants enhances both direct and indirect defense barriers. Experimental plants, exposed to this peptide by foliar spotting or root uptake through hydroponic culture, impaired larval growth and development of the noctuid moth *Spodoptera littoralis*, even across generations, reduced the leaf colonization by the fungal pathogen *Botrytis cinerea* and were more attractive towards natural herbivore antagonists. The induction of these defense responses was found to be associated with molecular and biochemical changes under control of the systemin signalling cascade. Our results indicate that the direct delivery of systemin, likely characterized by a null effect on non-target organisms, represents an interesting tool for the sustainable protection of tomato plants.

Keywords: biopesticide; endogenous defenses; insect herbivores; phytopathogenic fungi; parasitoids; tomato protection

1. Introduction

The use of synthetic pesticides has significantly fostered the success of modern agriculture, but has concurrently shown that their abuse generates a number of ecological, environmental and health problems. Growing awareness of these critical issues in public opinion and among policy makers was the background that a decade ago led to the definition of EU directive (2009/128) on sustainable use of pesticides. The consequent need to reduce pesticide use in agriculture has considerably promoted research efforts aiming to discover new plant protection tools with low impact on the environment and non-target organisms. These research efforts have increasingly shed light on the mechanisms underlying antagonistic interactions in nature, offering the opportunity to use the molecular weapons adopted by the fighting organisms, shaped by a long co-evolutionary history. We can define this approach as learning from nature to develop bio-inspired strategies of pest management [1,2].

The detection of invading organisms is a crucial step in plant immunity, which initiates the activation of defense responses. Herbivore-associated elicitors (HAE) are molecules recognized by the plant, each selectively inducing different segments of the defense reaction pathways [3]. Anti-herbivore defenses are induced not only by molecules produced by the invading organisms, but also by endogenous plant molecules, that are released upon damage caused by pest insects and pathogens and, therefore, are commonly referred as damage-associated molecular patterns (DAMP) [4]. These molecules, act as warning signals [5–7]. The effective amplification of this signal and of the triggered defense responses is under control of enzymatic cascades, which are up-regulated by the feeding damage. For example, reactive oxygen species (ROS) signals are produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [8], while cell wall fragments originate in the activity of polygalacturonase; both enzymes are induced by mechanical wounding or by biotic stress agents [9,10], which concurrently induce downstream genes encoding the precursors of endogenous peptide elicitors [7,11]. Peptides are the smallest biological molecules of the plant proteome that fulfill diverse roles in plant growth, development, reproduction, symbiotic interactions, and stress responses [12–14]. For example, Pep1 from *Arabidopsis thaliana* (*Arabidopsis*) was shown to activate defense genes associated with the innate immune response [11]. Systemin (Sys) was the first peptide signal discovered in plants [7]. It is an 18 amino acid peptide, which derives from the carboxy terminus of a 200 amino acid precursor called prosystemin (ProSys). The *prosystemin* gene evolved in species of the Solaneae subtribe of the *Solanaceae* family, including tomato, potato, bell pepper, nightshade, but it is not found in tobacco or *Arabidopsis* [7]. Upon wounding or insect attack, the expression of *ProSys* is increased and the encoded protein precursor is processed to release the Sys peptide, which interacts with a membrane-bound receptor to initiate a complex signalling cascade that leads to the production of defense compounds [15–17]. Perception of Sys at the cell surface stimulates cell membrane depolarization, which induces an efflux of K^+ and influx of Ca^{2+} into the cell [15].

Sys and JA appear to contribute to the propagation of the long-distance signal; systemin acts at the site of wounding to trigger the production of JA that, in turn, promotes a long distance defense response [18,19]. The regulation of Sys production and release is still largely unknown, but the enzymatic processing of its precursor appears to be mediated by phytaspases [20]. SR160/BRI1 has previously been postulated as the systemin receptor in tomato [16,21]), a rejected hypothesis recently replaced by the systemin receptor 1 and 2 (SYR1 and SYR 2) proposed by [17].

This defensive cascade has been described for tomato where Sys is not the unique signaling peptide. For example, the hydroxyproline-rich systemin glycopeptides (HypSys) are 18–20 amino acids in length, released from larger precursors, isolated from tomato and other plant species and active in plant defense [22,23]. Sys and HypSys work cooperatively to upregulate the systemic wound defense response in tomato [24].

Moreover, other genetically distinct families of plant defense signal peptides have been described in several species [11,14,22,25–28].

The modulation of direct and indirect defenses exerted by Sys in tomato plants under insect attack has been widely characterized [15,29–32]. Sys signaling flows into the promotion of direct and indirect defense responses against Lepidoptera larvae, aphids and phytopathogenic fungi [14,30,33] that include the production of protease inhibitors (PIs) and other compounds interfering with herbivore larval growth and survival and fungal colonization of the plant [29,30,34,35]. In addition, the Sys-mediated indirect defenses involve the modification of the composition of the volatile blend emitted by tomato plants with the consequent increase of attractiveness towards herbivore natural antagonists [36,37].

It was previously demonstrated that the constitutive expression of the prosystemin cDNA promotes the up-regulation of an array of defense genes, controlled by different signaling pathways conferring protection against both biotic and abiotic environmental challenges [30,35,38] although the plants showed some differences in phenotypes and physiology as, for example, reduced internodes elongation, delayed flowering time, reduced leaf area and stomatal conductance [38,39]. Thus, a single peptide hormone is capable of eliciting multiple defense pathways to counteract a wide range of unfavourable conditions for the plant. Therefore, the over-expression of *ProSys* in tomato plants is a valuable tool to reduce the loss inflicted by different biotic stressors. However, the continuous activation of the prosystemin gene that are normally induced by pests is costly, affecting the growth and the physiology of tomato plants. To develop an alternative delivery strategy, not relying upon transgenic plants, we investigated the effect of the exogenous application of the Sys peptide on the defense responses and its potential use as a plant protection strategy in tomato.

Here we demonstrate that Sys-treated plants, by spotting the peptide on intact leaves of healthy plants or by supplying it through hydroponic cultures, are resistant to the noctuid moth *Spodoptera littoralis* (*S. littoralis*) and to the fungal pathogen *Botrytis cinerea* (*B. cinerea*) and show an increased production of volatile compounds able to attract insect natural enemies. The resistant phenotype of treated plants is associated with the expression of an array of defense-related genes induced upon systemin treatment. These results prove that the use of the exogenous supply of Sys to tomato plant represents an interesting approach for the protection of the crop.

2. Results

2.1. Sys Supply Promotes Direct Defenses against Spodoptera littoralis

In order to assess the impact of Sys supply on the growth and mortality of *S. littoralis* larvae, a feeding bioassay was carried out, by comparing Sys-treated plants with untreated or Scp treated controls. Based on the gene expression results (see below), we decided to use 100 pM Sys solution, and larvae were fed with tomato leaves treated with this concentration of the peptide. The reduced weight gain was already evident after 5 days of feeding, and this consistent trend over time generated significant differences after day 15 (One Way ANOVA test: $p < 0.0001$, $F = 14.9$) (Figure 1A). Moreover, the survival rate of experimental larvae was significantly lower when fed on treated leaves than on controls (Log-rank (Mantel-Cox) test: $p < 0.0001$, $dF = 2$, $\chi 2 = 51.16$) (Figure 1B). After 25 days of feeding, the survival rate was as low as 25% in larvae fed on Sys-treated plants, compared to 90% and 97% for Scp and control plants, respectively (Figure 1B). Thus, Sys foliar application impairs both growth and survival of *S. littoralis* larvae.

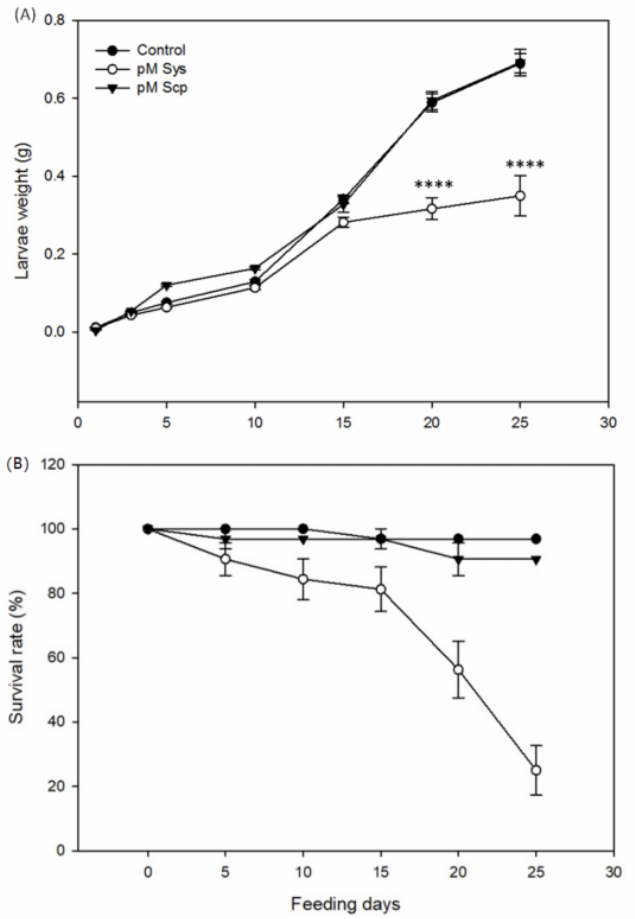

Figure 1. Systemin (Sys) foliar applications on *S. littoralis* larvae. (**A**) Mean weight (± S.E., standard error) of *S. littoralis* larvae feeding on control and treated leaves. (**B**) Survival rate of experimental *S. littoralis* larvae. Asterisks denote statistically significant differences (one-way Analysis of Variance, ANOVA: **** $p < 0.00001$). A group of 32 larvae was used for each experimental condition and the experiment was repeated twice.

Similarly, Sys supply in hydroponic cultures determined negative effects on larval growth and survival (Figure 2). Larvae fed with leaves from tomato plants kept on Sys-enriched hydroponics showed a significant reduction in weight starting 5 days after the onset of the bioassay (one-way ANOVA: $p < 0.0001$, $F(2.93) = 67.837$) (Figure 2A); the survival rate of larvae fed on hydroponics was significantly reduced if compared with the other two control groups (Log-rank (Mantel-Cox) test: $p < 0.023$; $df = 1$; $\chi 2 = 5.164$) (Figure 2B).

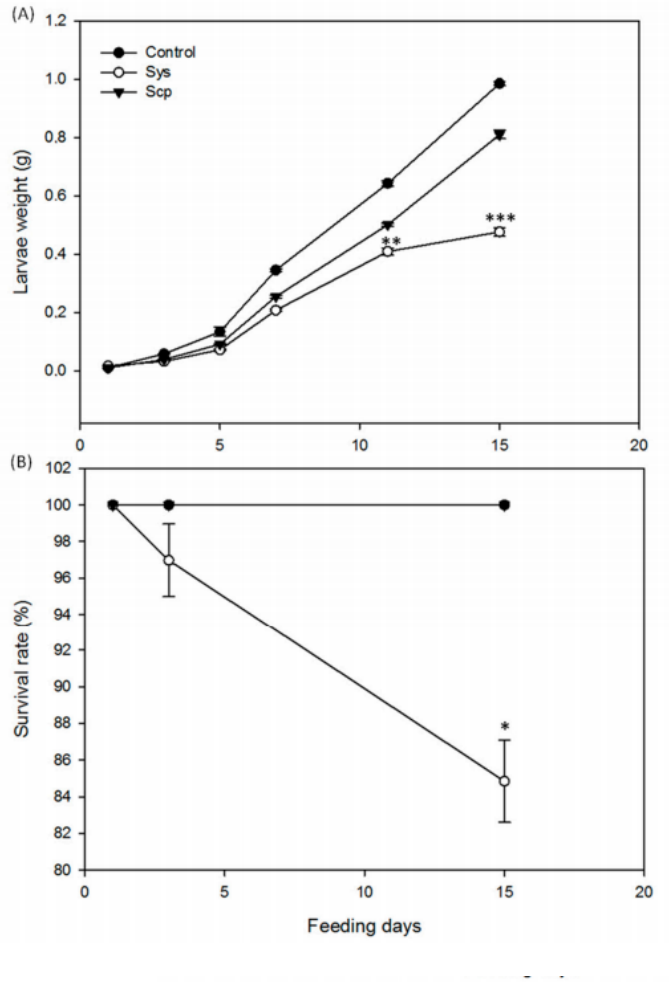

Figure 2. Effect on insect performance of systemin peptide supplied via hydroponics. Tomato plants were grown in hydroponic culture and supplied with 100 pM Sys or 100 pM Sys-scramble (Scp) or PBS1X. (**A**) Mean weight (± S.E.) of *S. littoralis* larvae feeding on tomato leaves. (**B**) Survival rate of experimental *S. littoralis* larvae. Asterisks denote statistically significant differences (one-way ANOVA: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). A group of 32 larvae was used for each experimental condition and the experiment was repeated twice.

The surviving experimental larvae were monitored for pupal development, adult survival and reproduction. Indeed, the time required by the experimental larvae to pupate was significantly higher in Sys treated plants (Kruskal-Wallis Test: $p < 0.0001$; $KW = 71.170$; $n = 32$) (Figure 3A). In addition, the emerged adults showed a significantly reduced survival rate (log-rank (Mantel-Cox) test: $p < 0.0001$, $dF = 2$, $\chi 2 = 45.04$) (Figure 3B) and a significantly lower fecundity (one-way ANOVA test $p < 0.0001$; $F(2.37) = 37.496$) (Figure 3C).

