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Small signals lead to big changes: Deciphering the mechanisms behind Peptide-Induced Resistance in plants

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Memòria presentada per Julia Pastor Fernández per optar al grau de doctora per la Universitat Jaume I

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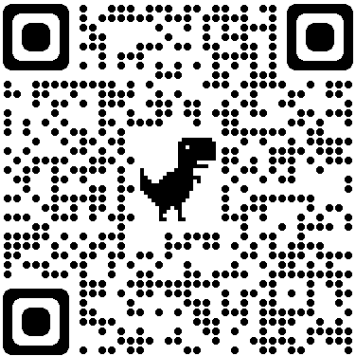

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Pero hui ja passe del drama
Vull tocar el foc, sentir que crema
Jo tinc sed de vida, no de fama
No vull que un error siga un problema

(Estiu-ZOO)

TABLE OF CONTENTS

ABBREVIATIONS

4MI3G: 4-hydroxyindol-3-ylmethyl glucosinolate

ABA: abscisic acid

AlaAT: alanine aminotransferase

ANOVA: analysis of variance

AMF: arbuscular mycorrhizal fungi

AspAT: aspartate aminotransferase

ATP: adenosine triphosphate

BABA: ®-aminobutyric acid

cDNA: complementary DNA

CDPK: calcium-dependent protein kinases

CHO: carbohydrate

DAMP: damage-associated molecular pattern

DH: dehydrogenase

DNA: deoxyribonucleic acid

dpt: days post treatment

ESI: electrospray ionization

ET: ethylene

ETI: effector triggered immunity

ETS: Effector triggered susceptibility

F1,6BPase: fructose 1,6-biphosphatase

FA: fatty acid

FBP: fructose biphosphastase

FK: Fructokinase

G6PDH: Glucose 6-phophate dehydrogenase

GAPDH: glyceraldehyde 3-phophate dehydrogenase

GK: glucokinase

GLS: glucan synthase like

GluDH: glutamate dehydrogenase

GPA: guanine nucleotide-binding protein alpha-1

GPI: glucose-6-phosphate isomerase

HAMP: herbivore-associated molecular pattern

hpi: hours post infection

hpt: hours post treatment

HR: hypersensitive response

I3CA: indole-3-carboxylic acid

IAA: indole acetic acid

IAM: indole-3-acetamide

IAN: indole-3-acetonitrile

IAOx: indole-3-Acetaldoxime

inf: infected

IR: induced resistance

ISR: induced systemic resistance

JA: jasmonic acid

JA-Ile: jasmonic acid-isoleucine

LAP A: leucine aminopeptidase

LC-MS: liquid chromatograph-mass spectrometer

LOX: lipoxygenase

LSD: least significant difference

MAMP: microbe-associated molecular pattern

MAPK: mitogen-activated protein kinase

MDH: malate dehydrogenase.

MeJA: methyl jasmonate

MeSA: methyl salicylate

MIR: mycorrhiza induced resistance

nM: nanomolar

OPDA: 12-oxophytodienoic acid

OPP: oxidative pentose phosphate pathway

PAMP: pathogen-associated molecular pattern

PCR: polymerase chain reaction

PDS: Phytoene desaturase

Pep: peptide

PEPC: phosphoenolpyruvate carboxylase

Pep-IR: peptide-induced resistance

PFK: phosphofructokinase

PGA: phosphoglycerate

PGI: phosphoglucose isomerase

PGK: phosphoglycerokinase

PGM: phosphoglycerate mutase

PGPR: plant-growth-promoting rhizobacteria

PGPF: plant-growth-promoting fungi

PI: protease inhibitor

PK: pyruvate kinase

PR: pathogenesis related

PS: prosystemin

PTI: pattern triggered immunity

RNA: ribonucleic acid

ROS: reactive oxygen species

RT: retention time

RT-qPCR: real-time quantitative PCR

SA: salicylic acid

SAR: systemic acquired resistance

sPLSDA: sparse partial least squares-discriminant analysis

Sys: Systemin

Sys-IR: systemin induced-resistance

TCA: tricarboxylic acid cycle

Trp: tryptophan

UPLC: ultraperformance liquid chromatography

VIGS: Virus Induced Gene Silencing

VOCs: volatile organic compounds

SUMMARY

SUMMARY

In nature, plants are constantly challenged by environmental biotic and abiotic factors. To cope with biotic stresses, such as pest and pathogen attacks, they have evolved a broad variety of adaptative defense strategies. Sometimes plant perception of external stimuli induces an enhanced resistance state that confers protection against a future attack in local and distal tissues. This state is known as "Induced Resistance" and can be triggered by both biological and non-biological stimuli. Recently an increasing number of plant peptides were described as defense elicitors that act as secondary danger signals or phytocytokines. They are released upon pest or pathogen attack and bind to membrane receptors triggering an amplification of immune responses. However, their potential as defense elicitors is poorly studied.

In this thesis, we found that peptides from different species can induce resistance against the necrotrophic fungus *Plectosphaerella cucumerina* in the taxonomically distant species *Arabidopsis thaliana* at very low concentrations. This induction of resistance was due to the stimulation of the plant immune system since antifungal in vitro assays revealed that they do not display direct antifungal activity. An analytical method for multiple peptides identification and quantification was developed. Noteworthy, of the tested peptides Systemin conferred a high degree of protection in Arabidopsis from very low concentrations, showing an optimal threshold of action, resembling the mode of action of a phytohormone or other IR elicitors. Thus, the following analyses were focused on deciphering the mechanisms of Systemin-Induced Resistance (Sys-IR). Systemin is a short peptide that regulates the plant response against herbivores in tomato plants. It is released upon wounding or pathogen attack and induces a cascade of plant defenses that produce the accumulation of protease inhibitors in local and systemic tissue. There is also evidence of the involvement of Systemin in tomato defenses against pathogens such as the necrotrophic fungus *Botrytis cinerea*. However very little is known about the perception and function of Systemin in heterologous species.

Analysis of the hormonal regulation of Systemin triggered defenses in Arabidopsis revealed that, like in tomato, JA but not SA was implicated in Sys-IR. In an attempt to unveil the perception of Systemin in Arabidopsis we found that Pep1 receptor PEPR1, the homolog of Systemin in Arabidopsis, was not responsible for the Systemin signal

transduction in this species. Regarding early signaling triggered by Systemin upon infection, *BAK1*, *BIK1*, *AGG2*, *RBOHD*, *MPK3*, and *MPK6* gene expression displayed a priming profile. In addition, Systemin primed MPK3 and MPK6 phosphorylation and ROS production upon a PAMP challenge. Lost of function mutants of the mentioned genes were impaired in the enhanced resistance triggered by Systemin treatment, demonstrating their key role in Sys-IR against *P. cucumerina*. To understand the metabolic fingerprint of Systemin in Arabidopsis and the possible mechanisms behind Sys-IR, we performed a non-targeted metabolomic analysis of Systemin-treated plants before and after fungus infection in Arabidopsis plants. This analysis revealed, on the one hand, that phenolic compounds were overaccumulated upon Systemin treatment and, on the other hand, that Systemin primed specific indolic compounds. By gene expression and knock-out mutant analysis, we confirmed that flavonoids and tryptophan-derived compounds were essential elements in expressing functional Sys-IR.

Our next goal was to shed light on the mechanisms of Sys-IR in its species of origin, tomato, in order to find metabolic fingerprints of the Systemin mode of action as a resistance inducer. Previous studies by other authors have demonstrated how endogenous levels of the Systemin precursor, ProSystemin, influence plant resistance against pests and pathogens, however, knowledge of the effects of exogenously applied Systemin is very scarce. In the present study, we found that exogenous treatment of Systemin has a great impact on the plant metabolism at different metabolic levels and triggered enhanced resistance to the necrotrophic fungi *Botrytis cinerea* through priming of callose deposition. Systemin treatment strongly affected the behavior of proteins and enzymatic activities of the primary metabolism participating in the photosynthesis, carbohydrate metabolism, TCA cycle, glycolysis, and amino acid metabolism. These changes lead to the accumulation of available sugars monomers and carbon structures, including tricarboxilic acids. The overaccumulation of a starch phosphorylase, a glucan synthase-like, and callose synthase-like proteins together with a higher starch degradation in Systemin-treated plants could explain the observed priming of callose. Additionally, proteins involved in redox homeostasis and the biosynthesis of the phenolic compound were induced by Systemin. Conversely, after infection, very few changes were observed in the proteomic profile. However, these few proteomic changes were very specific for pathogen defense including pathogen-related proteins 1 and 4 (PR1 and PR4) and a 1,3-**β**-glucanase (PR2). Unlike in the proteomic profile, major changes in the metabolome

were observed after infection, showing a clear priming profile. Some amino acids, phenolic compounds, and alkaloids were identified among the metabolites induced or primed by Systemin, which correlated well with the results obtained in the proteomic analysis. On the other hand, many metabolites showed a buffering effect towards infection, while they over-accumulated in control plants upon infection, they remained at the same levels of uninfected control or even lower in Systemin-treated plants following infection. A similar pattern was observed in the enzymatic activities in infected plants, especially at late time points after pathogen challenge.

Finally, we aim to find early signaling events that enable downstream Systemin triggered responses to ensure Sys-IR against *B. cinerea* in tomato plants. Tomato MPK1, MPK2, and MPK3, orthologs of Arabidopsis MPK3 and MPK6, which were previously reported to be involved in tomato plants' defense responses, were selected for further analysis. In the present work, we found that MPKs phosphorylation was primed by Systemin treatment upon a fungal PAMP challenge demonstrating its involvement in Sys-triggered defense responses. Analysis of MPK1/2 and 3 silenced plants by using the Virus-Induced Gene Silencing (VIGS) technique revealed that MPKs act upstream Systemin triggered induction of defense genes in the absence and presence of infection, including JA-related genes and genes involved in the Systemin production and release. Additionally, silenced plants in either MPK1 and 2 or MPK3, showed impaired Systemin response and protection against *Botrytis cinerea*, confirming that MPKs are essential signaling elements to ensure functional Sys-IR.