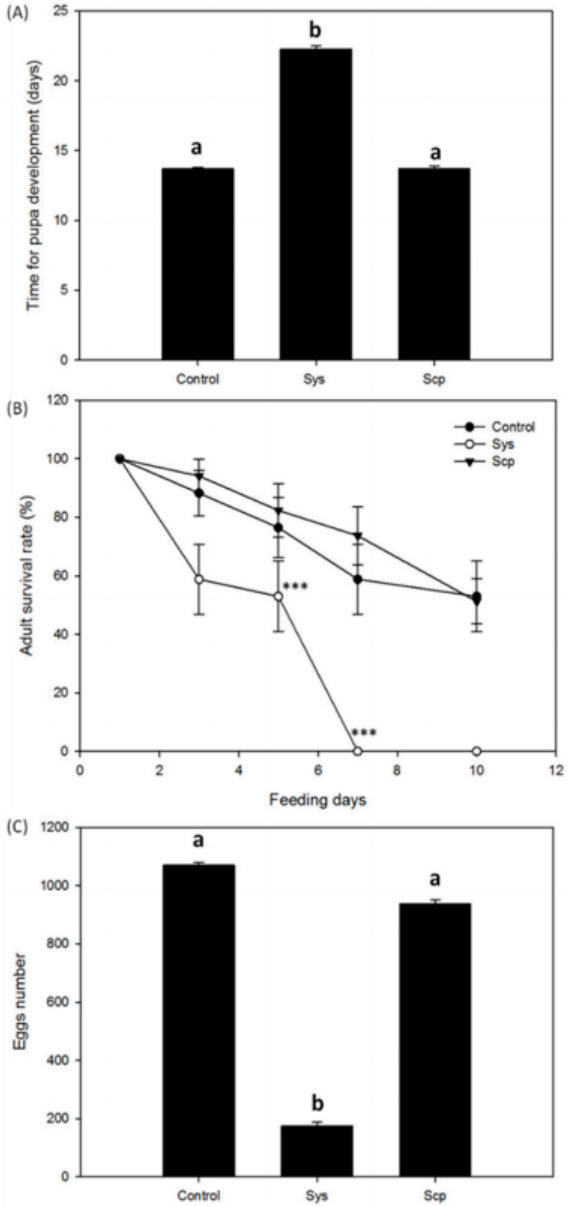

Figure 3. Systemin effect on development and reproduction of *Spodoptera littoralis* larvae. Tomato plants supplied with 100 pM Sys, or 100 pM Scp or PBS1X in hydroponics were used to feed *S. littoralis* larvae, on which the following parameters were scored: duration of pupal development (**A**), adult survival rate (**B**) and number of laid eggs (**C**). Letters and asterisks denote statistically significant differences (*** $p < 0.001$; one-way ANOVA). A group of 32 larvae was used for each experimental condition and the experiment was repeated twice.

2.2. *Sys Supply Enhances Plant Tolerance against Botrytis Cinerea*

Since tomato transgenic lines with over-expression or reduced expression of ProSys showed respectively increased resistance or increased susceptibility to *B. cinerea*, we evaluated the performance

of Sys-treated plants against this necrotrophic fungus, at four different time points (1, 3, 6 and 9 days pi). Disease severity was quantified by measuring necrotic areas. Sys-treated leaves displayed a marked reduction of *B. cinerea* induced lesions at all the time points considered (highest significant differences at six and nine days post inoculum with $p < 0.00001$) (Figures 4 and S1), similarly to what observed following the fungal inoculum on plant grown in hydroponic media enriched with the same concentration of Sys ($p < 0.05$) (Figure 5). Hydroponic supply of Scp did not produce any difference with controls. These results demonstrate that the hydroponic supply of the Sys peptide interferes with fungal growth following leaf colonization and reduce disease severity.

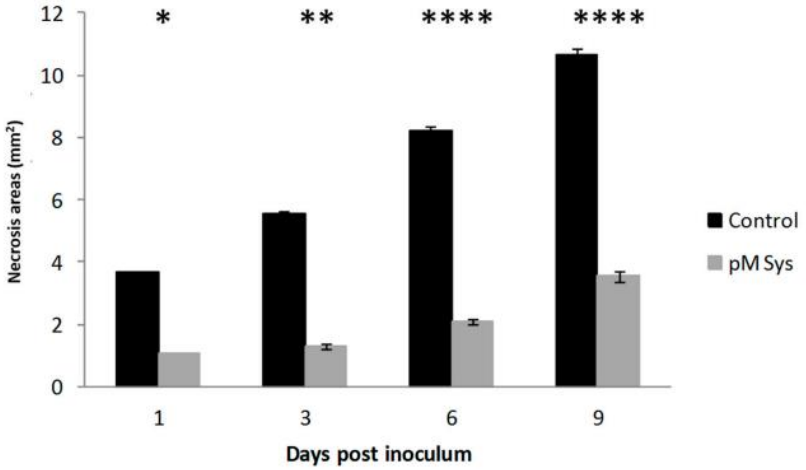

Figure 4. Enhanced resistance to *Botrytis cinerea* of Sys treated leaves. Response to *B. cinerea* infection by leaves of plant treated with 100 pM Systemin. The graphs display the average (± S.D.) of the lesion size at 1, 3, 6 and 9 days post inoculum. Asterisks denote statistically significant differences (T-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.00001$).

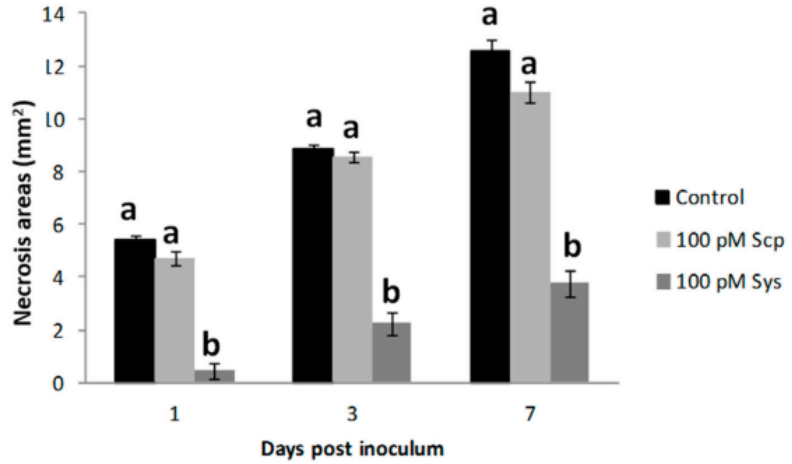

Figure 5. Enhanced resistance to *Botrytis cinerea* of tomato plants supplied with systemin via hydroponics. Response to *B. cinerea* infection by leaves from plants treated with 100 pM Sys, or 100 pM Scp or PBS1X in hydroponics. The graphs display the average (± S.D.) of the lesion size at 1, 3 and 7 days post inoculum. Letters denote statistically significant differences (One-way ANOVA, Tukey test).

2.3. Sys Supply Promotes Indirect Defenses by Increasing the Emission of VOCs

Tomato plants treated with Sys on intact leaves showed an increased attractiveness towards *A. ervi* females compared to the control (Figure 6A). *A. ervi* females showed 45% of oriented flights and 40% of landings on Sys-treated plants in comparison to 9.5% (G test, $\chi 2 = 31.35$, $df = 1$, $p < 0.01$) and 4.8% (G test, $\chi 2 = 27.60$, $df = 1$, $p < 0.01$) observed for controls, respectively. Similarly, plants grown in the presence of Sys-enriched hydroponic solution elicited 46.2% of oriented flights and 31.6% of landings on targets in comparison to 20% (G test, $\chi 2 = 17.01$, $df = 1$, $p < 0.01$) and 9.6% (G test, $\chi 2 = 15.72$, $df = 1$, $p < 0.01$) recorded for the controls (Figure 6B). No significant difference in parasitoid attraction was noted for Scp-treated plants, both on leaves and in hydroponics in respect to controls (Figure 6A,B). In order to experimentally support the observed increased attractiveness towards the parasitoid, we analyzed the volatile blend emitted by leaf-treated plants with the experimental peptides with the aim to identify volatile signals known to be involved in indirect defense. Under the described experimental conditions, we registered a quantitative variation in volatile blends released by treated plants (Table 1).

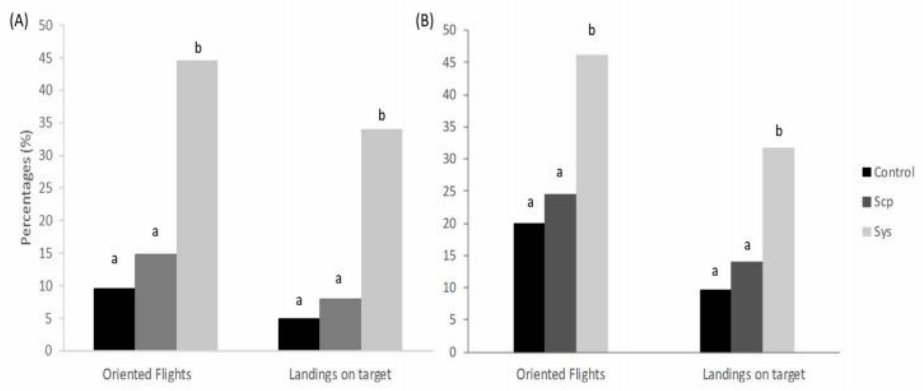

Figure 6. Flight behaviour of the aphid parasitoid *Aphidius ervi* towards tomato plants treated with Sys, Scp, and untreated (control) on intact leaves (**A**) or in hydroponics (**B**). Values indicate the percentage of females showing oriented flights and landings on source. Each assay was conducted using at least 100 females tested against 9 plants. Different letters indicate significant differences (G-test, $p < 0.05$).

Table 1. Volatile organic compounds (VOCs) increase upon treatment with the systemin peptide. List of VOCs significantly improved by Sys foliar application in comparison to VOCs blend released by mock- and Scp-treated plants (* $p < 0.05$, Kruskal-Wallis One Way ANOVA).

			Concentration (PPb)		
Name	**Molecular Formula**	**Molecular Weight g/mol**	**Control**	**Sys**	**Scp**
Benzaldehyde *	C_7H_6O	106.124	$1.54 \times 10^6 \pm 1.2 \times 10^5$	$3.09 \times 10^6 \pm 2.8 \times 10^5$	$1.81 \times 10^6 \pm 1.18 \times 10^5$
Ethylbenzene, p-Xylene *	C_8H_{10}	106.168	$1.53 \times 10^6 \pm 1.43 \times 10^5$	$3.06 \times 10^6 \pm 2.7 \times 10^5$	$1.81 \times 10^6 \pm 1.16 \times 10^5$
β-Ocimene *	$C_{10}H_{16}$	136.238	$1.01 \times 10^6 \pm 8.1 \times 10^4$	$1.22 \times 10^7 \pm 1.46 \times 10^5$	$1.19 \times 10^6 \pm 0.97 \times 10^5$
α-pinene *	$C_{10}H_{16}$	136.238	$1.01 \times 10^6 \pm 8.1 \times 10^4$	$1.22 \times 10^7 \pm 1.46 \times 10^5$	$1.19 \times 10^6 \pm 0.97 \times 10^5$
limonene *	$C_{10}H_{16}$	136.238	$1.01 \times 10^6 \pm 8.1 \times 10^4$	$1.22 \times 10^7 \pm 1.46 \times 10^5$	$1.19 \times 10^6 \pm 0.97 \times 10^5$
Methyl Jasmonate *	$C_{13}H_{20}O_3$	224.300	$6.4 \times 10^5 \pm 4.2 \times 10^4$	$1.16 \times 10^6 \pm 5.17 \times 10^4$	$5.68 \times 10^5 \pm 4.02 \times 10^4$
β-caryophyllene *	$C_{15}H_{24}$	204.357	$1.75 \times 10^5 \pm 2.5 \times 10^4$	$0.95 \times 10^6 \pm 7.6 \times 10^4$	$1.16 \times 10^5 \pm 1.37 \times 10^4$

A group of compounds associated with attractiveness towards insect natural enemies (Benzaldehyde, Ethylbenzene, p-Xylene, β-Ocimene, α-pinene, Limonene, Methyl-Jasmonate and β-caryophyllene) were found to be strongly increased (around 10 folds) by Sys application, while no differences were observed for mock- and Scp-treated plants (Table 1). In order to directly address the effect of Sys exogenous supply on the promotion of JA-mediated direct and indirect defenses, the absolute quantification of MeJA was carried out (Figure S2). Sys-treated plants released 2.57×10^8 ppbv of MeJA, significantly higher in comparison to Control and Scp (around 1×10^8 ppbv).

2.4. Systemin Supply on Leaves of Intact Plants Induce the Expression of Defense Genes

To investigate the effect of the exogenous supply of Sys at molecular level, we monitored the expression of defense-related genes in plantstreated by spotting a Sys solution on the abaxial face of fully expanded healthy leaves or adding the peptide in the hydroponic medium. Then we quantified the transcripts of early (signaling related: *Prosystemin*, *ProSys*, and *Allene Oxide Synthase*, *AOS*) and late (defense-related: *wound-induced proteinase inhibitors I* and *II*, *Pin I* and *Pin II*) genes on Sys- and Scp-treated plants. The expression of target genes was analyzed in a time-course assay by qRT-PCR, on plants exposed to two different concentrations of the experimental peptides. Relative quantification of treated samples was referred to the mock-treated control (relative quantification; $RQ = 1$). An enhanced transcription of the selected genes, both in the treated leaves (Figure 7) and in distal leaves (untreated leaves of treated plants) (Figure 8) was observed. In the treated leaves (Figure 7), *ProSys* transcripts significantly increased and maximal accumulation occurred within 3 h ($F = 0.0124$; $p = 0.00276$), while *AOS* transcripts doubled after 90 min and remained constantly transcribed at higher levels at all experimental time-points. A different transcript profile was observed for *Pin I* ($F = 0.00813$; $p = 0.00312$) and *Pin II* ($F = 0.047$; $p = 0.00272$), which showed a gradual increase, to reach a peak after 6 h. A dose-dependent effect of Sys treatment was observed for *Pin II* transcription after 6 h. In the distal leaves (Figure 8), no *ProSys* transcript up-regulation was observed, while *AOS* transcript greatly increased after 6 h. *Pin I* and *Pin II* transcripts showed a moderate up-regulation after 3 h and a high increase after 6 h. Similarly, to what observed for the expression of the early genes, following the application of the two different Sys concentrations, a different level of expression of the late genes was registered: the 100 pM concentration had the strongest induction effect on the gene transcription. No significant variation in the transcript levels of the tested genes was registered in leaves treated with Scp (Figure S3). Thus, the observed transcriptional enhancement of selected genes is unequivocally associated with the leaf application of the Sys peptide. The same transcripts were monitored in the leaves of plants grown under hydroponics enriched with 100 pM Sys. All the transcripts were significantly up-regulated (p value: *ProSys*, $p = 0.0219$; *AOS*, $p = 0.02037$; *Pin I*, $p = 0.0001$; *Pin II*, $p = 0.0038$) (Figure 9), while no significant transcript increase was observed following Scp application (Figure S4). These results demonstrate that hydroponic supply of Sys is able to induce the transcription of defense-related genes associated with the Sys signaling pathway.