RESUM

En la naturalesa, les plantes són constantment desafiades per factors biòtics i abiòtics ambientals. Per fer front als factors biòtics, com els atacs de plagues i patògens, han desenvolupat una àmplia varietat d'estratègies de defensa adaptativa. De vegades, la percepció vegetal d'estímuls externs indueix a un estat de resistència reforçat que confereix protecció contra un atac futur en teixits locals i distals. Aquest estat és conegut com a "resistència induïda" i pot ser desencadenat tant per estímuls biològics com no biològics. Recentment, un nombre creixent de pèptids de les plantes van ser descrits com a inductors de defensa que actuen com a senyals de perill, també coneguts com a fitocitoquines. S'alliberen en el moment d'atac de plagues o patògens i s'uneixen als receptors de membrana provocant una amplificació de les respostes immunitàries. No obstant això, el seu potencial com a inductors de la defensa està molt poc estudiat.

En aquesta tesi, es va trobar que pèptids de diferents espècies poden induir resistència contra el fong necrotròfic *Plectosphaerella cucumerina* en l'espècie taxonòmicament distant *Arabidopsis thaliana* a concentracions molt baixes. Aquesta inducció de resistència es va deure a l'estimulació del sistema immunitari de la planta, ja que els assajos antifúngics *in vitro* van revelar que no mostraven activitat antifúngica directa. A més, es va desenvolupar un mètode analític per a la identificació i quantificació de múltiples pèptids. Cal destacar que, dels pèptids provats, Sistemina va conferir un alt grau de protecció en Arabidopsis des de concentracions molt baixes, mostrant un llindar òptim d'acció, semblant al mode d'acció d'una fitohormona o altres inductors resistència. Així, els anàlisis següents es van centrar en el desxifratge dels mecanismes de la Resistència Induïda per Sistemina (Sys-IR). Sistemina és un pèptid curt que regula la resposta de la planta contra els herbívors en les plantes de tomàquet. S'allibera després de produir-se una ferida o un atac d'herbívor i indueix una cascada de defenses vegetals que produeixen l'acumulació d'inhibidors de proteases en teixit local i sistèmic. També hi ha evidència de la implicació de la Sistemina en les defenses de tomàquet contra patògens com el fong necrotrofòtic *Botrytis cinerea*. No obstant això, se sap molt poc sobre la percepció i la funció de la Sistemina en espècies heterològues (que no la produeixen de forma natural).

L'anàlisi de la regulació hormonal de les respostes defensives induïdes per la Sistemina va revelar que, com en la tomata, el àcid jasmònic (JA) però no el salicílic (SA) està implicat en Sys-IR. En un intent de revelar la percepció de la Sistemina en Arabidopsis,

vam trobar que el receptor de Pep1, PEPR1, l'homòleg de la Sistemina en Arabidopsis, no era responsable de la transducció de senyals de Sistemina en aquesta espècie. Pel que fa a la primera línia de senyalització provocada per la Sistemina després de la infecció, l'expressió gènica de *BAK1, BIK1, AGG2, RBOHD, MPK3* i de *MPK6* va mostrar un perfil de priming. A més, Sistemina va induir priming en la fosforilació de MPK3 i 6 i en la producció de ROS tres l'aplicació d'un PAMP. La majoria dels mutants de funcions dels gens esmentats es van veure perjudicats en la resistència reforçada provocada pel tractament de la Sistemina, demostrant el seu paper clau en Sys-IR contra *P. cucumerina*. Per entendre l'empremta metabòlica de la Sistemina en Arabidopsis i els possibles mecanismes darrere de la resistència induïda, realitzarem un anàlisi metabolòmic no dirigit de les plantes tractades per Sistemina abans i després de la infecció de fong en les plantes d'Arabidopsis. Aquest anàlisi va revelar, d'una banda, que els compostos fenòlics s'acumulaven en excés pel tractament de la Sistemina i, d'altra banda, que la Sisteminaa indueix priming de compostos indolics específics. Per l'expressió gènica i l'anàlisi de mutants, vam confirmar que els flavonoides i els compostos derivats del triptòfan eren elements essencials per expressar la resistència induïda per Sistemina.

El nostre següent objectiu va ser tractar de dilucidar els mecanismes de Sys-IR en la seva espècie d'origen, el tomàquet, per tal de trobar empremtes metabòliques de la manera d'acció de la Sistemina com a inductor de resistència. Estudis previs d'altres autors han demostrat com els nivells endògens del precursor de la sistèmica, la ProSistemina, influeixen en la resistència de les plantes contra els plagues i patògens, però el coneixement dels efectes de la Sistemica aplicada exògenament és molt escàs. En l'estudi actual, es va trobar que el tractament exogen de la Sistemina té un gran impacte en el metabolisme de les plantes a diferents nivells metabòlics i va desencadenar una resistència millorada als fongs necrotrof *Botrytis cinerea* a través d'un augment en la deposició de cal·losa. La inducció de cal·losa sembla estar relacionada amb una degradació més ràpida de midó en plantes tractades amb Sistemina. El tractament va afectar fortament el comportament de les proteïnes i les activitats enzimàtiques del metabolisme primari que participa en la fotosíntesi, el metabolisme dels carbohidrats, el cicle TCA, la glicòlisi i el metabolisme dels aminoàcids. Aquests canvis condueixen a la producció de sucres i estructures de carboni disponibles. La sobreacumulació d'una fosforilasa de midó, una glucosa-sintasa, i proteïnes semblants a la cal·losa-sintasa juntament amb una major degradació de midó en les plantes tractades podrien explicar el

primin observat en la deposició de cal·losa. Addicionalment, les proteïnes implicades en les reaccions redox i la biosíntesi de compostos fenòlics van ser induides per Sistemina. Al contrari, després de la infecció, es van observar molt pocs canvis en el perfil proteòmic. No obstant això, aquests pocs canvis eren molt específics per a la defensa front a patògens, incloent-hi les proteïnes patògenes 1 i 4 (PR1 i PR4) i una 1,3-se-glucanasa (PR2). A diferència del perfil proteòmic, es van observar canvis importants en el metaboloma després de la infecció, mostrant un perfil de priming clar. Alguns aminoàcids, compostos fenòlics i alcaloides van ser identificats entre els metabòlits induïts o amb perfil de priming, que estaven vinculats amb els resultats obtinguts en l'anàlisi proteòmic. D'altra banda, molts metabòlits van mostrar un efecte amortiguador front a la infecció, es a dir, mentre que estaven sobreacumulats en plantes de control amb la infecció, es van mantenir en els mateixos nivells de control no infectat o fins i tot més baixos en plantes tractades amb Sistemina després de la infecció. Es va observar un patró similar en les activitats enzimàtiques en les plantes infectades, especialment en temps tardans després de la inoculació del patogen.

Finalment, el nostre objectiu és trobar els primers esdeveniments de senyalització que permeten a la Sistemina provocar respostes per assegurar la inducció de resistència contra *B. cinerea* en les plantes de tomàquet. MPK1, MPK2, i MPK3 de tomata, ortòlogs de MPK3 i MPK6 d'Arabidopsis, que anteriorment es va informar que estaven implicats en les respostes de defensa de les plantes de tomàquet van ser seleccionats per a una anàlisi posterior. En el treball actual, es va trobar que la fosforilació de les MPKs va ser fomentada pel tractament de la Sistemina en plantes tractades amb un PAMP d'origen fúngic, fet que demostra la seva implicació en respostes de defensa induïdes per Sistemina. L'anàlisi de les plantes MPK1/2 i 3 silenciades mitjançant l'ús de la tècnica Virus-Induced Gene Silencing (VIGS) va revelar que les MPK actuen aigües amunt del la inducció de gens de defensa en absència i presència d'infecció, inclosos els gens relacionats amb la JA així com aquells implicats en la producció i alliberament de la Sistemina. Addicionalment, les plantes silenciades en MPK1 i 2 o MPK3, van mostrar una deteriorada protecció per Sistemina contra *Botrytis cinerea*, confirmant que són elements de senyalització essencials per assegurar la funcionalitat de la Sys-IR.

INTRODUCTION

INTRODUCTION

1. The plant immune system

In order to cope with invading attackers, plants have evolved a variety of complex strategies. Despite plants facing hundreds of noxious interactions every day, none or only very few attackers succeed in penetrating plant tissues.

As the first layer of interaction with insects or pathogens, plants have evolved strong constitutive barriers that are present before the interaction (Agrios et al., 2005). Among these constitutive barriers, there are physical structures such as trichomes, the cuticle and the cell wall that result in very efficient protection. In addition to physical structures, either inserted in the cell wall, contained in trichomes or in specialised cells, plants accumulate chemical constitutive defenses. These defenses are known as phytoanticipins and the most studied ones are the saponins and glucosinolates (Agrios, 2005). In Arabidopsis, one of the plant species extensively studied in the present thesis, glucosinolates are known to protect against insects and also pathogens. These compounds are stored in glycosylated forms in specialised cells, that keep them out of the reach of the myrosinase enzymes that cleave the sugar moiety and release the subproducts such as isothiocyanates, thiocyanates or nitriles that are highly toxic for insects and pathogens (Van Etten et al., 1994; González-Lamothe et al., 2009). Alternatively, cell walls contain hydroxycinnamic acids that are released when the cell wall is degraded as a consequence of an interaction, these compounds are also detrimental to pathogens (Ferguson et al., 2005).

Plants are able to recognise non-self molecules belonging to a specific type of insect or microbe that are termed Pathogen/ Microbe/ Herbivore Associated Molecular Patterns (PAMPs/ MAMPs/ HAMPs) (reviewed in Yu et al., 2017). These molecular signatures belong to bacteria, fungi, oomycetes or insects and are often peptides, fatty acids or oligosaccharides. Some well-studied MAMPs are the bacterial flagellin peptide flg22 or the protein EF-Tu (Felix et al., 1999), lipopolysaccharides, and chitin, a component of the fungal cell wall (Shinya et al., 2015; Couto and Zipfel 2016). On the other hand, herbivore oral secretions are an example of HAMPs (Mithöfer and Boland, 2008). PAMPs are exogenous danger signals that are recognized by cell surface receptors so-called pattern

recognition receptors (PRRs). Following the binding of their substrates, these receptors trigger the first layer of plant innate immunity known as pattern-triggered immunity (PTI). PTI is a general response that contributes to defense against a broad range of attackers. PTI responses are characterised by a cascade of signalling events that trigger the production of reactive oxygen species (ROS), increase in intracellular calcium (Ca^{2+}), activation of Mitogen-activated and/or calcium-dependent protein kinases (MPKs and CDPKs) and transcriptional changes (Hou et al., 2019).