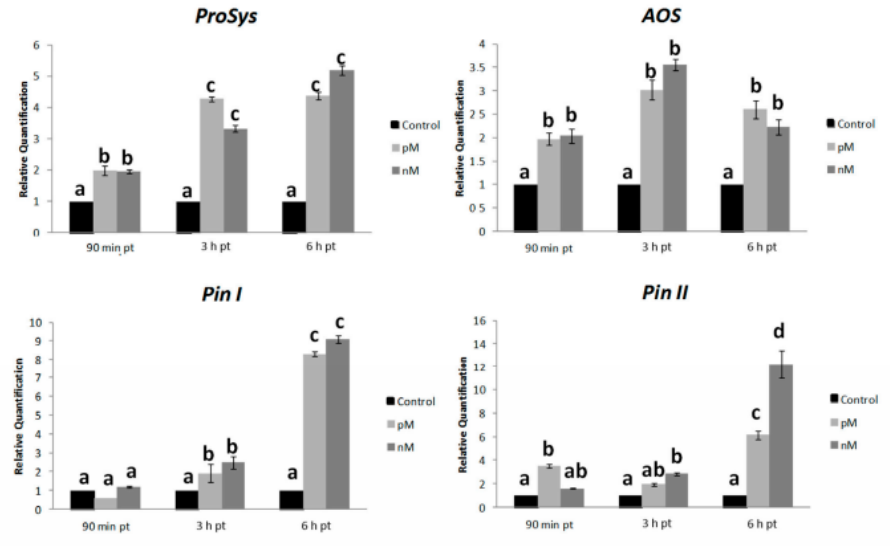

Figure 7. Gene expression analysis in leaf treated with Sys (local). Quantification of transcripts of early (*ProSys*, *AOS*) and late genes (*Pin I*, *Pin II*) by Reverse-Transcription-Polymerase Chain Reaction (RT-PCR) after 90 min, 3 h and 6 h following 100 pM and 100 nM systemin peptide treatment. Relative quantities are calibrated on samples obtained from tomato leaves spotted with PBS1X (Control). For each gene, relative quantification (*RQ*) variations have been analysed by two-way ANOVA. Different letters denote significantly different values ($p < 0.01$). Error bars indicate standard error.

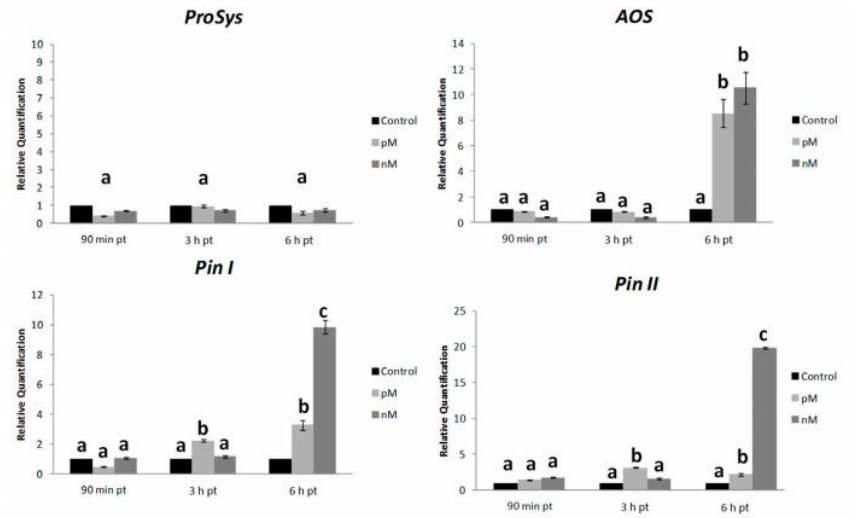

Figure 8. Systemic gene expression analysis in leaves upon Sys foliar treatment. Quantification of transcripts of early (*ProSys*, *AOS*) and late genes (*Pin I*, *Pin II*) in leaves distal from the treated ones by real time RT-PCR after 90 min, 3 h and 6 h following 100 pM and 100 nM systemin peptide treatment. Relative quantities are calibrated on samples obtained from tomato leaves spotted with PBS1X (Control). For each gene, *RQ* variations have been analysed by two-way ANOVA. Different letters denote significantly different values (*ProSys*: $p > 0.05$; *AOS*: $p < 0.01$ 6 h pt; *Pin I* and *Pin II*: $p < 0.05$ 3 h pt, $p < 0.01$ 6 h pt). Error bars indicate standard error.

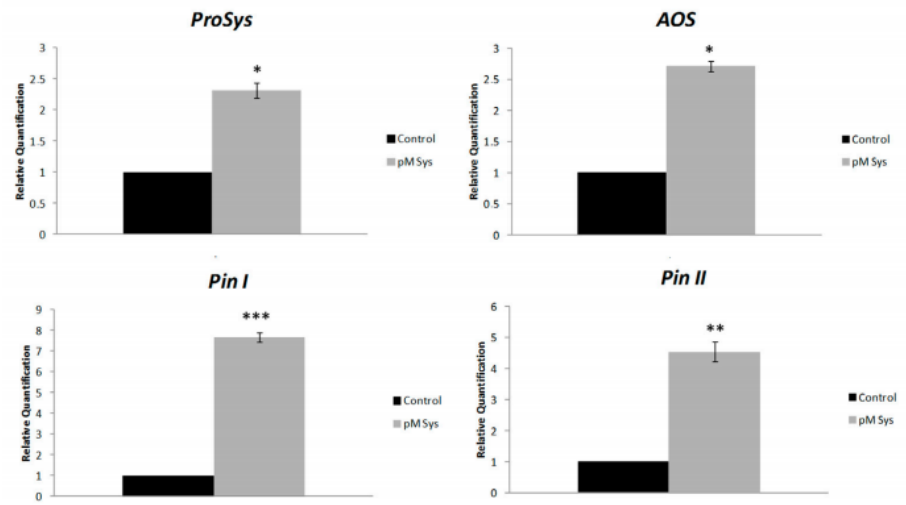

Figure 9. Gene expression in plants grown in hydroponic solution containing Sys. Quantification of transcripts of *ProSys*, *AOS*, *Pin I* and *Pin II* by Real Time RT-PCR detected in leaves of plants grown in a hydroponic system, 3 h after the addition of 100 pM systemin. Relative quantities are calibrated on samples obtained from tomato leaves of plant grown in a hydroponic system supplied with PBS1X. Asterisks denote statistically significant differences (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$; T-test). Error bars indicate standard error.

3. Materials and Methods

Two different peptides were produced: Sys and Sys-scramble (Scp), the latter was used as control. Peptides synthesis, purification and stability are described elsewhere [40]. Briefly, the peptides were obtained by solid phase synthesis following standard protocols [41]. Purification of the peptides was carried out by Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) (Shimadzu LC-8A, equipped with a SPD-M10 AV) on a semipreparative column (Jupiter 10µProteo 90A, 250 × 10.0 mm, Phenomenex, Torrance, CA, USA) using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 5 to 50% in 30 min at 5 mL/min. Peptides were characterized by mass spectrometry (LC-MS ESI-TOF 6230 Agilent Technologies, Milan, Italy). Systemin sequence: AVQSKPPSKRDPPKMQTD. Mass calculated: 2009.3 Mass found: 670.94 $[M + 3H]^{3+}$; 1005.60 $[M + 2H]^{2+}$.

Systemin scramble sequence: KSKMDRQPVQAPDKPSPT. Mass calculated: 2009.3 Mass found: 670.96 $[M + 3H]^{3+}$; 1005.53 $[M + 2H]^{2+}$.

Peptide stability was tested as previously described [40]. Analysis of the HPLC (Shimadzu LC-8A, equipped with a SPD-M10 AV) profiles and of the mass spectra collected indicates that the peptide is stable in all the tested conditions [40]. Stock solutions of the synthesized peptides were prepared as described in [42].

3.1. Plant Materials

The tomato (*Solanum lycopersicum* L.) cultivar used was "Red Setter". Seeds were germinated on sterile paper disks moistened with water and kept in the dark for three days in a climate chamber at 24 ± 1 °C. At the break of cotyledons, seeds were exposed to a 16:8 h light:dark photoperiod, for 48 h. Germinated seeds were transferred to sterile soil in a climate chamber, at 26 ± 1 °C, under a 16:8 h light:dark photoperiod. Four weeks-old plants were used for biological and molecular investigations, unless otherwise indicated. Intact leaves were treated with 2 µL of 100 pM and 100 nM Sys or Scp, by spotting the abaxial surface using a pipette. Both peptides were dissolved in phosphate buffer solution (PBS). Control plants were similarly treated with the buffer. Treated leaves (local leaves) were used for the expression analysis and bioassays with pests.

For hydroponics, tomato seeds, at two-cotyledon stage (5 days after sowing), were transferred into a hydroponic system, and grown for 4 weeks in a 5 L solution, containing $Mg(NO_3)_2 \cdot 6H_2O$ (384.0 mg/L), $Ca(NO_3)_2 \cdot 4H_2O$ (812.9 mg/L), KNO_3 (101.5 mg/L), K_2SO_4 (319.3 mg/L), KH_2PO_4 (204.8 mg/L), Hydromix (14.0 mg/L), and the experimental peptides to a final concentration of 100 pM.

3.2. Bioassay with Spodoptera Littoralis

Feeding bioassays with the phytophagous insect *S. littoralis* larvae were carried out as previously described [30]. Briefly, larvae were obtained from a laboratory population maintained at Isagro Ricerca (Novara, Italy) and reared in our laboratory for more than 10 generations, in a climate chamber at 25 ± 2 °C; 70 ± 5% relative humidity (RH); 16:8 h light:dark photoperiod. Larvae were fed with an artificial diet composed as follow: 41.4 g L^{-1} wheat germ, 59.2 g L^{-1} brewer's yeast and 165 g L^{-1} corn meal, supplemented with 5.9 g L^{-1} ascorbic acid, 1.8 g L^{-1} methyl 4-hydroxybenzoate and 29.6 g L^{-1} agar. Larvae grown on this artificial diet until the 2nd instar. Uniform second instar larvae were selected in groups of 32 individuals, and each group was used to evaluate larval weight and survival rate as affected by the treatment with 100 pM Sys compared to controls mock-treated (phosphate buffer; PBS) or supplied with 100 pM Scp. Every day, leaves from five control or treated plants (biological replicates) were harvested. Similar leaves, in terms of size and position on the plant, were used to produce leaf disks to feed experimental larvae. Tomato leaf disks were laid down on 2% agar (*w/v*) to create a moist environment required to keep them turgid in a tray well (Bio-Ba-32, Color-Dec, Lucca, Italy) covered by perforated plastic lids (Bio-Cv-4, Color-Dec, Lucca, Italy). Larvae were singly separated into each box and fed with the correspondent leaf disk (control or treated). These 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 incubated at controlled conditions (28 ± 1 °C; 70 ± 5% RH; 16:8 h light:dark photoperiod). Larval weight and mortality were recorded until pupation, which took place into plastic boxes containing vermiculite (25 × 10 × 15 cm).

For Sys supplied in hydroponics, the 3rd instar larvae were used for which larval weight and longevity were registered. In addition, the following reproduction-related parameters were recorded: time for pupa development (from the onset of the bioassay to pupation), adult longevity and fecundity. Briefly, pupae were collected, washed in a 50% solution of bleach (0.05% sodium hypochlorite), rinsed with distilled water and air dried, before they were sexed under a stereomicroscope (40×) by observing morphological characters, as described [43], separated in aerated plastic boxes (25 × 10 × 15 cm) and daily inspected until adult emergence. After emergence, adults had access to a 50% water solution of honey. Males and females were kept together (1 female:2 males) for 24 h, at 25 °C, to allow mating. Then, mated females were separated from males (marked with red ink) and singly transferred into a plastic cylinder (diameter 8 cm, height 9 cm), lined with paper where their egg laying activity was assessed on a daily basis, for the whole lifespan, by counting the number of eggs deposited on paper, under a stereomicroscope operating at 40× magnification. Longevity of the adults was also recorded. Each experiment was repeated two times.

3.3. Bioassay with Botrytis Cinerea

Four week-old plants, treated with 100 pM Sys directly delivered on the leaf surface or dissolved in the hydroponic solution (final Sys concentration was 100 pM), were tested for resistance to *B. cinerea*. Spores of the fungus were obtained as follow: suspension in sterile distilled water, filtration through sterile Kimwipes (Kimberly-Clark) to remove fragments of hyphae, and adjustment to a concentration of $1 \cdot 10^6$ conidia per mL. Six hours after Sys application, an aliquot of 10 µL of the fungus spore suspension was applied to the leaves. The assay was carried out using four plants per treatment, which were incubated in a growth chamber at 23 ± 1 °C, for a 16 h photoperiod and under 90% RH. The size of the lesions was measured at different days post inoculums (pi). Lesion dimensions were measured using a digital caliber (Neiko 01407A).

3.4. Aphidius Ervi Flight Behavior

Bioassays with *Aphidius ervi (A. ervi)* parasitic wasps were conducted in wind tunnel (100 × 50 × 50 cm), as previously described in detail [44]. Plants were tested 24 h after the treatment with Sys and Scp experimental peptides (100 nM), and control buffer applied directly on leaves or added in the hydroponic growth solution. *A. ervi* naïve females, 1–2 days old, mated and fed, were released singularly in the wind tunnel, 50 cm downwind from the target plant and observed up to 5 min to determine their flight orientations and landings on the plant. Insect behavior was recorded as "Oriented flight" when the females flew within 5 cm of plant or landed on it. Similarly, it was recorded as "Landing on target" when females landed on plant. Bioassays were conducted by observing at least 100 females on 6 different plants for each treatment on 6 different days. Plants were presented in random order each day to avoid any daily bias. The experimental conditions were a temperature of 20 ± 1 °C; 65 ± 5% RH; wind speed, 25 ± 5 cm/s; Photosynthetic Photon Flux Density (PPFD) at releasing point, 700 µmol m^2/s.

3.5. Volatile Organic Compounds (VOCs) Collection and Analysis

VOCs sampling and analyses were performed under controlled temperature, at 25 ± 1 °C. Leaf treated plants and control (100 pM Sys or 100 pM Scp or buffer) were used for headspace volatile collection and VOCs analysis. Headspace sampling was performed 1h after closing five plants in a glass box (60 × 60 × 60 cm) to accumulate VOCs. The collected headspaces were directly injected into the Proton Transfer Reaction ionization with a Time-of-Flight Mass Spectrometry (PTR-TOF-MS) drift tube hated (110 °C) peek inlet tube with a flow rate of 100 sccm for calculation.

VOCs were detected in real-time through proton transfer reactions using Proton Transfer Reaction-Quadrupole interface Time of Flight- Mass Spectrometry (PTR-Qi-TOF-MS) apparatus supplied by Ionicon Analytik GmbH (Innsbruck, Austria). The drift tube was kept under controlled conditions of pressure (3.8 mbar), temperature (80 °C) and voltage (1000 V), resulting in a field density ratio (E/N) of 141 Td (E being the electric field strength and N the gas number density; 1Td = 10−17 V cm^{-2}).

The raw data recorded by the PTR apparatus were acquired by the TofDaq software (Tofwerk AG, Thun, Switzerland), normalized per plant and subsequently evaluated with the PTR-MS Viewer 3.2.6 (Ionicon analytic GmbH, Innsbruck, Austria).

3.6. Calibration of Methyl-Jasmonate Standard

The absolute quantification of methyl jasmonate (*m/z* 152.15) was performed using the IONICON Liquid Calibration Unit (LCU) coupled with PTR-Qi-TOF-MS. LCU evaporates aqueous standards into a gas stream, resulting in a gas flow containing compounds at exactly know trace concentrations. To produce a calibration curve for MeJA, a gradient flow has been obtained by nebulizing both, the liquid standard (MeJA at concentration of 10^{-6}) and the distilled water, starting from 100% water to 100% MeJA. Nitrogen was utilized as a carrier gas at 1000 sccm (nitrogen with a purity of 5.0—i.e., 99.999%—purchased from Linde-Vienna-Austria) with a constant flow. The combined liquid from the two inlets was sprayed and evaporated inside the heated spray chamber at the temperature of 100 °C and was introduced in the inlet of PTR-Qi-TOF-MS. Finally, data were filtered to remove all peaks ascribed to water chemistry (*m/z* 21.022 and *m/z* 39.033 corresponding to$H_3{}^{18}O^+$ and $H_2OeH_3{}^{18}O^+$, respectively) or other interfering ions (e.g., oxygen, nitrogen monoxide).

3.7. Gene Expression Analysis

Three fully-expanded leaves per plant were treated and three plants for each treatment (Sys or Scp or buffer) were used as biological replicates. Treated leaves and un-treated leaves of treated plants (named as distal leaves) were harvested at different time points, immediately frozen in liquid nitrogen and stored at −80 °C until use. For experiments in hydroponics, plants were grown in three different

tanks and supplied with nutritive solution without (control plants) or with (treated plants) 100 pM Sys or 100 pM Scp. Three leaves per plant and three plants per each experimental condition were harvested 3 h after treatment and stored as described above. The isolation of total RNA from leaves, the synthesis of the first strand cDNA and real-time PCRs were performed according to standard procedures, as already described elsewhere [45]. For each sample, two technical replicates for each of the three biological replicates were used for the gene expression analysis. Relative quantification of gene expression was carried out using the $2^{-\Delta\Delta Ct}$ method [46]. The housekeeping gene EF-1α was used as endogenous reference gene for the normalization of the expression level of the target genes [47,48]. Primers and their main features are reported in Table S1.

3.8. Statistical Analysis

Differences in relative quantities of defense transcripts were analyzed by comparing ΔCt values by one-way or two-way ANalysis Of Variance (ANOVA), while for coupled comparisons a two-tailed Student's test was used. For the insect assay, larval weights were compared by one-way ANOVA or Kruskal-Wallis non parametric ANOVA, followed by Tukey-Kramer honestly significant difference (HSD) and Dunn's post test for multiple mean value comparisons. Survival curves of *S. littoralis* larvae and adults were compared by using Kaplan-Meier and log-rank analysis. The time required by larvae to pupate was compared by Kruskal-Wallis non parametric ANOVA followed by Dunn's post test for multiple mean value comparisons, while the number of laid eggs was compared by one-way ANOVA, coupled with Tukey-Kramer multiple comparisons test. For the evaluation of Sys effect on *B. cinerea* infection, necrosis area differences between control and 100 pM Sys-treated sample were analyzed by T-Student's test. Size differences of the necrotic areas, induced by fungal inocula on plants treated with Sys or Scp via root uptake, were analyzed by one-way ANOVA coupled with Tukey-Kramer honestly significant difference (HSD) test.

The number of parasitoids responding, as oriented and non-oriented flight, to each target plant was compared by a G-test for independence, as described in [49].

Differences in VOCs released by treated and control plants were compared using Kruskal-Wallis non parametric ANOVA.

4. Discussion

Plants have several strategies to counteract damage caused by insect and pathogens that include the induction, upon attack, of endogenous peptides, triggering defense responses against invaders. One of the major issues to address for exploiting at the best this source of molecular biodiversity for their possible use in agriculture, is the development of suitable delivery strategies of these defense molecules, which prevent environmental degradation and loss of biological activity. Here we contribute to this research area using systemin, a well-known octadecapeptide hormone of the tomato plant, that triggers plant defense pathways against different biotic stress agents and enhances defense barriers when constitutively expressed in plants [30,50,51]. Sys supply to tomato plants proved to be very effective in conferring measurable protection against *S. littoralis* and *B. cinerea*, demonstrating that both hydroponics and leaf spotting represent useful peptide delivery strategies for pest control.

Interestingly, it was recently demonstrated that the Sys peptide effectively spread throughout tomato stems and leaves following injection into the stem and leaves of tomato plants [52]. The transient pattern of gene expression registered upon Sys leaf treatment, proving that the treatment activates the octadecanoid pathway locally and systemically, is in general agreement to what expected for the early expressed defense signaling genes (*ProSys* and *AOS*), and the late downstream genes (*Pin I* and *Pin II*) encoding defensive molecules, which directly target the insect pests [11]. Our data show that *AOS* transcript increases in distal leaves after 6 h from Sys treatment. This result is apparently in contrast with the early involvement of this gene in the signaling events of the Sys-dependent defense pathway. However, since AOS is the first enzyme in the branch pathway leading to the biosynthesis of JA, the most straightforward interpretation of this result is that the enzyme contributes to the production of JA

in distant leaves to trigger the systemic activation of defense related genes [53]. This hypothesis is corroborated by the high increased production of Methyl-Jasmonate (MeJA) in Sys-treated plants.

The increased expression of genes of the octadecanoid pathway leads to the production of JA and to the activation of defense genes such protease inhibitors [7]. The increased transcription of this genes is possibly associated with an increased accumulation of the inhibitors known to be involved in the reduction of growth and life of chewing herbivores and necrotrophic fungi [30]. In addition, resistance to *B. cinerea* and *S. littoralis* could be dependent on a response to Sys mediated by the pepr1/2 ortholog receptor-like kinase1, a protein with biological functions in systemin signaling and tomato immune responses [54,55].

Considering that the plant cell wall is semi-permeable, it is possible to speculate that it allows Sys to pass through and interact with its receptor with the subsequent activation of the signaling cascade.

These results are corroborated by previous observations showing that plant treatment with peptides induced defense genes and metabolites [56,57]. For example in *Arabidopsis*, 4-week-old plants grown in soil sprayed withPep1 showed an increased expression of a gene encoding a defensin [58] while plant treatment with the bacterial peptide flagellin induces the expression of numerous defense-related genes and triggers resistance to pathogenic bacteria [59]. In addition, *Solanum pimpinellifolium (S. pimpinellifolium)* roots elongated in response to systemin treatment, thus suggesting that the root perceive the peptide [60].

A number of compounds associated with indirect defense were retrieved in treated plants 10 folds in respect to controls. These compounds are known to be signals acting as synomones as they bring an advantage to both the emitter plant and the receiver organism: be it an insect (i.e., a natural enemy that finds its prey) or another plant (i.e., a neighbour unchallenged plant) [36,61–63]. The results of behavioral bioassay with *A. ervi* are consistent with the volatile blend released by Sys-treated plants. Among these compounds, β-caryophyllene is reported to be identified at antennal level by *A. ervi* at a concentration as low as 0.01 mg/mL and to elicit a significant higher attractiveness towards this parasitoid in respect to control solvent when tested as purified compounds in wind tunnel bioassay [64].

Surprisingly, adults *S. littoralis* of emerged from larvae fed on Sys-treated plants had reduced survival rate and lower fecundity, suggesting that Sys treatment has a strong effect on the fitness of the insect population. Plant watering with Sys solution could be an interesting option for protection against pests and pathogens, as hydroponics is largely used for tomatoes, the most widely grown vegetable in the world, and other *Solanaceae* that may benefit from Sys supply. To exploit at the best this potential, a large array of Sys concentrations should be investigating in order to assess the lowest peptide levels able to confer effective protection to tomato and other *Solanaceae* crops.

We also demonstrated that Sys treatment of healthy plants increase the attraction of *A. ervi*, a natural antagonist of the aphid *Macrosiphum euphorbiae (M. euphorbiae)*, thus inducing a reinforcement of the indirect defense barriers. Interestingly, tomato plants constitutively expressing *ProSys* were moderately tolerant to *M. euphorbiae* attacks [30]. It is tempting to speculate that, in presence of *A. ervi*, Sys treated plants will show an increased tolerance to *M. euphorbiae* attacks.

The development of safe and sustainable crop protection strategies is a challenging goal facing our society. This is increasingly pursued through bioinspired research efforts, aiming to mimic natural mechanisms of pest suppression [2]. Application of plant endogenous peptides prompting defense responses that affect the fitness and behavior of herbivores and pathogens represents a very safe approach of plant protection, due to the expected low or null toxicity of these molecules on non-target organisms. However, the evaluation of the cost of the treatment on plant physiology should be further investigated.

Although Sys homologues have been described only in solanaceous plants belonging to the subtribe Solaneae, like tomato, potato, black nightshade, and pepper [65], other genetically distinct families of plant defense signal peptides have been identified in different species reviewed in [14]. In *Arabidopsis* Pep1 is released from the C-terminus of a longer precursor protein (ProPep) and is perceived as a DAMP by specific receptors with the consequent amplification of the plant innate

immune responses against pathogens. The constitutive expression of the precursor confers resistance to *Arabidopsis* plants against the oomycete plant pathogen *Pythium irregulare (P. irregulare)* [11]. Conversely, *Zea mays (Z. mays)* Pep3 regulates direct and indirect anti-herbivore defenses, likely by modulating the downstream signaling response to insect oral secretions [66]. ProPep orthologous were identified in numerous species [11] and, interestingly, a functional orthologous was also found in tomato, where it is involved in defense against a root pathogen [67]. In addition, our unpublished results suggest the presence of the recently identified systemin receptor 1 and 2 (SYR1 and SYR2), responsible of Sys perception in species of *Solanaceae*, not only in representative species of the sister subfamily *Nicotianoideae* [17], but also in other higher plants. Interestingly, the SYR and SYR-like genes are close relatives of the plant elicitor peptide receptors (PEPRs) [28,68] suggesting that the defensive signal transduction could be mediated by the same or similar players in higher plants, despite their phylogenetic distances.

5. Conclusions

The presented data, collectively, indicate that 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 powerful tool for sustainable agriculture that could reduce the use of chemical inputs while providing food quality and safety. This goal can be further pursued by developing bioformulations able to modulate plant defense. An example of such a formulation is represented by Messenger®, a Trade Mark product, that enhances disease and pest resistance in treated plants. These enhancements are based on the activity of naturally occurring proteins, the active ingredients in Messenger®, that trigger natural defense systems against many diseases and pests [69]. Towards this aim we are presently producing recombinant Sys in bacteria (unpublished) in order to greatly reduce the cost and increase the feasibility of the proposed approaches. In addition, despite the continuous exposition of pests to Sys within the naturally occurring tomato-pests interaction no pest's resistance to the peptide was observed thus suggesting a good durability of the proposed approaches.

Although the involvement of Sys in the activation of tomato plant defenses was proven previously, to our knowledge this is the first work proving that that the treatment of healthy unwounded tomato plant with Sys confers resistance against pests representing a promising strategy for pest control. The effect of this peptide on multiple stress agents, both biotic and abiotic (i.e., salt tolerance) and the efficacy of different delivery strategies is very promising from an applied perspective representing a significant addition towards the field use of defense peptides in crop protection.