Adapted pathogens are able to bypass PTI and suppress the first layer of plant defense by delivering effector molecules that promote virulence (Dodds et al., 2010). In these interactions, the effectors succeed in counteracting plant defenses driving to a compatible interaction resulting in the Effector Triggered Susceptibility (ETS). In order to survive hostile pathogens, plants have evolved strategies to counteract pathogen effector molecules. They recognize these virulence molecules via resistance (R) proteins and activate the second layer of immune defense called effector-triggered immunity (ETI) (Jones and Dangle, 2006). ETI or gene for gene resistance is a horizontal defense that involves specific pathways and leads to the so-called hypersensitive response (HR) which often triggers programmed cell death and the production of antimicrobial molecules to deter pathogen invasion (Spoel et al., 2012).

Although it is not recognized as a constitutive defence, the degradation of cell walls and membranes has important consequences in the interaction between plants and micro/organisms. The release of fragments or plant structures is key for the cell to identify an upcoming attack and accordingly mount an effective defense. In addition to PAMPs recognition, pest or pathogen invasion also triggers the production and release of host-derived molecules that are also perceived by PRRs to trigger PTI. Pathogens and insects produce lytic enzymes to degrade plant tissues and access host cells leading to the release of degradation products. These molecules are commonly known as Damage-Associated Molecular Patterns (DAMPs; Heil, 2009; Albert et al., 2013). Recently, a more accurate classification for endogenous danger signals was proposed. Those host molecules that are passively released after cell damage and disruption are primary endogenous danger signals and include the “classical” DAMPs (Gust et al., 2017). Examples of classical DAMPs are plant cell wall fragments such as oligogalacturonides or cellulose fragments (Hou et al., 2019). On the other hand, peptides that are produced actively by cells under

biotic attack are secondary endogenous danger signals termed phytocytokines (Gust et al., 2017). Production of phytocytokines often involves processing from a larger precursor that leads to the release of the mature peptide which is perceived by neighbouring cells to spread the danger alarm. Thus, unlike classical DAMPs, phytocitokine peptides may be present at the site of infection even if there is no cell disruption and they can be released in adjacent intact cells (Ma et al., 2013).

Following perception, either of self or non-self molecules, plants activate signals that are amplified in the cytoplasm of damaged or adjacent cells leading to hormone-regulated defense responses. Hormones such as SA, ET, JA and ABA regulate many resistance responses associated not only with basal immunity but also with gene-for-gene and systemic resistance. Some time ago, Thomma et al (1998) defined for the first time that SA-dependent responses are effective to resist biotrophic pathogens whereas JA/ET-regulated defenses are active against necrotrophs. This view is an oversimplification of the hormone-regulated resistance, since many later studies have profiled a much more complex view of the phytohormones and their cross-interactions in response to a pathogen or an insect attack. In an updated review, Pieterse et al (2009) described that SA indeed contributes to resisting biotrophs, this resistance is mediated by EDS1 and PAD4 participating in a positive loop for SA accumulation. Increased concentrations of SA in the cytoplasm lead to catalase inhibition and changes in redox imbalance triggering NPR1 monomerization that allows its penetration into the nucleus to induce SA-responsive genes. This transcriptional activation is mediated by a family of *TGA* and *WRKY* transcription factors that play a dual role by inducing SA-dependent genes such as *PR1* but also tuning hormonal crosstalk by inactivation of the JA/ET-dependent responses. This crosstalk optimises the energy imbalance in the plant upon pathogen attack.

Conversely, when a necrotroph is detected by the plant, FADs and LOX enzymes provide the oxylipin precursors driving JA biosynthesis. Either in the attacked or adjacent cells, JA is perceived by COI1 that activates the JAZ ubiquitination and degradation leading to transcription of JA-responsive genes mediated by *MYC2* and *ERF1* transcription factors. As an example of the complex hormonal regulation, it was demonstrated that plants can distinguish between chewing insects and necrotrophs both resisted through JA signalling (Verhage et al., 2010). In fact, there is negative crosstalk on *ERF1* regulated by *MYC2*

following wounding that prioritises JA/ABA pathway leading to *VSP2* expression and alternatively represses JA/ET-dependent pathway (Verhage et al., 2010).

The defense gene transcription is regulated by hormones. Following gene transcription and translation, a plethora of enzymes participate in different defense strategies. Among them, the biosynthesis of phytoalexins is one of the most relevant defences. The term phytoalexins includes secondary metabolites that are accumulated in response to a pathogen attack and their accumulation must result in a reduction of the infection. Phytoalexins can be synthesised through alternative biosynthetic pathways. Terpenic phytoalexins are generated from mevalonic acid that is transformed in the master precursor isopentenyl pyrophosphate. This compound is the monomer for the synthesis of terpenic phytoalexins such as capsidiol in pepper and rishitin in potatoes. Another major group of phytoalexins are those synthesised from shikimic acid. This aromatic acid is synthesised by conjugation of the erythrose-4-P and phosphoenolpyruvate. Subsequently, shikimic acid is transformed into two amino acids, Phe and Tyr, which by deamination generate the hydroxycinnamic acid, trans-cinnamic acid and p-coumaric acid. Further biosynthesis steps lead to the formation of phenylpropanoid phytoalexins present in a multitude of plant species. Brassicaceae species generate a very specific sort of phytoalexins termed glucosinolates (Glawischnig, 2007). Among these phytoalexins, there are two major groups, aliphatic and aromatic glucosinolates. Although these compounds play a major role as phytoanticipins, they can also be actively synthesised and accumulated in response to a pathogen attack. The main phytoalexin characterised in Arabidopsis is camalexin which contains an indolic moiety. This compound is synthesised from Trp that is converted into indole-3-acetaldoxime, then into indole-3-acetonitrile and following additional biosynthetic steps generate the precursor dihydrocamalexic acid that is decarboxylated by PAD3, the ultimate gene responsible for the camalexin biosynthesis. Attending to the relevance of the Trp derivatives pathway, a more detailed explanation of this biosynthetic pathway will be provided along this thesis.

The above mentioned description of the plant immune responses refers mostly to within cell responses after a PAMP or a DAMP perception. However, plants as pluricellular organisms must warn as well adjacent cells and distal tissues. Local responses affecting cells surrounding penetration sites suffer alteration in secondary metabolic pathways, cessation of the cell cycle, synthesis of pathogenesis-related proteins, accumulation of

either SA or JA as well as reinforcement of cell walls by papillae formation (Ellinger and Voigt, 2014). A 1,3-beta-glucan called callose is one of the main components of the papillae. This glucose polymer is rapidly assembled in the inner side of the cell wall by the GLS5 callose synthase named PMR4 (Nishimura et al., 2003; Ellinger and Voigt, 2014). If the infection progresses, callose infiltrates through the cell wall and accumulates on the outer side generating a very efficient barrier that blocks fungal penetration in locally infected cells, but also generates a protection halo in the immediate surrounding of the damaged cells.

Contrastingly to local responses, distal tissues do not accumulate callose but also respond to the infection by activating glucanases, chitinases and peroxidases and PR proteins (Schilmiller and Howe, 2005; Vlot et al., 2020). These responses in systemic tissues are however rather complex and prepare the distal tissues for an upcoming attack generating a phenomenon known as systemic resistance.

2. Induced Resistance in plants (IR)

Local induction of PTI and ETI upon danger signals perception can trigger a plant state of enhanced resistance against future attackers known as “Induced Resistance” (IR). When resistance is triggered in systemic tissues, it is called systemic resistance. Plants under the IR state show augmented defense responses at several metabolic levels and better performance upon different challenges (Pieterse et al., 2012; Walters et al., 2013; Mauch-Mani et al., 2017; De Kessel et al., 2021). For a long time, many researchers have performed studies that reconfigure both induced resistance and systemic resistance. To refer to one of the first discoveries related to enhanced defensive state it was used the term Systemic Acquired Resistance (SAR; Ross 1961; Durrant and Dong 2004; Spoel et al 2012). The establishment of SAR involves the transport of several mobile signals from the initial site of infection to distal parts of them that are perceived in the systemic tissues leading to the accumulation of salicylic acid (SA), which mediates the production of antimicrobial proteins through the activation of pathogen-related genes (Durrant and Dong, 2004; Spoel et al., 2012). Despite the general agreement that SAR is mediated by a rapid H_2O_2 accumulation leading to SA-dependent defences that involves hypersensitive response, the systemic signal that transports the resistance to distal tissues was elusive for many years. Time ago, the hypothesis that H_2O_2 or SA were the signals

moving through the vascular system was discarded. Finally, Park et al. (2009) discovered that MeSA was the mobile signal of SAR in tobacco. SA is locally methylated and transported to distal tissues. In damaged systemic tissues, the SABP2 esterase hydrolyzes the methyl group by releasing active SA. Despite this clear evidence, in 2012 Návarová et al. (2012) demonstrated that pipecolic acid was also a systemic signal in Arabidopsis suggesting that SAR may be mediated by multiple signals. In fact, since then, several compounds have been shown to participate in the transport of systemic signalling of induced resistance such as N-hydroxipipecolic acid, azelaic acid, dehydroabietal, glicerol-3-P, imprimatins etc (Park et al., 2007; Jung et al., 2009; Chanda et al., 2011).

Another relevant issue related to systemic resistance is 'plant memory'. Recent findings suggest that histone acetylation regulates intragenerational memory, since acetylation in specific Lys9 residue of the H3 leads to chromatin opening in those sites associated to PR1 promoter which generates a faster transcriptional machinery access. In the same line of evidence, it was demonstrated that SAR is also transgenerational (Luna et al., 2012). The progeny of Arabidopsis plants that have been exposed to *Pseudomonas syringae* pv tomato DC3000 are more resistant compared to their parents. The inheritance of SAR is conducted by hypomethylation of SA-dependent genes that presumably direct through the RdDM pathway the acetylation of Lys-9 of the His3 in the descendants (Luna et al., 2012).

Another variation of Induced Resistance is the Induced-Systemic Resistance (ISR). This specific long distance enhanced resistance is achieved in plants interacting with beneficial microbes such as *Pseudomonas simiae*, *Trichoderma spp.*, *Bacillus spp*., or Arbuscular Mycorrhizal Fungi (AMF; Pieterse et al., 2014). As a specific trait that differs from SAR, the mechanism of ISR is mainly mediated by the root-specific transcription factor *MYB72* in the onset of ISR in Arabidopsis and in shoot distal tissues by the hormones jasmonic acid (JA) and ethylene (ET) and it is effective against herbivores and necrotrophic pathogens. The interaction at the root interface with beneficial microorganisms triggers a root reprogramming (Pieterse et al., 2014; Rivero et al., 2015) that is transported to the shoots by an yet unknown mechanism to activate enhanced defense against necrotrophs. Note that this resistance has as common traits the mediation of NPR1 upstream *MYC2* leading to priming of JA/ET-dependent defenses and ABA-mediated callose accumulation. Recent advances suggest that lignans such as yatein and

secoisolariciresinol, that are detected in elevated concentrations in mycorrhiza-associated roots, are transported to the shoots and participate in enhanced resistance against *Botrytis cinerea* (Sanmartín et al., 2020).

Plant Growth Promoting Rhizobacteria (PGPR) have been extensively used to identify specific stimuli triggering ISR. Not only beneficial bacteria, but aslo fungi trigger ISR, such as in the case for AMF (Jung et al., 2012). However, the "growth-promoting" action may be considered contradictory when it turns into induced resistance. Most studies in which these beneficial organisms are used focus on Induced Resistance and dismiss the growth promotion assays. In fact, despite it being clear that IR should not have a fitness cost, there are many examples in which there is no growth promotion or in which this growth is highly context dependent. Some plant-evolutionary ecologists have argued against this terminology. It seems clear that from the plant-associated microbe perspective, it may be beneficial to ensure or manipulate the plant metabolism to grow more in order to get more carbohydrate resources. But from the host plant side, the growth promotion may also depend on growth limiting conditions, either biotic or abiotic, in which the association with a beneficial microbe may result or not in a growth improvement. Clearly, the beneficial output is visible in stress-mitigation when pathogens or insects are interacting with the host plant. Hence a reasonable proposal by T. Pavlo, although not accepted yet, is to avoid the terms PGPR and PGPF's and replace them with other more neutral terms like "(potentially) beneficial plant-associated microorganisms'' (PBPaMs).

Induced resistance can be achieved not only by exposing plants to beneficial organisms but also by treating plants with proteins, xenobiotics, natural extracts, DNA, volatile organic compounds (VOCs), physical damage or chemicals (Conrath et al., 2006; Pastor et al., 2013; Mauch-Mani et al., 2017; Kesel et al., 2021). For example, SA and its analogues are potent resistance inducers (Conrath et al., 2006). Similarly, β-aminobutyric acid (BABA) has been greatly studied as a resistance inducer in plants against a great variety of stressors (Pastor et al., 2013; Cohen et al., 2016). Other recent examples of non-biological defense elicitors are glutamic acid and D-Lactic acid which have been shown to induce resistance against pathogens in Arabidopsis (Goto et al., 2020; Lee et al., 2020). Regarding the use of external agents to induce resistance it must be clearly stated whether

these stimuli trigger a primed state in the plant or immediate resistance gene expression. In this regard, it is important to test whether the treatments have fitness costs for the plant.

Note that all the terminology used above aims to keep the definitions as close as possible to the original cited literature. However, a recent publication by De Kessel et al. (2021) has suggested important changes in the terminology with the aim to gain clarity and homogenise the naming of such varied and complex interactions between plants, stimulus and attackers. Induced Resistance is considered a global term and it is encouraged to be used in favour of homogeneity of terminology. ISR should be used when defense responses are systemically spread. However, Induced Resistance must be used when the stimulus itself is distributed along with the plant such as mobile endophytes or systemic chemicals like BABA. The IR phenomenon is associated with both a direct induction of defences and also with primed defense responses which are intrinsically linked to local and systemic responses. In local tissues, direct resistance gains relevance whereas in systemic tissues priming has a major contribution to defence which is also linked to responses delayed in time. Another relevant issue stated in the glossary by De Kessel et al (2021) is the terminology to be used to refer to the stimulus inducing priming or resistance. Since the pathway and the final output of the interaction between the stimuli and the resistance phenotype is highly dependent on the pathosystem and also on the age of the plant, it is proposed to use the general term IR stimulus. As a final consideration, the mechanisms underlying IR or priming are strongly context-dependent, hence it is strongly recommended to use a general stimulus-phenotype terminology such as I3CA-IR, BABA-IR, mycorrhiza-IR etc, but this must not be interpreted as a specific mechanistic naming rather than including information closer to the working system such as the name of the pathogen in which the resistance is observed, Eg: I3CA-IR against *Plectosphaerella cucumerina*.

2.1. Priming

Sometimes plant perception of an IR stimulus does not trigger major changes in the plant metabolism directly but rather shows an augmented response only when the challenge appears. This state of enhanced resistance is known as “defense priming” (Conrath et al., 2006, Martínez-Medina et al., 2016; Mauch-Mani et al., 2017). Primed plants exhibit a faster and stronger defense response that leads to enhanced disease protection against a

broad range of pathogens (Martínez-Medina et al., 2016). Interestingly, plant perception of the priming stimuli entails a very low associated fitness cost (Conrath et al., 2006; Hulten et al., 2006). What differentiates priming from other types of induced resistance is that instead of directly inducing defense responses upon stimuli perception, plants accumulate inactive defense molecules ready to be activated upon challenge (Mauch-Mani et a., 2017). Thus, when the biotic stressor is present, primed plants show a more robust response and better performance (Martínez-Medina et al., 2016).

The first reports of priming in plants showed no impact on plant metabolism following perception of the priming stimuli (Zimmerli et al 2000, 2001). Note that these experiments monitored the accumulation of callose and trailing necrosis on the one side, and the *PR1* expression on the other. Reasonably, in the absence of infection, the priming stimulus cannot trigger callose accumulation. The use of northern blots to determine gene expression is not sensitive enough to detect small changes in the genes. For these reasons, the first reports of priming in plants stated the total lack of changes and fitness costs in the absence of challenge. In time, we learnt that plants reasonably perceive the priming stimuli by readjusting their metabolism with minimal energetic and metabolic costs. This is what has been defined as the pre-challenge priming state (Gamir et al., 2014) or the priming phase (Mauch-Mani et al., 2017). At this stage, the plant does not show major defensive responses but it adjusts its metabolism to prepare for an upcoming stress. The major changes described during this stage are increases in the cytosolic Ca^{2+}, increases in specific tricarboxylic acids, augmented H_2O_2 production, conjugation of hormones and free sugars. Additionally, increases in inactive MPK3 and 6 were also shown (Beckers et al., 2009). All these changes prepare the plant to rapidly respond once the pathogen or the insect is present. In fact, primed plants experience a strong increase in glycosyl hydrolases and active SA compared with naive plants. Also, the levels of phosphorylated MPK3 and 6 rise much faster in BTH primed plants. Indeed, during the post-challenge primed state, plants accumulate faster transcripts of defence proteins such as *PR1*. This is possible due to the previous opening of the chromatin during the priming phase (Luna et al., 2012, Slaughter et al., 2012; Pastor et al 2013). In addition, as with other kinds of IR, priming is also inheritable (Martínez-Medina et al., 2016, Pastor et al., 2013). A helpful flowchart to decipher whether there is direct induction of resistance or not is shown by Martinez-Medina and coworkers (2016).

Recently a consensus was achieved on that the IR phenotype is considered to be a sum of both direct and primed defence activation (De Kessel et al., 2021). In fact, it has been observed that sometimes the same stimuli can trigger either direct induced resistance or primed defenses depending on the concentration (Conrath et al., 2006). SA can directly activate defense gene expression whereas at low doses, it has a priming effect upon future challenges (Mur et al., 1996; reviewed in Conrath et al., 2006). Similarly, the induced resistance state triggered by BABA is also dose-dependent (van Hulten et al., 2006). Moreover, some resistance inducers can even have negative effects at high doses. For instance, high doses of BABA induce sterility in Arabidopsis (Jakab et al., 2001). This suggests the importance of establishing an optimal dose threshold for achieving beneficial effects when using a resistance elicitor.

3. Phytocytokines

Accumulating studies reveal the importance of small secreted peptides in cell-to-cell signalling to coordinate cellular function including defense response in plants. Phytocytokines are small peptides secreted after damage perception that induce the amplification of immune responses in damaged and undamaged cells (Gust et al., 2017). Tomato Systemin was the first signalling peptide found in plants (Pearce and Ryan, 1991). Later, many peptides having a defense signalling effect were identified in different plant species, such as Peps from Arabidopsis, maize, and soybean (Huffaker et al., 2006; Huffaker et al., 2011; Yamaguchi et al., 2011). Recently there's an emerging number of studies reporting the discovery of new peptides involved in plant defense against a variety of biotic stressors in different plant species (Table 1; Hou et al., 2014; Chen et al., 2014; Gully et al., 2019).

Defense peptides can be very short in their amino acid length with examples of biologically active peptides from 5aa in length such as phytosulfokine (Zhang et al., 2018). Moreover, they can be active at concentrations as low as femtomolar (Roy et al., 2018). Regarding these mentioned features and their ubiquitous participation in the plant physiological events and cell-to-cell communication, they have been considered by many authors as peptidic hormones (Matsubayashi and Sakagami, 2006; Roy et al., 2018) and making them suitable candidates to be used as IR elicitors.