Supplementary Materials: The following are available online at http://www.mdpi.com/2223-7747/8/10/395/s1, Figure S1: Symptoms of *B. cinerea* infection on tomato leaves. Necrosis caused by *B. cinerea* spores 3 and 9 days post inoculums are shown in control (A, C) and Sys-treated (B, D) leaves, Figure S2: Absolute quantification of methyl-jasmonate (MeJA) released by systemin-treated plants. Standard curve and calculation of released amount of MeJA in tomato plants treated with Sys, Scp or mock on intact leaves, Figure S3: Relative quantification of defense transcripts upon Scp foliar treatment. Expression analysis of *ProSys* and *Pin I* by Real Time RT-PCR 6 h following Scp treatment. Relative quantities are calibrated on samples obtained from Red Setter leaves spotted with PBS1X. No significant differences were registered (One-way ANOVA). Error bars indicate standard error, Figure S4: Effect of 100 pM Scp added in hydroponics. Relative quantities of defense transcripts by Real Time RT-PCR detected in leaves after 3 h of hydroponics. Relative quantities are calibrated on samples obtained from tomato leaves of plant grown in a hydroponic system supplied with PBS1X. No significant differences were registered (One-way ANOVA). Error bars indicate standard error.

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Ethical Statement: The plant species and variety used in this experiment is a widely cultivated variety and we have followed all proper ethical standard. All of the reagent and fertilizers used are properly recommended by the authority.

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Rapid Identification of Protein-Protein Interactions in Plants

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Enzyme-enzyme interactions can be discovered by affinity purification mass spectrometry (AP-MS) under in vivo conditions. Tagged enzymes can either be transiently transformed into plant leaves or stably transformed into plant cells prior to AP-MS. The success of AP-MS depends on the levels and stability of the bait protein, the stability of the protein-protein interactions, and the efficiency of trypsin digestion and recovery of tryptic peptides for MS analysis. Unlike in-gel-digestion AP-MS, in which the gel is cut into pieces for several independent trypsin digestions, we uses a proteomics-based in-solution digestion method to directly digest the proteins on the beads following affinity purification. Thus, a single replicate within an AP-MS experiment constitutes a single sample for LC-MS measurement. In subsequent data analysis, normalized signal intensities can be processed to determine fold-change abundance (FC-A) scores by use of the SAINT algorithm embedded within the CRAPome software. Following analysis of co-sublocalization of "bait" and "prey," we suggest considering only the protein pairs for which the intensities were more than 2% compared with the bait, corresponding to FC-A values of at least four within-biological replicates, which we recommend as minimum. If the procedure is faithfully followed, experimental assessment of enzyme-enzyme interactions can be carried out in Arabidopsis within 3 weeks (transient expression) or 5 weeks (stable expression).

Basic Protocol 1: Gene cloning to the destination vectors
Alternate Protocol: In-Fusion or Gibson gene cloning protocol
Basic Protocol 2: Transformation of baits into the plant cell culture or plant leaf
Basic Protocol 3: Affinity purification of protein complexes
Basic Protocol 4: On-bead trypsin/LysC digestion and C18 column peptide desalting and concentration
Basic Protocol 5: Data analysis and quality control

Keywords: affinity purification mass spectrometry • fold change abundance • protein-protein interaction

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INTRODUCTION

Affinity purification mass spectrometry is a highly effective method for isolating and identifying protein-binding partners of a target protein under in vivo conditions. Protein complexes can be captured by antibodies specific for the bait proteins or for tags fused to the bait proteins via recombinant DNA technologies. These complexes are thereby "pulled-down" onto immobilized-protein agarose beads via affinity purification, prior to their detection and identification via mass spectrometry. Given that AP-MS experiments have been widely used to generate meaningful interaction networks, it follows that they could also be used to produce information-rich data concerning extra-pathway protein-protein interactions (Bürckstümmer et al., 2006; Morris et al., 2014; Puig et al., 2001; Zhang, Beard, et al., 2017; Zhang, Sun, Zhang, Brasier, & Zhao, 2017; Zhang, Swart et al., 2018). Such interactions could aid in the characterization of the functions of the interacting proteins, provide detailed catalogs of proteins involved in protein complexes and biological processes, or reveal networks of biological processes on both the local and proteome-wide scale (Morris et al., 2014). In order to better understand these interactions, AP-MS can be readily performed in many plant species, with the main prerequisites being the ease of genetic transformation and availability of a sequenced reference genome. However, presently, these features apply to a multitude of plant species. The basic procedure can be divided into five stages: (i) gene cloning into the destination vectors (see Basic Protocol 1 and Alternate Protocol); (ii) plant cell culture transformation (see Basic Protocol 2); (iii) affinity purification of protein complexes (see Basic Protocol 3); (iv) on-beads trypsin/LysC digestion and C18 column–based peptide desalting and concentration (see Basic Protocol 4); and (v) data analysis and quality control (see Basic Protocol 5). The entire workflow is summarized in Figure 1.

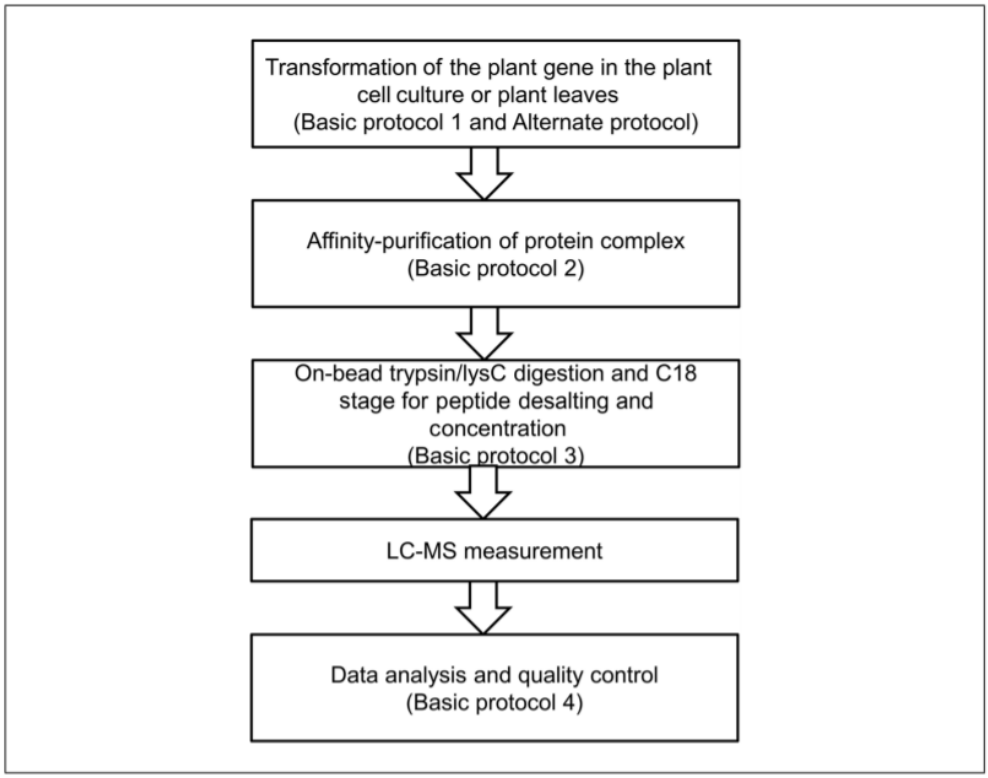

Figure 1 Workflow for characterization of protein-protein interactions by affinity-purification mass spectrometry in plants.

STRATEGIC PLANNING

The Arabidopsis plant cell culture (PSBD, ABRC stock, CCL84840, Background: Ler, Landsberg erecta) can be obtained from the ABRC stock center and maintained according to a published protocol (Menges & Murray, 2002). Similarly, Arabidopsis seeds can be obtained from stock centers (ABRC), and Arabidopsis plants growth can be carried out as described in the literature (Sanchez-Serrano & Salinas, 2014; Zhang, Swart, et al., 2018).

GENE CLONING TO THE DESTINATION VECTORS

A variety of protocols have been described over the years in regard to gene cloning (Curtis & Grossniklaus, 2003; Katzen, 2007; Walhout et al., 2000). Described here is the gene cloning protocol used in our lab. A two-step polymerase chain reaction (PCR) is used to clone the genes of interest and link them to the donor vector using the Gateway BP reaction. As some genes cannot be linked to the donor vector by the Gateway BP reaction, we have alternatively used In-Fusion or Gibson assembly to sub-clone these genes (see Alternate Protocol). Next, the genes of interest are recombined into the destination vector by the Gateway LR reaction, under the control of the plant ubiquitin 10 promoter (Grefen et al., 2010).

Materials

Nuclease-free water

Phusion High-Fidelity DNA polymerase (ThermoFisher Scientific, F530L) and corresponding 5× buffer

dNTP mix (New England Biolabs, cat. no. N0447)

Template-specific primers: design the following template-specific primers; include 12 bases of the attB1 or attB2 site on the 5′ end of each primer, as appropriate:

Gene forward: 5′-AAAAAAGCAGGCTCCACCNNNNNNNNN-

Gene reverse: 5′-CAAGAAAGCTGGGTcatagccNNNNNNNN-

NNNNNNNNNNN represents the gene-specific primer with at least 20 bp; we strongly recommend using Primer, version 7 (*https://www.primer-e.com/our-software/primer-version-7/*) to evaluate the primers

Template DNA: cDNA from seedling of Arabidopsis (RevertAid First Strand cDNA Synthesis Kit, ThermoFisher Scientific, K1622)

Adapter primers: design the following adapter primers (required to install the complete attB sequences):

attB1 adapter: 5′-G GGGACAAGTTTGTACAAAAAAGCAGGCTCCACC-3′

attB2 adapter: 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTcatagcc-3′

attB2St adapter: 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTcttagcc-3′ to amplified gene with stop code

1% agarose gel with RedSafe nucleic acid staining solution (Chembio Ltd., 21141; also see Current Protocols article: Voytas, 2000)

Nucleic acid gel extraction and purification kit (Qiagen, cat. no. 28704)

Donor vector: Gateway™ pDONR™221 vector (Thermo Fisher Scientific, 12536-017) or pDONR™207 vector (Thermo Fisher Scientific, 12213-013)

TE buffer, pH 8.0 (Current Protocols, 2001)

Gateway® BP Clonase® II enzyme mix (Thermo Fisher Scientific, 11789013, 11789020) including 2 µg/µl proteinase K solution

DH5α *E. coli* competent cells (Thermo Fisher Scientific,18265017)

Lysogeny broth (LB) medium (see recipe)

LB agar plates (see recipe) with 50 µg/ml kanamycin

M13F (GTAAAACGACGGCCAG) and M13R universal primers (CAGGAAA CAGCTATGAC)

Gateway® LR Clonase® Enzyme Mix—to create a Gateway™ expression clone (Thermo Fisher Scientific, 11791019)

200-µl PCR tubes

Thermal cycler

42°C water bath for heat shock

37°C shaking incubator

Gene cloning

1. Prepare gene-specific PCR mix (20 µl/reaction):

Component	20 µl reaction	Final concentration
Nuclease-free water	To 20 µl	
5× Phusion HF buffer[a]	4 µl	1×
10 mM dNTPs	0.4 µl	200 µM
10 µM gene-specific forward primer	0.2 µl	0.01 µM
10 µM gene-specific reverse primer	0.2 µl	0.01 µM
Template DNA	0.2 µl	<250 ng
DMSO (optional)[b]	(0.6 µl)	3%
Phusion DNA polymerase	0.2 µl	0.02 U/µl

[a]Optionally 5× Phusion GC buffer can be used.
[b]Addition of DMSO is recommended for GC-rich amplicons. DMSO is not recommended for amplicons with very low % GC or amplicons that are >20 kb.

2. Perform the first-step PCR in a thermal cycler using the following machine settings:

Step	Time	Temperature	Cycles
Initial denaturation	30 s	98°C	1×
Denaturation	10 s	98°C	15×
Annealing	20 s	60° to 72°C	
Extension	30 s/kb	72°C	

3. Transfer 10 µl of the PCR reaction to a 40-µl PCR mixture containing 40 pmol each of the *att*B1 and *att*B2 adapter primers (note that attB1 and attB2St adapters are for the gene with stop code).

Component	40 µl reaction	Final concentration
Nuclease-free water	add to 40 µl	
5× Phusion HF or GC buffer (see step 1)	8 µl	1×
10 mM dNTPs	0.8 µl	200 µM
10 µM adapter forward primer	2 µl	0.5 µM
10 µM adapter reverse primer	2 µl	0.5 µM
DMSO (optional; see step 1)	(1.2 µl)	3%
Phusion DNA polymerase	0.4 µl	0.02 U/µl

4. Perform the second-step PCR in a thermal cycler using the following machine settings:

Step	Time	Temperature	Cycles
Initial denaturation	1 min	98°C	1×
Denaturation	10 s	98°C	5×
Annealing	20 s	55°C	
Extension	30 s/kb	72°C	
Denaturation	10 s	98°C	29×
Annealing	20 s	68°C	
Extension	30 s/kb	72°C	
Final extension	5–10 min	72°C	1×

5. Use agarose gel electrophoresis (see Current Protocols article: Voytas, 2000) to check quality and yield of the *att*B-PCR product, and then purify the PCR products for the BP reaction using a nucleic acid gel extraction and purification kit (e.g., Qiagen).

6. Perform a BP recombination reaction between an *att*B-flanked DNA fragment and an *att*P-containing donor vector (pDONR221 or pDONR207) to generate an entry clone.

   a. Add the following components to a 1.5-ml microcentrifuge tube at room temperature and mix::

Clone (*att*B-PCR product, from step 5; ≥30 ng/μl; final amount up to 100–150 ng/μl)	1–3.5 μl
pDONR™ vector (supercoiled, 150 ng/μl)	0.5 μl
TE buffer, pH 8.0	To 4.5 μl

   b. Vortex Gateway® BP Clonase® II enzyme mix briefly. Add 0.5 μl to the components above and mix well by vortexing briefly twice.
   c. Incubate the reaction at 25°C for at least 1 hr (can be overnight).
   d. Add 1 μl of 2 μg/μl proteinase K solution (included with the Clonase enzyme) and incubate at 37°C for 10 min.