The release of small defense peptides often involves processing of larger precursors which differ in structure, indicating different processing mechanisms (Yamaguchi and Huffaker, 2011). According to their precursor structure, there are peptides derived from precursors having an N-terminal secretion signal; derived from precursors not having an N-terminal secretion signal; and derived from proteins that have a different biological function (Yamaguchi and Huffaker, 2011; Albert., 2013). Systemin precursor, Prosystemin, or the precursors of Arabidopsis Peps, PROPEPS, are examples of proteins not having an N-terminal secretion signal (Pearce et., 1991; Huffaker et al., 2006). Recently, some research studies have shed light on the mechanism by which these peptides precursors are being processed in plants; PROPEP1 is processed via calcium-dependent metacaspases whereas ProSystemin processing is mediated by phythaspases and a leucine aminopeptidase (Beloshistov et al., 2017; Hander et al., 2019) On the other hand, HypSys peptides derive from a precursor with an N-terminal secretion signal (Pearce et al., 2021; Pearce and Ryan, 2003) and GmSubPep from soybean derives from a protein with distinct primary function (Pearce et al., 2010). However, the processing mechanisms of HypSys and GmSubPep are poorly understood. Besides proteolytic processing, some peptides require posttranslational modifications (PTMs) to be biologically active and to interact with their receptor (Matsubayashi, 2014). Posttranslational modifications include tyrosine sulfation, proline hydroxylation and hydroxyproline arabinosylation (Matsubayashi, 2014). Phytosulfokine (PSK) was the first identified peptide with posttranslational modifications, having a sulfation at the two tyrosine residues (Matsubayashi, 1996). Later HypSys peptides were identified in tobacco and tomato as having proline hydroxylations (Pearce et al., 2001; Pearce and Ryan, 2003).

Once the mature peptide is released it triggers a cascade of signalling events and defense responses upon its perception by a membrane receptor. Peptides' perception, signal transduction and triggered defense responses are reviewed in the following sections.

3.1. Peptides Perception and signal transduction

A fast and efficient perception of plant surroundings is indispensable for plant survival. Similarly to classical DAMPs or PAMPs, phytocytokines perception by membrane receptors of damaged and adjacent cells is crucial to ensure danger alarm spread that leads

to the amplification of immune signalling in undamaged tissues and resistance to pest and pathogens.

As other danger signals, plant defense peptides are perceived by membrane receptors that are usually receptor-like kinases (RLKs) with an extracellular domain that binds the peptide ligand, a transmembrane domain, and an intracellular kinase domain that ensures the initiation of an intracellular signalling cascade (Li et al., 2020). These cell surface receptors often form complexes with coreceptors that enable activation of downstream signalling upon ligand perception (He et al., 2018). The receptor-like Kinase BRI1-associated receptor Kinase (BAK1) function as coreceptor of multiple PRRs including those perceiving phytocytokines (Table 1). In addition, some Receptor-like cytoplasmatic kinases such as Botrytis-induced kinase (BIK1) interacts with PRRs complexes to initiate the signal transduction upon complex activation in response to danger signals (Liu et al 2010; Liu et al., 2013a).

An increasing number of peptide-receptors pairs have been discovered in the last few years (Table 1). Arabidopsis Plant elicitor peptide 1 (Pep1) is perceived either by PEP RECEPTOR 1 (PEPR1) or 2 (PEPR2) whereas Arabidopsis Pathogen induced peptide 1 (PIP1) is perceived by RLK7 (Yamaguchi et al., 2006; Hou et al., 2014). Both PEPR1 and RLK7 form a complex with BAK1, although early signalling triggered by PIP1 is only partially dependent on BAK1 (Liu et al., 2013; Hou et al., 2014). Similarly, PEPR1 can directly phosphorylate BIK1, without relying on BAK1 (Liu et al., 2013). However, PIP1 signal transduction was demonstrated to be BIK1-independent (Hou et al., 2014). Recently, MIK2 was demonstrated to be the SCOOP12 receptor in Arabidopsis. Very interestingly it not only perceives SCOOPs but can also sense microbial proteins from fungi and bacteria (Hou et al., 2021). MIK2 also associates with BAK1 and its close homolog SERK4 and relies on BIK1 and PBL1 for the downstream signalling events (Hou et al., 2021).

On the other hand, tomato Systemin is perceived by both SYR1 and SYR2, but it seems that SYR1 is a high affinity receptor whereas SYR2 is a low affinity receptor (Wang et al., 2018). The PEPR tomato ortholog PORK1 is also necessary to trigger Systemin-induced signalling since plants with silenced PORK1 but intact SYRs lack some Systemin responses (Xu et al., 2018). However, it is not known yet if PORK1 directly binds Systemin or functions as a coreceptor of SYRs similarly to the Arabidopsis receptor protein complexes mentioned above. Interestingly some peptides can be perceived by

more than one receptor. This is the case of IDL6 which is perceived by both HAE and HLS2 (Wrzaczek et al., 2009 and 2014). The peptide-perception complex RALF23-FER-BAK1 has been demonstrated to negatively regulate plant immunity (Stregmann et al., 2017). Remarkably, although BAK1 associates with multiple PRRs upon danger perception enabling the signal transduction, it has been shown that pathogens are able to induce BAK1 depletion in order to hijack PTI responses (Macho and Zypfel, 2014). When this happens, it was demonstrated that PEPR pathway ensures basal resistance inducing cell death and salicylate-related defenses (Yamada et al., 2016). This suggests that phytocytokines-triggered immune responses can also occur independently of common PTI signalling.

Although many peptide-receptor complex pairs have been elucidated in the past few years, there are many phytocytokines for which perception mechanisms are still elusive. These include the maize ZmPeps and Zip1, soybean Peps, and tomato CAPE1(Table 1). Further research is needed to address this issue and improve our knowledge of phytocytokines perception and signal transduction. Techniques and methods for finding new peptide ligand receptor pairs are extensively reviewed elsewhere (Roy et al., 2018; Olsson et al., 2019)

3.2. Intracellular signalling and defense responses triggered by phytocytokines

Binding of phytocytokines to their receptor triggers a cascade of defense signalling that leads to an amplification of the plant immune system in order to mount a defense response against invading attackers (Figure 1A). Defense peptides share common intracellular signalling elements with other self and non-self defense elicitors (Table 1). Although there's specific recognition of peptides by PRRs, triggered defense responses and intracellular signalling often resemble responses to exogenous patterns (Yu et al., 2017). Resistance inducers and priming agents also trigger typical PTI defense responses and primed plants have potentiated defense in response to a challenge (Mauch-Mani et al 2017). In the next sections present the defense responses that are triggered by plant defense peptides and their natural role against biotic stresses.

Increase of Cytosolic Ca^{2+} is one of the earlier responses triggered by some phytocytokines as well as by other PAMPs and DAMPs during PTI, occurring within a few minutes, or even seconds after perception, upstream of subsequent immune responses

(Yu et al., 2017). Tomato Systemin perception triggers an increase in the intracellular calcium in mesophyll cells (Moyen et al., 1998). Similarly, Pep1, Pep3 and SCOOPs treatment induce an increase of cytosolic calcium in Arabidopsis (Ma et al., 2012; Ma et al., 2013; Rhodes et al., 2021).It was recently reported that PROPEP1 processing for the release of Pep1 is performed by Ca2+ dependent metacaspases (Hander et al., 2019). In tomato, PSK not only induces a Ca^{2+} increase but also Ca^{2+} dependent auxin responses for the protection against *Botrytis cinerea* (Zhang et al 2018). These findings demonstrate the importance of cytosolic Ca^{2+} in the phytocytokines-triggered defense signalling.

Opening of ion channels and extracellular alkalinization is a hallmark response occurring after peptide treatment (Figure 1). Media alkalinization occurs also very rapidly (after 1 min) upon flg22 or elf18 treatment (Jeworutzki et al., 2010). Rapid alkalinization factors (RALFs) peptides owe their name to their ability to alkanize the extracellular media when applied to a cell suspension culture (Pearce et al., 2001). Similarly, tobacco and tomato HypSys as well as peptides from soybean (GmSubPep, GmPep914 and GmPep890) also induce extracellular alkalinization when supplied to suspension-cultured cells (Table 1; Pearce et al., 2001; Pearce and Ryan 2003; Pearce et al 2010; Yamaguchi et al., 2011). In addition, opening of ion channels by modulation of plasma membrane H^+ATPase activity is a Systemin-triggered early event (Schaller et al., 1999).

Reactive Oxygen Species (ROS) production is one of the earliest cellular responses upon pathogen recognition and it mediates other defense responses in the plant (Torres et al., 2006; Fichman and Mittler 2020). PAMPs, defense elicitors and many phytocykines perception produces an oxidative burst (Table1). In Arabidopsis, exogenous application of PEP1 as well as PIP1 to leaves causes the production of H_2O_2 (Huffaker et al., 2006; Hou et al., 2014). Pep3 induces H_2O_2 and NO production , which are essential for Pep3-triggered immunity against *PstDC3000*, evidenced by compromised in *rbohD/F* and *noa1* mutants (Ma et al., 2013). Similarly, SCOOP12 induces ROS as well as Phosphatidic acid (PA) in Arabidopsis, suggested to be in involved in ROS production, MAPK activation and defense gene induction (Testerink et al., 2011; Gully et al., 2019). In addition, in Arabidopsis GRIM REAPER peptide (GRI) was shown to regulate ROS-dependent cell death (Wrzaczek et al., 2009; 2014). In tomato, both CAPE1 and Systemin treatment trigger H_2O_2 formation (Chen et al., 2014; Wang et al., 2018), whereas in potato HypSys also elicits H_2O_2 generation (Bhattacharya et al., 2013). Conversely, RALF23 is a negative regulator of PAMP-induced ROS (Stregmann et al., 2017).

Table 1. Main features of phytocytokines

Phytocytokine	Species of origin	Signal transduction	Induced defense responses and signalling		References
Peps	Arabidopsis	PEPR1 and PEPR2	media alkalinization, H_2O_2	*PDF1.2* and *PROPEPs* gene expression	*Huffaker et al., 2006*
		BAK1	Ca^{2+}	ET, callose	*Ma et al., 2013*
		BIK1/PBL1	Ca^{2+}, H_2O_2 NO	MPK3 and WRKY33 gene expression	*Bartels and Boller, 2015*
ZmPep1	Maize			JA, ET, defense gene expression, defense metabolites accumulation	*Huffaker et al., 2011*
ZmPep3	Maize			JA, ET, defense gene expression, volatiles emission, phytoalexin	*Huffaker et al., 2013*
PIP1	Arabidopsis	RLK7	ROS	*FRK1, WRKY30, WRKY33, WRKY53, MYB51* and *PR1* gene expression	*Hou et al., 2014*
		partially BAK1-dependent	MAPK	Callose, Stomatal closure	
SCOOP12	Arabidopsis	MIK2-BAK1/SERK4	ROS	Callose	*Gully et al., 2019*
			Phosphatidic acid (PA)	*FRK1* gene expression	*Rhodes et al., 2021*
SCOOPs		BIK1/PBL1	Ca^{2+}, ROS, MAPK	ET, defense gene expression	*Hou et al., 2021*
PNP-A	Arabidopsis	PNP-R2		antagonizes SA-responses, stomatal closure	*Lee et al., 2020*
RALF23	Arabidopsis	FER-BAK1	Ca^{2+}, Media alkalinization		*Stregmann et al 2017*
			Antagonizes PAMP-induced ROS		
IDL6	Arabidopsis	HAE and HSL2		Poligalacturonase gene *ADPG2*	*Wang et al., 2017*
GRI	Arabidopsis	PRK5		ROS-dependent Cell death, hormones	*Wrzaczek et al., 2009 and 2014*

Continues Table 1. Main features of phytocytokines

Phytocytokine	Species of origin	Signal transduction	Induced defense responses and signalling		References
Systemin	Tomato	SYR1	Opening of ion chanels, Ca^{2+}, MAPKs	JA, defense genes	*Pearce et al., 1991*
		SYR2	CDPKs, ROS	Protease inhibitors	*Zhang et al., 2020*
		PORK1	CAT and APX activity	Volatiles emission	*Molisso et al., 2021*
PotSys1 and 2	Potato	SYR1 and SYR2		Protease inhibitors	*Constabel el et., 1998*
PepSys	Pepper				
NishSys	Nightshade				
HypSys1, 2 and 3	Tomato		Media alkalinization	JA, PI-I and PI-II	*Pearce and Ryan, 2003*
	Potato		H_2O_2	PIs, JA, defense-related genes, antioxidant defensive enzymes	*Bhattacharya et al., 2013*
TobHypSys 1 and 2	tobacco		Media alkalinization, MAPK	Protease inhibitors	*Pearce et al., 2001*
CAPE1	Tomato		H_2O_2	SA, defense gene expression	*Chen et al., 2014*
PSK	Arabidopsis	PSRKs	Ca^{2+}	IAA and Auxin-dependent responses	*Shen and Diener, 2013*
	Tomato				*Zhang et al., 2018*
PSY1	Arabidopsis	PSY1R			*Shen and Diener, 2013*
SubPep	Soybean		Media alkalinization	*Chitinase1b*, *CYP93A1*, *chalcone synthase* and *PDR12* gene expression	*Pearce et al., 2010*
Pep914	Soybean		Media alkalinization	*CYP93A1*, *Chib1-1* and *chalcone synthase* gene expression	*Yamaguchi et al., 2011*
Pep890					
Zip1	Maize			SA, SA and JA marker genes, defense-related genes	*Ziemann et al., 2018*

Activation of protein kinases cascades is a hallmark of PTI responses. Mitogen-activated protein kinases (MAPKs) cascades are essential signalling elements that ensure defense signaling activation downstream of pattern recognition receptor complexes (Yu et al 2017). PIP1, SCOOPs, Systemin and Hypsys peptides induce MAPK activation in their respective species of origin (Hou et al 2014; Lee et al., 2020; Stratmann 1997; Pearce et al 2001). Parallelly, Calcium-dependent protein kinases (CDPKs), which are Ca^{2+} sensor protein kinases, are also activated upon several danger signals perception and trigger downstream defense responses (Boudsocq and Sheen, 2013). However, very little is known about their involvement in peptide-induced defenses. In this regard, Pep3 induction of MPK3 and *WRKY33* and Pep-triggered immunity against *PstDC3000* is CDPK-dependent since it is impaired in the *cpk* mutants or when a kinase inhibitor is applied (Ma et al., 2013).

Most phytocytokines induce the ***expression of a variety of defense related genes*** in different plant species (Table 1). Although there are peptide-specific transcriptomic fingerprints, transcriptional changes triggered by defense peptides often overlap. In Arabidopsis, treatment with Peps induced the expression of plant defensin *PDF1.2*, *MPK3* and *WRKY33* transcription factor (Huffaker et al., 2006; Ma et al., 2013). PIP1 treatment induces gene expression of immune-related *Flg22-INDUCED RECEPTOR KINASE1* (FRK1), the transcription factors *WRKY30, WRKY33, WRKY53* as well as expression of pathogen related *PR1* in protoplasts and the transcription factor *MYB51* in roots (Hou et al., 2014). Similarly, SCOOPs also induced *FRK1, WRKY30, and WRKY33* gene expression as well as *CYP81F2*, involved in glucosinolate metabolism and resistance to fungi (Gully et al., 2019; Hou et al., 2021). Systemin treatment induces the expression of defense related genes, especially genes involved in the synthesis of JA, such as AOS and JA marker genes PI-I and PI-II (Coppola et al., 2019). Similar to Systemin, the HypSys peptides activate the expression of octadecanoid pathway genes as well as essential pathogen- and hervibore-related genes (Bhattacharya et al., 2013). CAPE1 activates the expression of pathogen-related genes PR1b, *BETA-1,3-GLUCANASE* (PR2), *CYS PREOTEASE* (PR7), a chitinase, *ETHYLENE RESPOSE FACTOR5* (ERF5) and *AvrPto-DEPENDENT Pto-INTERACTING PROTEIN3* (Adi3) among others (Chen et al., 2014). In soybean, GmSubPep, GmPep914 and GmPep890 peptides induce *CYP93A1* gene expression, involved in synthesis of a phytoalexin, a chitinase and chalcone synthase gene expression (Pearce et al., 2010; Yamaguchi et al., 2011). In Maize, ZmPep1 induced

expression of genes encoding for defense proteins which include endochitinase A, PR-4, PRms, and SerPIN, and a gene involved in the biosynthesis of the phytoalexin benzoxazinoid (Huffaker et al., 2011). On the other hand, ZmPep3 increases the expression of indole biosynthetic genes together with genes encoding proteins associated with herbivory defense and biosynthetic enzymes for production of volatile terpenes and benzoxazinoids (Huffaker et al 2013). Also in maize, Zip1 induce the expression of SA and JA marker genes and other defense-related genes such as *WRKY* transcription factors (Ziemann et al., 2018).

Some peptides also induce the expression of their precursors. This is the case of AtPep1, which activates the expression of *PROPEP1* (Huffaker et al., 2006). PEPR activation also mediates *PROPEP2*/*PROPEP3* activation (Yamaguchi & Huffaker, 2011). SCOOP12 as well as Pep1 trigger the expression of *PROSCOOPS* (Gully et al., 2019). Similarly, CAPE1 induces the expression of its precursor protein PR1b (Chen et al 2014) and Systemin induces the expression of ProSystemin (Coppola et al., 2019). As another exemple, Zip1 maize peptide induces the activity of the proteases that process its precursor PROZIP1 (Ziemann et al., 2018). These findings suggest that peptides promote their own release in a positive feedback loop in order to keep amplifying defense responses.

A very common response triggered by defense peptides in tomato and other solanaceous species is the ***induction of Protease inhibitors (PIs)***. PIs inhibit insect digestive enzymes and are key elements in plant defense against herbivory (Green and Ryan, 1972). Systemin, the first discovered signalling peptide, was identified when looking for signals that induced PIs accumulation in tomato (1991). Later, it was found that Systemin was also present in potato, pepper and nightshade where it also induced the accumulation of PIs. Similarly, Hydroxyproline rich Systemins (HypSys) found in tobacco, tomato and potato have also the ability to induce PIs for insect defense (Bhattacharya et al., 2013). On the other hand, CAPE1 a tomato peptide embedded in PR1b was found to induce the expression PIs (Chen et al., 2014). In addition, induction of PIs biosynthetic genes was also observed in maize after ZmPep3 treatment (Huffaker et al., 2013).

Phytohormones are well known for their involvement in plant defense and their production in plants under attack is a conserved response across species. SA, ET, JA and ABA are the main hormones regulating many resistance responses associated with basal

immunity as well as gene-for-gene and systemic resistance. In the literature are some examples of phytohormonal production upon defense peptide perception. In Arabidopsis, Pep1 and SCOOPs induce the accumulation of ET in Arabidopsis (Gully et al., 2019). In maize both ZmPep1 and ZmPep3 induce JA and ET (Huffaker et al., 2011; 2013) whereas Zip1 was observed to induce both JA and SA marker genes and strong accumulation of SA (Ziemann et al., 2018). In Solanaceous species, Systemin induce the release of linolenic acid that leads to the production of JA and JA-Ile as well as the biosynthesis of ET (Sun et al., 2011; Wang et al., 2018). HypSys from tomato and potato were reported to activate the octadecanoid pathway and the production of JA (Pearce and Ryan, 2003; Bhattacharya et al., 2013). On the contrary, CAPE1 significantly induces SA accumulation in tomato (Chen et al., 2014). Other peptides seem to be involved in hormonal regulation upon different stresses. Arabidopsis PLANT NATRIURETIC PEPTIDE A, PNP-A, was shown to antagonize SA-mediated responses (Lee et al., 2020). Similarly, GRIM REAPER peptide was shown to be involved in hormonal regulation since SA and JA accumulation upon stress was strongly reduced in the *gri* knock-out plants (Wrzaczek et al., 2009). On the other hand, PSK induces IAA and auxin-dependent responses in tomato plants against *Botrytis cinerea* infection (Zhang et al., 2018).