E. coli transformation

7. Thaw 50 µl of chemically competent cells (DH5α or Top 10) for each transformation on ice. Add 5 µl of the BP recombination reaction to the competent cells and mix gently. Do not mix by pipetting up and down. Incubate the vial(s) on ice for 30 min. Heat-shock the cells for 45 s at 42°C without shaking. Remove the vial(s) from the 42°C bath and place them on ice for 2 min. Add 1 ml of room temperature LB medium to each vial. Cap the vial(s) tightly and put on a shaker (850 rpm) at 37°C for 1 hr. Microcentrifuge for 1 min at 14,000 × *g*, discard the supernatant, and resuspend the pellet by pipetting. Plate the bacteria onto the pre-warmed selective plate and incubate overnight at 37°C.

8. After sequencing by vector-specific primers (M13F and M13R for the pDONR221; see Current Protocols article: Shendure et al., 2011), perform an LR recombination reaction between an *att*L-flanked DNA fragment (produced before) and an *att*R-containing donor vector to generate a digestion vector:

a. Add the following components to a 1.5-ml microcentrifuge tube at room temperature and mix (*att*L-Vector t or linearized *att*L expression):

Donor vector (≥ 30 ng/μl; final amount 100−150 ng)	1–3.5 μl
Digestion vector (supercoiled, 150 ng/μl)	0.5 μl
TE buffer, pH 8.0	To 4.5 μl

b. Vortex Gateway® LR Clonase® II enzyme mix briefly. Add 0.5 μl to the components above and mix well by vortexing briefly twice. Incubate the reaction at 25°C for at least 1 hr (can be overnight). Add 1 μl of 2 μg/μl Proteinase K solution and incubate at 37°C for 10 min. Transform competent *E. coli* and select for the appropriate antibiotic-resistant digestion vector following the method mentioned above.

IN-FUSION OR GIBSON GENE CLONING

Although the BP reaction works for most genes, there are around 2% to 5% of genes that cannot be cloned by the BP reaction due to sequence-specific problems, among other reasons. Here, we provide an alternative protocol to sub-clone these genes into pDONR vector by In-Fusion and Gibson assembly.

Additional Materials *(also see Basic Protocol 1)*

In-Fusion HD cloning (Takara, 639650)
Gibson Assembly® Master Mix (NEB, E2611)
pDONR-IF-f: GACCCAGCTTTCTTGTACAAAGT
pDONR-IF-r: GGTGGAGCCTGCTTTTTTGT

1. Amplify the specific gene in a 50-μl PCR reaction as described in Basic Protocol 1, steps 1 and 2, using gene-specific primers.

2. Amplify the pDONR vectors using gene vector–specific primers (pDONR-IF-f/r) in a 50-μl PCR reaction at an annealing temperature of 58°C according to the manufacturer's instructions.

3. Use agarose gel electrophoresis (see Current Protocols article: Voytas, 2000) to check quality and yield of the products, then purify the PCR products using a nucleic acid gel extraction and purification kit.

4. Mix the two PCR products at a 1:1 ratio.

PCR products (≥30 ng/μl; final amount, 100-150 ng)	1-4 μl
pDONR vector (≥ 50 ng/μl; final amount, 100-150 ng)	1-4 μl
TE buffer, pH 8.0	To 8 μl

5. Vortex the In-Fusion or Gibson enzyme mix briefly. Add 2 μl of the enzyme mix to the components above and mix well by vortexing briefly twice. Incubate the reaction at 50°C for 30 min to 1 hr.

6. Transform competent *E. coli* and select for the appropriate antibiotic-resistant pDONR vector as described in Basic Protocol 1.

TRANSFORMATION OF BAITS INTO THE PLANT CELL CULTURE OR PLANT LEAF

This protocol has been optimized for overexpressing the bait protein in the plant. The plant destination vectors containing plant promoter (such as ubquitin or actin) can be transformed into the plant cell culture within 1 month (Fig. 2). The cell culture could be harvested directly following transformation for the AP-MS.

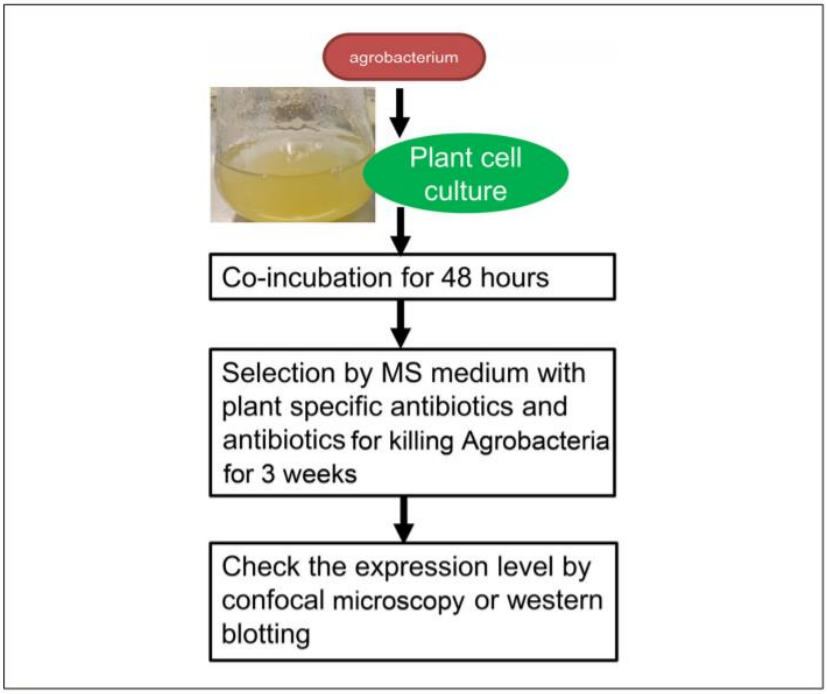

Figure 2 Workflow for plant cell culture transformation.

Materials

Yeast extract beef (YEB) medium and agar plates (see recipe)
Appropriate antibiotics:
- Carbenicillin (Sigma, 4800-94-6)
- Rifampicin (Sigma, R3501)
- Ticarcellin clavulanic acid (Sigma, T5639)
- Vancomycin (Sigma, V1130)
- Kanamycin (Sigma, 60615)
- Vector-specific selection antibiotics

Agrobacterium tumefaciens AGL1 (Intact Genomics, 1283-12)
MSCC medium (see recipe)
0.1 M acetosyringone (Sigma, D134406) dissolved in dimethyl sulfoxide (DMSO)

2.5-ml culture tubes
2-ml microcentrifuge tubes
Electroporation apparatus and electroporation cuvettes
25° and 28°C shaking incubator
15-ml conical tubes (e.g., Corning Falcon)
50-ml and 100-ml Erlenmeyer flasks

Additional reagents and equipment for confocal microscopy (see Current Protocols article: Rajwa, 2005)

Agrobacterium tumefaciens transformation

1. Pour 20 ml YEB medium with carbenicillin (20 μg/L) and rifampicin (50 μg/L) but no gentamicin in a 2.5-ml liquid culture tube, add 200 μl from the frozen stock of *Agrobacterium tumefaciens* AGL1, and incubate the cultures overnight with shaking at 28°C.
2. Add 2 ml of the *Agrobacterium* overnight culture to a 2-ml tube and centrifuge for 30 s at 2000 × *g*, 4°C. Discard the supernatant, removing as much as the liquid as possible.
3. Put the sample on ice. Add 2 ml of ice-cold water, vortex, and centrifuge for 30 s at 14,000 × *g*, 4°C. Discard the supernatant. Repeat these steps with 1 ml, 500 μl, and 200 μl of ice-cold water. Do not discard the 200 μl water; resuspend the pellet in this volume and put on ice (these are the *Agrobacterium* competent cells).
4. Add 1 to 5 μl of the expression clone DNA sample into a 2-ml tube and place it on ice. Add 45 μl of *Agrobacterium* competent cell suspension from step 3 and incubate on ice 5 min. Put the solution into cold electroporation cuvettes and leave on ice.
5. Electroporate cells using an electroporation apparatus according to the manufacturer's instructions. Following electroporation, directly add 1 ml of YEB medium (with 20 μg/L carbenicillin and 50 μg/L rifampicin, but no gentamicin) and transfer the solution back into a new 2-ml tube.
6. Shake for 1 to 2 hr at 28°C.

7. Microcentrifuge for 1 min at 14,000 × *g*, discard the supernatant, and resuspend the pellet by pipetting up and down. Plate the bacteria on pre-warmed YEB plates (with 20 µg/L carbenicillin and 50 µg/L rifampicin, and the appropriate antibiotic for specific selection of your gene of interest, but no gentamicin) and incubate at 28°C for 2 to 3 days.

Cell culture transformation

8. *DAY 1 (Wednesday):* Take one colony of the transformed *Agrobacterium* and plate it on a fresh YEB plate (with 20 µg/L carbenicillin, 50 µg/L rifampicin, and vector-specific antibiotics, but no gentamicin) with freshly grown *Agrobacteria*.

9. Incubate the YEB plate at 28°C for 2 days.

10. Dilute 10-ml 7-day-old Arabidopsis cell suspension cultures in 40 ml fresh MSCC (1/5 dilution).

11. *DAY 3 (Friday):* Shave off *Agrobacterium* culture (see step 9) from plate, dissolve it in 2 ml MSCC medium in a 15-ml conical tube, and check the OD_{600}. If OD_{600} is below 1.0, shave more *Agrobacterium* from plates and suspend in the same tube. Dilute the *Agrobacteria* to OD ~1 using MSCC medium.

12. *Co-cultivation/transformation:* Take a 50-ml autoclaved Erlenmeyer flask and mix 6 ml 2-day-old Arabidopsis cell suspension culture with 12 µl 0.1 M acetosyringone. Combine 6 ml of Arabidopsis cell suspension/acetosyringone culture with 200 µl (300 µl and 400 µl for tests) of *Agrobacterium* culture from plate (OD ~1.0). Close the flask and shake at 130 rpm for 72 hr at 25°C.

13. *DAY 10 (Monday):* Add 20 ml MSCC plus 250 mg/L ticarcellin clavulanic acid (killing *Agrobacteria*), 250 mg/L vancomycin (killing *Agrobacteria*), and 25 mg/L kanamycin (plant cell culture selection) to a 50-ml flask and shake at 130 rpm 25°C for 5 days.

14. *DAY 15 (Wednesday; only for direct transformation):* Transfer 10 ml (as much cells as possible) into a 100-ml flask containing 40 ml MSCC plus 250 mg/L ticarcellin clavulanic acid (killing *Agrobacteria*), 250 mg/L vancomycin (killing *Agrobacteria*)

    and 50 mg/L kanamycin (plant cell culture selection). Shake at 130 rpm at 25°C for 7 days.

15. *DAY 22 (Wednesday; only for direct transformation:* Transfer 10 ml of cells (after letting them sink down to the bottom) into 40 ml of MSCC plus 250 mg/L ticarcellin clavulanic acid (killing *Agrobacteria*), 250 mg/L vancomycin (killing *Agrobacteria*), and 50 mg/L kanamycin (plant cell culture selection) into a 100-ml flask. Shake at 130 rpm at 25°C for 7 days.

16. *DAY 29 (Wednesday) only for direct transformation:* Transfer as many cells as possible into 40 ml of MSCC with only 50 mg/L kanamycin (plant cell culture selection) in a 100-ml flask

17. Check the plants for protein expression via confocal microscopy (see Current Protocols article: Rajwa, 2005).

This is a critical step. By now, the Agrobacteria should be dead. You can check by streaking on a fresh YEB plate. If Agrobacteria grow, keep the culture in MSCC plus 250 mg/L ticarcellin clavulanic acid (killing Agrobacteria), 250 mg/L vancomycin (killing Agrobacteria), and 50 mg/L kanamycin (plant cell culture selection) for one week more

After 7 days of transferring 5 ml into 45 ml MSCC plus 50 mg/L kanamycin, expression analysis can be done.

Keep shaking at 25°C and 130 rpm and transfer the culture into new medium every week.

AFFINITY PURIFICATION OF PROTEIN COMPLEXES

Protein complexes can be isolated through in vivo immunoprecipitation methods by using specific antibodies recognizing the bait protein. Given that it is incredibly laborious to directly use specific antibodies against plant proteins, an affinity tag protein (GFP or GS tag) fused to the protein of interest facilitates the development of a high-throughput affinity purification method for protein complexes (Van Leene et al., 2015). The target protein could be inserted into pUBC-GFP-Dest and pUBN-GFP-Dest (Grefen et al., 2010) and transformed into plant cell culture as mentioned above. Total protein extracts are collected from the transformed plant materials and incubated with affinity beads in order to purify the protein complexes (Fig. 3). The purified protein complexes are then measured by LC-MS.