Plants under attack trigger a ***metabolic rearrangement*** in order to coordinate the biosynthesis of defense compounds with antimicrobial effect, such as phytoalexins. Very few reports have studied the production of defense metabolites downstream defense peptides perception. In maize it was found that ZmPep1 as well as ZmPep3 treatment induce the accumulation of benzoxazinoid phytoalexin, involved defense against lepidopteran herbivores(Huffacker et al 2011; Huffaker et al 2013). In tomato, constitutive expression of Prosystemin enhances the production of secondary metabolites (Chen et al 2006; Pastor et al., 2018).

For inducible downstream defense responses, there are a few reports of peptides inducing ***callose accumulation.*** Callose is a β-1,3 glucan polymer that accumulates in the plant cell wall in response to pathogen infection in order to strengthen the plant cell wall and restrict their entry (Luna et al., 2011). Augmented callose formation is an important feature of BABA-induced resistance against pathogenic fungi that leads to plant protection (Conrath et al., 2006). Regarding peptide-triggered responses, Pep1, PIP1 and SCOOP12 were reported to induce the production of callose in Arabidopsis plants

although to a much lesser extent than flagellin and chitin (Hou et al 2014; Gully et al 2019).

Stomatal closure is also among the inducible defenses triggered by plants under attack since stomata are sites of bacterial pathogen entry in the plant. In this regard, PIP1 was found to induce stomatal closure in Arabidopsis (Hou et al., 2014), whereas, PNP-A was reported to regulate stomatal closure upon bacterial infection (Lee et al., 2020).

Finally, sometimes plants are able to induce ***indirect defenses*** upon stimuli perception that includes the release of volatile organic compounds (VOCs) to attract pest natural enemies. Additionally, the release of VOCs can also serve to prime distal parts of the plant or alert neighbouring plants of an upcoming stress. In maize, ZmPep3 treatment triggered an enhanced emission of volatiles which included terpenes and shikimate pathway-derived compounds that made plants more attractive for lepidopteran herbivores parasitoids (Huffaker et al., 2013). In tomato, Systemin induces the emission of volatiles that attract pest natural enemies and alert neighbouring plants priming their defenses (Corrado et al., 2007; Coppola et al., 2017).

3.3. Role of endogenous phytocytokines in the defense response against pest and pathogens

Several studies have demonstrated that changing endogenous levels of some phytocytokines by overexpressing or silencing the peptide precursor produces changes in the natural resistance of plants against different attackers confirming their key role in plant defense.

Constitutive overexpression of the Pep1 precursor PROPEP1 confers resistance to the root pathogen *Pythium irregulare* in Arabidopsis (Huffaker et al., 2006). Similarly, overexpression of prePIP1 and prePIP2 in Arabidopsis induces resistance against *P. Syringae* and *Foc 699* (Hou et al., 2014*).* In tomato, Prosystemin overexpressing plants (PS+) are more resistant to several attackers including aphids, larvae and necrotrophic fungi (Coppola et al., 2015) as well as plant viruses (Bubici et al., 2017). HypSys overexpression in tobacco leads to enhanced resistance to *Helicovera armigera* larvae (Ren and Lu, 2006). On the contratry, plants expressing antisense Prosystemin were more susceptible to *Manduca sexta* larvae (Orozco-Cádenas et al., 1993). A knock out mutant of SCOOP12 precursor showed higher susceptibility to *Erwinia amylovora* but enhanced

resistance to *Alternaria Brassicicola (*Gully et al 2019). In a similar way, loss of PSK signalling reduces resistance against necrotrophic fungi (Mosher et al 2013), whereas at the same time increases resistance to biotrophic bacteria (Igarashi et al., 2012) and fungi (Shen and Diener, 2013). Similarly, mutants of GRI peptide show increased resistance to virulent bacteria due to an increase in cell death (Wrzaczek et al., 2009).

An antagonistic effect on resistance to biotrophic and necrotrophic pathogens is observed among phytocytokines. This suggests a specificity of the plant phytocytokines involvement in resistance against the type of pathogen lifestyles and might be correlated with the hormonal regulation. Thus, it makes sense that peptides involved in defense to herbivores may also defend against necrotrophs, however, the same peptide may not participate in defense against biotrophs. For instance, Systemin is effective against several types of herbivores, such as caterpillar and aphids, as well as against necrotrophs (Coppola et al., 2015), whereas although not tested it's likely not involved in defense against hemibiotrophs such as *Pseudomonas syringae*. On the contrary, in Arabidopsis, PNP-A was shown to antagonise SA-mediated and SA-primed defenses, thus overexpression of PNP-A resulted in compromised resistance to *P. syringae* (Lee et al., 2020). It is tempting to think that the same plants would be more resistant to necrotrophic pathogens or herbivores.

Table 2 . *Effect of overexpression of phytocytokines or their precursors*

Plant species of origin	Peptide /precursor	Recipient plant/organism	Effect	References
Arabidopsis	PROPEP1	Arabidopsis	resistance to *Pythium irregulare* and *Pseudomonas syringae*	Huffaker et al., 2006
Arabidopsis	PrePIP1	Arabidopsis	resistance to *foc 699*	Hou et al., 2014
Arabidopsis	SCOOP	Arabidopsis	resistance to *Alternaria brassicicola*	Gully et al., 2019
			susceptibility against *E. amylovora*	Rhodes et al., 2021
Arabidopsis	RALF23	Arabidopsis	susceptibility to *P. syringae* and *P.cucumerina*	Stregmann et al 2017
Arabidopsis	IDL6	Arabidopsis	succeptibility to *P. syringae*	Wang et al., 2017
Arabidopsis	GRI	Arabidopsis	succeptibylity to *P. syringae*	Wrzaczek et al., 2009 and 2014
tomato	ProSystemin	tomato	resistance to herbivore	Coppola et al., 2015
			resistance to aphids	Coppola et al., 2015
			resistance to *B. cinerea* and *A. alternata*	Coppola et al., 2015
			reduced susceptibility to *Cucumber mosaic virus*	Bubici et al., 2017
tomato	ProSystemin	Arabidopsis	resistance to *B. cinerea*	Zhang et al., 2017
tomato	PSK	Arabidopsis	susceptibility to *Fusarium oxysporum*	Shen and Diener, 2013
Arabidopsis	PSK	tomato	*Botrytis cinerea*	Zhang et al., 2018
Maize	Zip1	*Ustilago maydis*	resistance against *Ustilago maydis*	Ziemann et al., 2018
Tobacco	HypSys	tobacco	resistance to *Helicovera armigera*	Ren and Lu, 2006

4. Peptides as Induced Resistance elicitors

The biological function of small secreted peptides in plant innate immunity is extensively studied. We have seen that phytocytokines trigger a huge range of defense responses and signalling cascades upon cell damage by pest and pathogens to amplify the defense response (Figure 1A). Similar responses are observed when their precursor is overexpressed. But, what is the outcome of their exogenous application against a future attack? Because of their ability to activate the plant immune system and induce defensive responses at very low concentrations, they can be considered suitable candidates for defense elicitors (Figure1B).

In a natural environment, phytocytokines are released after plant perception of a biotic challenge during the activation of the first layer of immune responses, PTI. Then phytocytokines bind to their receptors to amplify and strengthen the already activated defenses and spread the danger alarm to adjacent cells (Figure 1A). On the contrary, if we use peptides as defense elicitors, we apply them previous to any challenge. The plant perceives them as danger signals and activates defense signalling, thus when a future biotic challenge occurs the plant poses an enhanced defensive response displaying peptide-IR (Figure 1B). However, the effect of exogenously applied peptides in the plant defensive responses may differ from that triggered naturally when the endogenous peptide is released after the challenge.

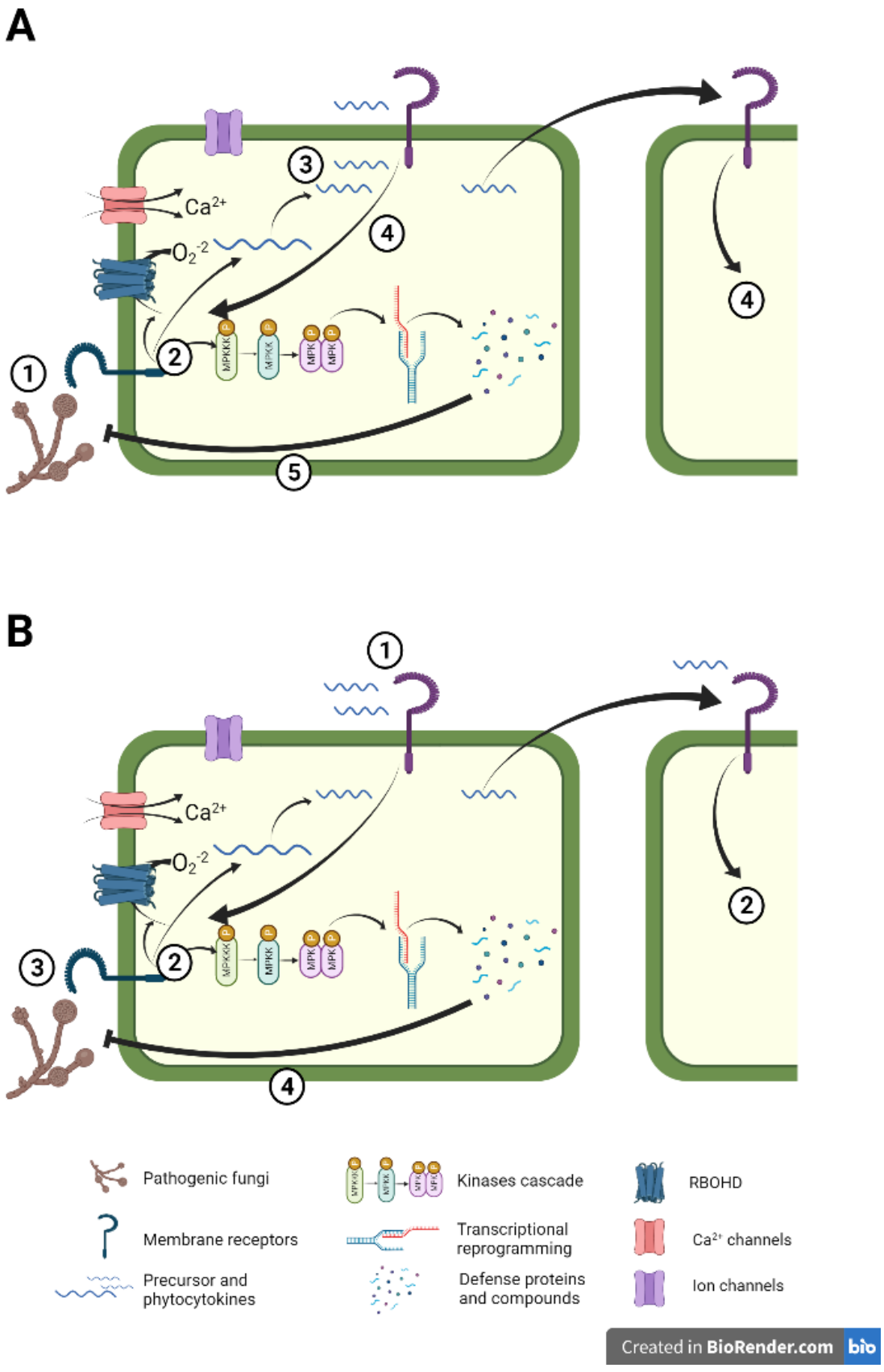
4.1. Peptide-Induced Resistance against pest and pathogens