Materials

Frozen plant cell power or plant tissue powder: grind plant cells or tissue into a fine powder with a mortar and pestle in the presence of liquid nitrogen; transfer powder to a tube and store in freezer
Extraction buffer (see recipe)
10× protease inhibitor cocktail (Sigma, P8340).
ChromoTek GFP-Trap Nanobody beads (*http://www.chromotek.com/products/nano-traps/*)
Wash Buffer I (same as the extraction buffer)
Wash Buffer II (extraction buffer with 250 mM NaCl)
Wash Buffer III (extraction buffer with 500 mM NaCl)

15-ml conical polypropylene tubes (e.g., Corning Falcon)
2-ml microcentrifuge tubes
Refrigerated centrifuge
Spin Columns (ChromoTek, *https://www.chromotek.com/products/detail/product-detail/spin-columns/*)
Rotating shaker

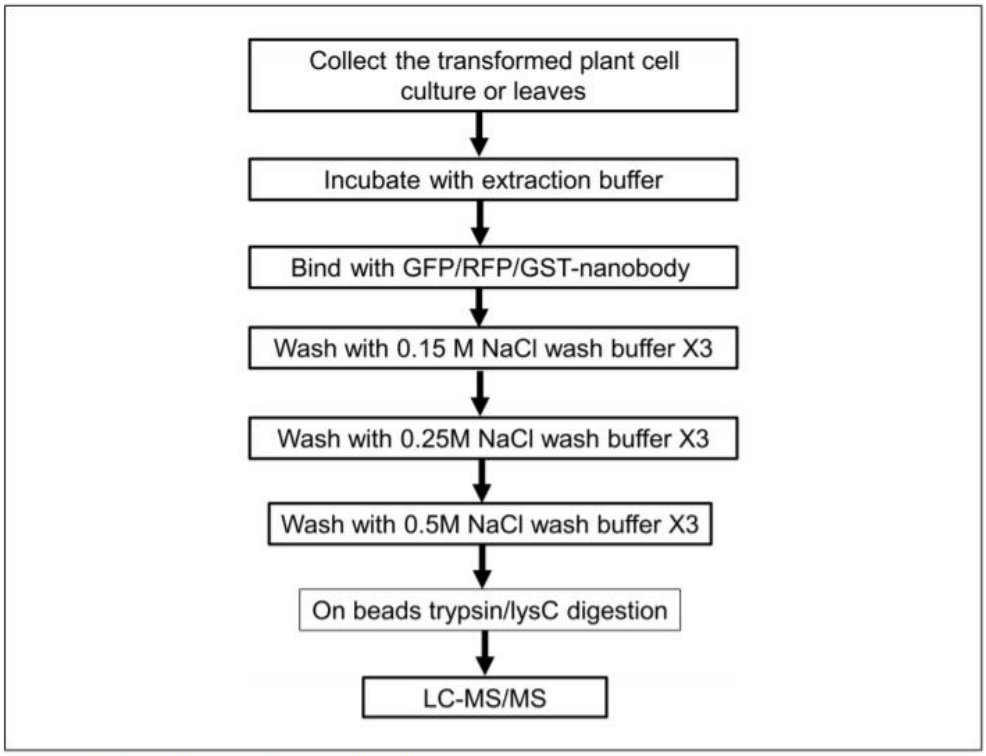

Figure 3 Workflow for affinity purification.

1. Place ~2 g of frozen plant cell powder or plant tissue powder in a 15-ml conical tube and add 2 ml extraction buffer. Use between three and four independent biological replicates.
2. Mix by vortexing for 1 min, incubate on ice for 5 min, add 100 µl 10× protease inhibitor, and repeat the vortexing three times.
3. Centrifuge 10 min at 3000 × *g*, 4°C, transfer 3 ml of the supernatant into 2-ml tubes, centrifuge at 20,000 × *g* at 4°C for 15 min, and transfer the supernatant to new 2-ml tubes. Repeat the centrifugation step and keep the supernatant for the following pull-down assays.
4. Wash 25 µl of GFP beads with 500 µl of extraction buffer three times in 2-ml tubes (remember to cut the ends of the pipette tips to widen the opening and mix well when taking the GFP beads), each time by centrifuging 1 min at 3000 × *g*, room temperature.
5. Transfer 2 ml of the supernatant from step 3 to a tube containing 25 µl of GFP beads, mix gently, and incubate at 4°C for 1 hr with rotation.

6. Centrifuge 1 min at 3000 × *g*, 4°C, and take out 1.6 ml of the supernatant. Using a cut-off pipet tip, transfer the rest of the supernatant and beads to a spin column. Centrifuge the columns 1 min at 3000 × *g*, 4°C.

   Before adding beads to a spin column remove the upper cap of a new spin column and snap off the plug from the bottom of the spin column. Keep cap and plug.

7. Wash the spin columns containing the beads three times, each time for 1 min at 3000 × *g*, 4°C, using 500 µl of Wash buffer I.

8. Wash three times, each time for 1 min at 3000 × *g*, 4°C, using 500 µl of Wash buffer II.

9. Wash three times, each time for 1 min at 3000 × *g*, 4°C, using 500 µl of Wash buffer II.

   The pull-down beads can be used for Western blotting. For in-solution trypsin digestion, the column should be closed by insertion of the bottom plug.

ON-BEAD TRYPSIN/LysC DIGESTION AND C18 COLUMN PEPTIDE DESALTING AND CONCENTRATION

In-solution enzymatic protein digestion is a useful, and sometimes necessary, alternative to in-gel digestion. For samples of low content, or for samples not amenable to SDS-PAGE, in-solution digestion can be used and will provide similar results to in-gel digestion. However, protein folding can protect the amino acid chain from enzymatic cleavage, so denaturation is necessary for efficient cleavage. The conundrum with in-solution digestion is finding conditions to denature the sample without denaturing the protease. Detergents cannot be used in the denaturation process, since they will interfere with subsequent MS analysis. Common denaturants that we use in our laboratory for in-solution digestions include 8 M urea in 10 mM Tris·Cl (pH 8.0), 8 M guanidine HCl (pH 8.0), and 6 M urea/2 M thiourea in 10 mM Tris·Cl (pH 8.0). Unfortunately, trypsin, the most common protease for MS analysis, is not stable under any of these conditions, but fortunately another enzyme, LysC protease, is. LysC cleaves on the carboxyl side of lysine residues, while trypsin targets both lysine and arginine residues.

Materials

Protein sample of interest
6 M urea/2 M thiourea in 10 mM Tris·Cl, pH 8
Reduction buffer: 1 µg/µl (6.5 mM) dithiothreitol (DTT) in water
Alkylation buffer: 5 µg/µl (27 mM) iodoacetamide in water
10 mM Tris·Cl, pH 8
Trypsin/LysC proteases, modified sequencing grade (Promega): 0.4 µg/µl; i.e., in 50 µl, 20 µg)

100% methanol
80% (v/v) acetonitrile/0.1% trifluoroacetic acid (TFA) in distilled deionized water
0.1% and 2% (v/v) TFA in distilled deionized water
Resuspension solution: 0.2% (v/v) TFA/5% acetonitrile
Equilibration buffer A (100% H_2O/0.2% TFA)
Elution buffer B (100% acetonitrile/0.2% TFA)

Bath sonicator
Refrigerated centrifuge
pH strips
C_{18} SepPak columns, 100 mg/ml
Visiprep™ 12- Port Vacuum Manifold (Sigma, 57044)
Vacuum pump
SpeedVac evaporator
Nano LC 1000 liquid chromatograph (ThermoFisher Scientific) with reversed-phase C18 column (Acclaim PepMap RSLC, 75 µm × 150 mm, C18, 2 µm, 100 Å; ThermoFisher Scientific)

In-solution digestion

1. Dissolve sample in a small volume of 6 M urea/2 M thiourea (pH 8.0). Use as low a volume as is compatible with your sample. Sonicate for 10 min to solubilize using a bath sonicator.

   In this procedure, all steps are performed at room temperature to reduce unwanted derivatization of amino acid side-chains by the denaturants.

2. Centrifuge samples 10 min at 8000 × *g*, room temperature, to pellet any insoluble material.

   The pH of the final solution should be near 8.0 for optimal trypsin digestion. Check this with pH strips.

3. Add 1 µl reduction buffer for every 50 µg of sample protein and incubate for 30 min at room temperature.

   Only a very rough estimate of protein content is necessary—where sample amount is limited, it is better to sacrifice accuracy than waste sample on a protein assay.

4. Add 1 µl alkylation buffer for every 50 µg sample protein and incubate for 20 min at room temperature in the dark.

5. Dilute sample with four volumes of 10 mM Tris·Cl, pH 8.

   This step is absolutely necessary to dilute the urea concentration, as trypsin/LysC is very sensitive to high concentrations of salt.

6. Add 1 µl of 0.4 µg/µl trypsin/LysC per 50 µg sample protein and incubate overnight at 37°C.

C18 Stage- SepPak for peptide desalting and concentration

7. Put the C18 Stage-SepPak columns in the Visiprep™ 12- Port Vacuum Manifolds and attach the vacuum pump.
8. Equilibrate the C18 SepPak columns with 1 ml 100% methanol and switch on the pump.
9. Equilibrate the C18 SepPak columns with 1 ml 80% acetonitrile/0.1% TFA in distilled, deionized water and switch on the pump.
10. Equilibrate with 1 ml of 0.1% TFA in distilled deionized water and switch on the pump. Repeat this step.
11. Dissolve samples in 0.1% TFA (add 1/10 volume of 2% TFA to reach pH 2.0). If the pH is too high, add 2% TFA, until it reaches a pH of 2.0.

    It is very important to reach pH 2.0 for the peptide to bind to the column.

12. Load the sample onto the SepPak columns, and switch on the pump.
13. Wash the tube that contained the digested sample with 200 µl 0.1% TFA, centrifuge 1 min at 1000 × *g*, and load this onto the column.
14. Wash the column with 1 ml of 0.1% TFA. Repeat this step.
15. Elute the peptides with 800 µl of 60% acetonitrile and 0.1% TFA into a new 1.5-ml microcentrifuge tube.
16. Dry the peptides in a SpeedVac evaporator.

    Samples can be stored dry at −80°C for a long time.

17. Using a Nano LC 1000 liquid chromatograph with a reversed-phase C18 column (Acclaim PepMap RSLC, 75 µm × 150 mm, C18, 2 µm, 100 Å), perform mass spectrometric analysis as required by the experiment.

    Here, we suggest using neutral-loss scanning and multistage activation.

18. Add a final volume of 40 µl of resuspension solution (0.2% TFA/5% acetonitrile) to the sample and transfer it to a microtiter plate for mass spectrometric analysis.

    LC-MS/MS analysis can be performed on a Q Exactive Plus (ThermoFisher Scientific).

    A Nano LC 1000 (ThermoFisher Scientific) and reversed-phase C18 column (Acclaim PepMap RSLC, 75 µm × 150 mm, C18, 2 µm, 100 Å) are used to resolve peptides.

    A gradient is prepared using Equilibration buffer A (100% H_2O/0.2% TFA) and elution buffer B (100% acetonitrile; 0.2% TFA). Gradient should be run as follows: 5 min from 0 up to 10% buffer B with 300 nl/min flow, 30 min up to 20% buffer B with flow 300 nl/min, 8 min up to 40% buffer B with flow 300 nl/min, followed by wash for 2 min with 80% buffer B at flow of 300 nl/min, 5 min with 80% buffer B at flow of 500 nl/min, and 5 min with 0% buffer B at flow of 500 nl/min.

Q Exactive Plus Full MS scan settings are resolution 60,000, AGC target 3e6, maximum IT 100 ms, scan range 150 to 1600 m/z.

MS2 scan settings are resolution 15,000, AGC target 2e5, loop count 15, isolation window 2 m/z, collision energy.

Data-dependent acquisition settings are apex trigger on, charge exclusion 1.5-8, >8.

Quantitative analysis of MS/MS measurements is performed using the Progenesis IQ software (Nonlinear Dynamics, Newcastle, U.K.).

Proteins are identified from spectra using Mascot (Matrix Science, London, UK). Mascot search parameters are set as follows: TAIR10 protein annotation, requirement for tryptic ends, one missed cleavage allowed, fixed modification: carbamidomethylation (cysteine), variable modification: oxidation (methionine), peptide mass tolerance = ±10 ppm, MS/MS tolerance = ±0.6 Da, allowed peptide charges of +2 and +3. A decoy database search is used to limit false discovery rates to 1% on the protein level. Peptide identifications below rank 1 or with a Mascot ion score below 25 are excluded. Mascot results are imported into Progenesis QI, quantitative peak area information extracted, and the results exported for data plotting and statistical analysis.

DATA ANALYSIS AND QUALITY CONTROL

The ribosome protein and translation-related protein could be deleted at this step. The normalized signal intensities are processed to determine fold-change abundance (FC-A) scores by use of the SAINT algorithm embedded within the CRAPome software (Mellacheruvu et al., 2013). Compared with the GFP control, the background protein could be deleted at this step by FC-A values of at least four within at least three replicates (Morris et al., 2014). MS/MS information from the SUBA4 database (Hooper, Castleden, Tanz, Aryamanesh, & Millar, 2016) could give the subcellular localization of the bait and prey, and thus improve the reliably of the interactions. Finally, we consider only the protein pairs for which the intensities are in the top 2% compared with the bait intensity to represent positive interactions.

1. Following the LC-MS measurement, the ribosomal and translation-related proteins can be deleted unless there is a specific interest in translation-regulatory proteins.

2. The normalized intensity of all the bait and tag control lines can be analyzed by the CRAPome software (you need to follow the introduction to the software to prepare the interaction files (Fig. 4A).

3. After getting the resulting lists of candidate interactors with the corresponding fold change (FC; Fig. 4B and C), interactions with FC greater than 4 can be selected as

(A) Examples to prepare the excel files for the CRAPome software analysis.

PROTID	PROTLEN	Cont-GFP_NUMSPECSTOT	Cont-GFP_NUMSPECSTOT	Cont-GFP_NUMSPECSTOT	Cont-GFP_NUMSPECSTOT	Cont-PGM3_NUMSPECSTOT	Cont-PGM3_NUMSPECSTOT	Cont-PGM3_NUMSPECSTOT	Cont-PGM3_NUMSPECSTOT
NA	NA	CONTROL	CONTROL	CONTROL	CONTROL	PGM3	PGM3	PGM3	PGM3
AT2G42910	337	21249637,41	21621943,17	23370834,78	32378427,60	1155836,67	1237408,23	12100093,78	413810,96
AT1G56070	134	5783886,34	6679244,61	1492147,07	10111859,18	3985997,59	2942070,26	1882101,23	1956779,62
AT1G48920	106	2540713,27	4089461,93	2628127,04	3799826,41	2006734,94	1520180,84	4561941,77	1814627,52
AT1G56110	135	2209645,16	5626975,26	3485770,79	8505429,98	1211525,27	864350,09	856764,89	227541,89

The value is the the normalized intensity from LC-MS measurement.

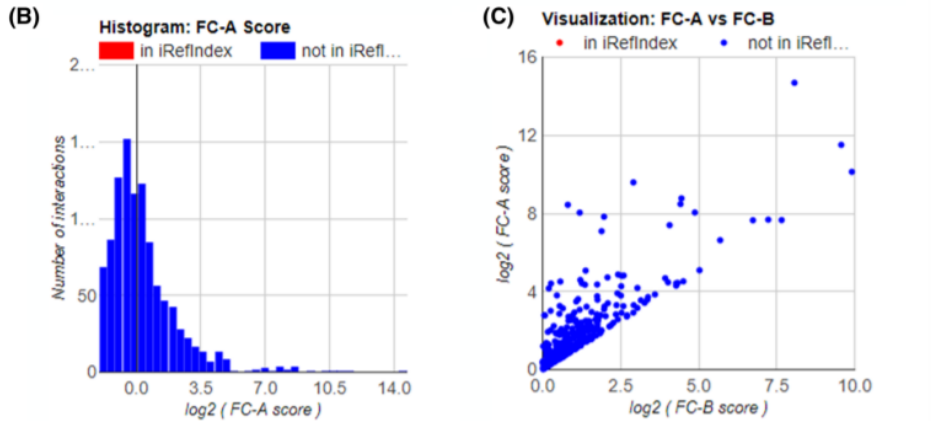

Figure 4 Data analysis. (**A**) Examples to prepare the Excel files for the CRAPome software analysis. (**B**) Histogram of FC-A score. Data are displayed in a table format and in different graphical formats. The standard primary fold-change calculation (FC-A) averages the counts across all controls, while the more stringent secondary fold-change score (FC-B) takes the average of the top three highest spectral counts for the abundance estimate. (**C**) Visualization: FC-A versus FC-B. The conservative FC scores readily distinguish between the contaminant and true interaction partner.

possible positive interactions. Only the intensities of interactors that have greater than 2% of the intensity of the GFP protein are selected.

4. The possible target prey proteins also need to be analyzed by the SUBA4 database to get the right subcellular localization with the bait protein.
5. The protein interaction network can be presented by Cytoscape (*https://cytoscape.org/*; Shannon et al., 2003).

REAGENTS AND SOLUTIONS

Extraction buffer

	Final concentration	For 10 ml	Stock
Tris·Cl, pH 7.5	25 mM	500 µl	0.5 M
$MgCl_2$	15 mM	150 µl	1 M
EGTA	5 nM	250 µl	200 mM
DTT	1 mM	10 µl	1 M
PMSF	1 mM	100 µl	0.1 M
NaCl	150 mM	300 µl	5 M
H_2O		8.69 ml	

Store up to 6 months at 4°C

Lysogeny broth (LB) medium and agar plates

10 g/L tryptone
5 g/L yeast extract
10 g/L NaCl
For LB agar plates, add 15 g agar per liter
Store medium or plates up to 3 months at 4°C

MSCC medium

Prepare 4.43 g Murashige & Skoog salts with minimal organics (Sigma, M6899) with 30 g sucrose in a volume of 1 liter and adjust pH to 5.7 with KOH. Autoclave. Before use, add freshly prepared 50 µl kinetin [1 mg/ml kinetin (Sigma, K0753)/ 0.1 M NaOH] and 500 µl NAA [1 mg/ml α-naphtaleneacetic acid (NAA; Sigma, N0640)/0.1 M NaOH]. Also add the following antibiotics:

500 µl kanamycin (50 mg/ml, plant cell culture selection) or 500 µl hygromycin (10 mg/ml, plant cell culture selection)
2000 µl ticarcellin clavulanic acid, (125 mg/ml, killing *Agrobacteria*)
2500 µl vancomycin (100 mg/ml, killing *Agrobacteria*).

All antibiotics, NAA, and kinetin must be filter-sterilized using a 0.2-µm filter. The medium can be stored up to 6 months at 4°C after adding antibiotics.

Yeast extract beef (YEB) medium and plates

1.0 g/L yeast extract
5.0 g/L beef extract
5.0 g/L peptone
5.0 g/L sucrose
20 g/L agarose (for plates)
1000 ml distilled water
Autoclave
Freshly add antibiotics:
Rifampicin (50 µg/ml final concentration)
Gentamicin (20 µg/ml final concentration)
Vector-specific antibiotics
Store up to 6 months at room temperature

Agrobacterium tumefaciens AGL1 carries the hypervirulent, attenuated tumor-inducing plasmid pTiBo542 from which T-region DNA sequences have been precisely deleted, allowing optimal DNA transformation of many dicotyledonous plants (Lazo, Stein, & Ludwig, 1991).

Background Information

A protein complex is a group of two or more proteins associated by different or the same functional polypeptide chains by non- covalent interactions in vivo (Hartwell, Hopfield, Leibler, & Murray, 1999). They are usually organized into functional modules to play central roles in regulating DNA replication, transcription, translation, RNA splicing, protein secretion, cell cycle con- trol, signal transduction, and intermediary metabolism (Bontinck et al., 2018). As they are the basis of many biological processes, studying these complexes and exposing their intricate interaction networks are thus of fundamental importance to understand not only basic cellular processes but also complex developmental programs. Several methods for analyzing protein-protein inter- actions (PPIs) are available, such as yeast two-hybrid (Y2H; Parrish, Gulyas, & Finley, 2006), co-immunoprecipitation followed by western blotting (co-IP; Antrobus & Borner, 2011), or protein-fragment complementation assays such as bimolecular fluorescence complementation (BiFC; Kerppola, 2008) and split luciferase (Chen et al., 2008; Fujikawa & Kato, 2007; Li, Bush, Xiong, Li, & McCormack, 2011). However, most of these methods allow testing PPIs only in a pairwise fashion or as three-protein interactions, and require prior knowledge to determine which combinations to test. Thus, a complementary method that is more suited to study co-complex memberships is AP-MS (Mellacheruvu et al., 2013). This method can affinity-purify protein complexes under near- physiological conditions to maintain PPIs intact, which is followed by their detection with mass spectrometry (Choi et al., 2011). In AP-MS, PPIs can be captured by anti- bodies specific for the bait proteins or for tags that were introduced on the bait proteins and pulled down onto immobilized protein agarose beads or magnetic agarose beads (Zhang, Sun, et al., 2017). The affinity-purified protein com- plexes can be further digested into peptides by trypsin/LysC and identified by quantification of the resulting peptides via mass spectrom- etry. As the specific interactors are enriched in the bait sample, this method can produce a large amount of information-rich data that detail protein-protein interactions in different organisms and biological systems or different conditions and treatment (see Current Proto- cols article: Adelmant, Garg, Tavares, Card, & Marto, 2019). Using the putative interac- tions information, we can further confirm in- teractions by other binary interaction methods in order to characterize the functions of pro- teins and provide detailed catalogs of proteins involved in protein complexes and biological processes. These interactions could also reveal networks of biological processes at local and proteome-wide scales to further help us un- derstand the genetic, epigenetic, and protein- based associations of these proteins. To estab- lish a reliable protein interaction network, a well-established procedure is needed includ- ing sample preparation, diverse interaction scoring and clustering algorithms, methods for graph theory and data mining, and biologi- cal networks. In this article, we describe plant cell culture transformation, sample prepara- tion, affinity purification, in-solution diges- tion, mass spectrometry detection, and, finally, data analysis required to produce meaningful networks. In addition, the success of AP-MS depends on several factors, including high expression levels of bait protein, the extraction of pro- tein complexes, the antibody to enrich the bait protein and preserve the protein complexes, the efficiency of trypsin digestion, and the re- covery of the tryptic peptides for MS analysis (Oeffinger, 2012; Varjosalo et al., 2013). Here, we suggest using a GFP tag for the bait protein, which facilitates the detection of the protein lo- calization by confocal microscopy (Dunham, Mullin, & Gingras, 2012; Keilhauer, Hein, & Mann, 2015). The efficiency of the trypsin digestion and the recovery of the resulting digested peptides for MS analysis are very important for the success of AP-MS (Zhang, Swart, et al., 2018). Instead of in-gel digestion for performing global proteomics profiling (Huang et al., 2016; Van Leene et al., 2015), several studies have used the improved effi- ciency of in-solution digestion on the beads, reducing time and steps (Leo´n, Schwa¨mmle, Jensen, & Sprenger, 2013; Zhang, Sun, et al., 2017). These studies have shown that the choice of chaotropic agent, surfactant, or or- ganic solvent has a significant impact on the ef- ficiency, reproducibility, and completeness of trypsin digestion, and hence affects sequence coverage of protein identification by MS anal- ysis. Here, we use the simplified method of digesting the protein in the urea/thiourea so- lution with both LysC and trypsin, and desalt the tryptic peptide in a C18 Stage-SepPak for the mass spectrometry.

Critical Parameters and Troubleshooting

The following troubleshooting guide does not include common issues that may arise when using reagents other than those rec- ommended in the protocols (such as using anti-GFP-agarose and anti-RFP-agarose beads from suppliers other than ChromoTek), when a different tag is used for the bait protein (such as GS tag, GST tag), or when a different di- gestion method is used.

Protein expression in the plant cell culture

The protein expression level should be checked by confocal microscopy before start- ing the affinity purification. For subcellularly localized proteins (nuclear or membrane), the specific procedure for breaking the nucleus or membrane should be perfomed first and the protein expression level can be confirmed by western blotting before the AP-MS. Normally, the ubiquitin 10 promoter can express enough protein, while the 35S promoter can result in many false-positive interactions because of the higher expression. Plant cell cultures can be treated with different buffers or environments for different pull-down conditions.

Tag used for the affinity purification

As they enable protein expression level to be easily detected, GFP-, RFP-, or mCherry- tagged baits are suggested in this procedure. The GSrhino-TAP and glutathione S-transferase (GST) tags can also be used in affinity purification from plant, while the HA, FLAG, and His tags are not suggested for affinity purification from plant materials, since these three tags result in very strong background sig- nals in plant.

Biological replicates and statistical analysis

In order to obtain a reliable protein interac- tion network, we recommend using more repli- cates (at least three) for the statistical analysis. Normalized intensities are used for the data analysis. For some poor reproducibility sam- ples, six replicates are suggested. In addition, the proteins that only have intensity in bait samples are suggested as the candidate inter- actors.

Negative control

Given that the AP-MS produces large amounts of information-rich data, both the type of bead and the tag affect the affinity- purified interactors. Using the same subcellu- lar localization of GFP as negative control, it can very importantly exclude the false-positive interactions. Proteins localized to subcellular compartments different from the bait protein are likely false positives and should be excluded. Here, we suggest using SUBA4 (http://suba.live/) to exclude subcellular local- izations that are different from the bait protein. Given that large amounts of ribosomal proteins and proteins related to translation are detected from the AP-MS, these interactors should also be excluded. It is important to note that using a different plant material as control will result in lots of false-positive or false-negative interactors, e.g., if using transformed plant cell culture for the samples analysis while using the transformed seedling GFP lines as control.

Data analysis

In the data analysis, normalized signal intensities are processed to determine fold- change abundance (FC-A) scores by use of the SAINT algorithm embedded within the CRAPome software (Morris et al., 2014). Compared with intensity of bait, only the pro- teins for which the intensity score was more than 2%, corresponding to FC-A values of at least 4 within at least three replicates, should be regarded as positive interactions. Screening the SUBA4 database (Hooper et al., 2016), only pairs sharing the same subcellular local-ization are selected as positive interactors. In addition, transient interactions also play an im- portant role within protein-protein interaction networks, especially post-translational modi-fications (Perkins, Diboun, Dessailly, Lees, & Orengo, 2010). The proteins that have low in- tensity in bait samples and no intensity in the negative control can be selected.

Understanding result

The affinity purification protocol presented has been used by us and our collaborators to characterize binding partners of proteins spanning a variety of functional categories. This protocol should enable both novices and skilled biochemists alike to obtain valuable and meaningful information about interaction partners and help generate novel hypotheses.

TCA cycle interaction network

The 38 mitochondrial proteins of *Arabidop-sis thaliana* were transformed into a PSB-D Arabidopsis plant cell culture, and a GFP-tag-based modified AP-MS procedure was im- plemented based on at least three biological replicates (Zhang, Beard, et al., 2017; Zhang, Swart, et al., 2018). Unlike normal AP-MS in which the gel is cut into pieces for several independent trypsin digestions (Morris et al., 2014), we used a proteomics-based in-solution digestion method to directly digest the pro- teins on the beads following affinity purifi- cation (Zhang, Sun, et al., 2017). Thus, an AP-MS experiment constitutes a single sam- ple for the LC-MS measurement. In the subse- quent data analysis, normalized signal intensi-ties were processed to determine fold-change abundance (FC-A) scores by use of the SAINT algorithm embedded within the CRAPome software (Morris et al., 2014). A total of 3421 protein-protein interactions were obtained dis- playing in excess of four-fold changes in the five independent experiments. We considered only the protein pairs for which the protein in-tensity was in the top 2% compared with bait protein, corresponding to FC-A values of at least 4 within at least three of the replicates as positive interactions. A total of 449 poten- tial positive protein-protein interactions were obtained according to these criteria, including those interactions with several ribosomal and protein-translation proteins.

As we are interested in mitochondrial in- teractions, only the mitochondrially targeted proteins were selected for network genera- tion. It is, however, important to note that given that many of the enzymes of the TCA cycle have isoforms (exhibiting high iden- tity), in more than one compartment the non-mitochondrial interactions, while not directly physiologically relevant, may well provide hints to interactions that do occur in vivo albeit extra-mitochondrially. Screening of the SUBA4 database (Hooper et al., 2016) re- vealed a total of 257 interactions between mitochondrially localized TCA cycle proteins and 37 of the proteins comprising the mito- chondrial interaction network. Of these 257 interactions, 132 interactions between the en-zymes of TCA cycle had already been reported (Zhang, Beard, et al., 2017), while there were 125 novel interactions between subunits of en- zymes and other pathway enzymes or proteins (Zhang, Swart, et al., 2018).

Time considerations

Identification of protein-protein interaction networks comprises multiple steps which can be accomplished within 1 to 2 months. The project is easily divided among the following independent stages: gene cloning will require 1 week, plant cell culture transformation will require 1 month, and finally the affinity purifi-cation with mass spectrometry and subsequent data analysis will require 2 to 4 weeks of work. The time needed for processing of protein interaction networks depends on the (i) trans-formation of plant cell culture, (ii) transient expression of plant leaves, and (iii) mass spec- trometry measurement. In our experiment, a full run that includes all the steps can be finished within 2 months.

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