Although the natural function of phytocytokines is currently under study, their potential in induced-resistance when applied exogenously needs further research. Nevertheless, there are some promising evidences of their positive effect.

Exogenous treatment with Pep3, PIP1 or SCOOP12 in Arabidopsis leads to *P. syringae* resistance (Ma et al., 2013; Hou et al., 2014; Gully et al 2019). In maize, ZmPep1 treatment confers resistance to necrotrophic fungal pathogens *Cochliobolis heterostrophus* and *Colletotrichum graminicola (*Huffaker et al., 2011) whereas ZmPep3 treatment reduced *Spodoptera exigua* larval growth (Huffaker et al., 2013). In tomato, CAPE1 application induces resistance to both the herbivore *Spodoptera litura* and the biotrophic pathogen *P. syringae* (Chen et al., 2014). Similarly, exogenously applied

phytosulfokine (PSK) as well as Systemin enhance resistance to the necrotrophic fungus *Botrytis cinerea* in tomato (Zhang et al., 2018; Coppola et al., 2019). In addition, Systemin treatment also impairs larval growth of *Spodoptera litoralis* (Coppola et al., 2019). Hence, it seems clear that peptides induce resistance against herbivores and necrotrophs.

On the other hand, peptide treatment can confer susceptibility to pathogens in some cases. PNP-A exogenous application results in compromised resistance against *P. syringae* (Lee et al 2020). In maize, Zip1 treatment induces susceptibility to *B. cinerea* (Ziemann et al., 2018). This suggests again the specificity of phytocytokines-induced resistance that function in pathosystem-dependent manner. However, this is not always true, since some peptides such as CAPE1 confer resistance to both herbivores and biotrophic pathogens, suggesting that some phytocytokines are effective against a broader spectrum of biotic challenges.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Pathogenic fungi
Membrane receptors
Precursor and phytocytokines
Kinases cascade
Transcriptional reprogramming
Defense proteins and compounds
RBOHD
Ca2+ channels
Ion channels
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Figure 1. Natural function of defense peptides vs Peptide-Induced Resistance. (A) *Cellular reponses against a pathogenic fungi infection.* ***1****, Pathogen tries to penetrtate the cell and it is perceived by plant membrane receptors.* ***2****, Intracellular signalling and defense responses are activated, including ROS production, opening of ion and* Ca^{2+} *channels and MAPK cascades activation that lead to downstream transcriptional reprograming and defense compounds production; paralelly, peptide precursos are sythesiszed and phytocytokines are released.* ***3****, mature peptides are released to the apoplast were they are perceived by damaged and adjacent cells.* ***4****, phytocytokines trigger the amplification of defense responses.* ***5,*** *the battery of defensive elements impairs pathogen success.* ***(B)*** *Mechanisms of Peptide-IR.* ***1,*** *cell membrane receptors perceived the exogenously applied phytocytokine.* ***2****, Immune responses are activated as in (A).****3,*** *an invading fungal pathogen is perceived.* ***4,*** *the plant is already prepared to counteract the infection, displaying a faster and stronger defense response that leads to enhanced resistance.*

4.2. Crosspecies perception and peptide-Induced Resistance

Interestingly, a few studies have reported heterologous peptide sensing and signaling in taxonomically distant plant species. Although a report claims that tobacco cells do not respond to exogenous Systemin treatment (Scheer et al., 2003), a later study showed that tobacco calli and suspension cells responded to Systemin by both MAPK activation and weak-medium alkalinization (Malinowski et al., 2009). In addition, constitutive expression of the tomato prosystemin gene in tobacco considerably affected the plant metabolism by inducing the synthesis of host proteins, several of which are involved in protection against pathogens, suggesting the ability of tobacco to reproduce Systemin signalling (Rocco et al., 2008). More surprisingly, Zhang et al. (2017), reported that tomato Systemin was sensed by Arabidopsis plants, leading to an inhibition of seedling root growth and the expression of the plant defensin *PDF1.2*. Tobacco cells transformed with the Arabidopsis Pep1 receptor PEPR1 responded to nanomolar concentrations of Pep1, producing a strong alkalinization of the cell culture medium, suggesting again a capacity of tobacco to activate signalling upon an heterologous peptide treatment (Yamaguchi et al., 2006). Later, Huffaker and coworkers (2013) found ZmPep orthologs in rice (OsPep2) and sorghum (SbPep1) and tested their ability to induce volatile emission

in maize plants. They found that both peptides elicited a full spectrum of herbivore-associated volatiles at the same level as those induced by maize Peps. This suggests that Peps from rice and sorghum species might be able to induce resistance in maize similarly to ZmPeps.

However, evidences of peptide induced resistance in heterologous species are very scarce (Table 3). Heterologous peptides, including Sytemins from Solanaceae and AFPs from radish confer Arabidopsis resistance to the necrotroph *Plectosphaerella cucumerina* (Pastor-Fernández et al., 2020). In addition, very recently it was demonstrated that Systemin is also able to induce resistance against necrotrophic fungi in the taxonomically distant species *Vitis vinifera,* as well as in *Solanum melongea,* which is taxonomically closer but still does not produce the peptide (Molisso et al., 2021). The functionality of crosspecies-IR could mean that peptides can be used as general agents of biocontrol and thus deserves further research.

Table 3. Effects of exogenous peptide applications in resistance against pest and pathogens

Plant species of origin	Peptide	Recipient plant	effect	References
Arabidopsis	Pep3	Arabidopsis	Resistance to *P. syringae*	Ma et al., 2013
Arabidopsis	PIP1	Arabidopsis	Resistance to *P. syringae*	Hou et al., 2014
Arabidopsis	SCOOP12	Arabidopsis	Resistance to *P. syringae*	Gully et al., 2019
maize	ZmPep1	Maize	Resistance to *Cochliobolis heterostrophus* and *C. graminicola*	Huffaker et al., 2011
maize	ZmPep3	Maize	Resistance to *Spodoptera exigua*	Huffaker et al., 2013
tomato	CAPE1	Tomato	Resistance to *Spotoptera litura*	Chen et al., 2014
			Resistance to *P. syringae*	
tomato	PSK	Tomato	Resistance to *B. cinerea*	Zhang et al., 2018
tomato	Systemin	Tomato	Resistance to *Spodoptera litoralis*	Coppola et al., 2019
			Resistance to *B. cinerea*	
Arabidopsis	PNP-A	Arabidopsis	Susceptibility to *P. syringae*	Lee et al., 2020
maize	Zip1	Maize	Susceptibility to *B. cinerea*	Ziemann et al., 2018
tomato	Systemin	Arabidopsis	Resistance to *P. cucumerina*	Pastor-Fernández et al., 2020
potato	PotSysII			
pepper	PepSys			
nightshade	Nishsys			
tomato	HypSys			
radish	AFPs			
Arabidopsis	Pep1	Arabidopsis	Resistance to *P. cucumerina*	Pastor-Fernández et al., 2020
tomato	Systemin	Eggplant	Resistance to *B.cinerea*	Molisso et al., 2021
		Vitis vinifera		

5. Cooperative functioning of peptides in innate immunity

As previously mentioned, defense peptides function as amplifiers of the "warning alarm". The increasing number of identified peptides functioning as phytocytokines within the same plant species such as Arabidopsis, maize or tomato suggests a possible interaction between them in order to coordinate the immune response. In fact, Marmiroli and Maestri (2014) found a complex network of interconnected peptides in the plant response to stress and defense by performing an *in silico* analysis of the predicted peptide interactome.

Later, some studies showed evidences of peptide cooperation to amplify the defense response. PIP-RLK7 and Pep1-PEPR1 were found to cooperate to amplify the immune response triggered by the PAMP flagellin in Arabidopsis (Hou et al., 2014). Both SCOOP12 and Pep1 induce the expression of several of the SCOOP precursors genes, PROSCOOPs, (Gully et al., 2019), suggesting that Pep1 is cooperating with SCOOPs to amplify its feedback loop. In tomato, Systemin and HypSys function together in the regulation of the long-distance wound signalling response in tomato through the upregulation of the octadecanoid pathway and the synthesis of jasmonates (Narvaéz-Vázquez et al., 2007). Similarly, CAPE1 was found among the signals induced upon wounding combined with MeJA treatment together with Systemin, both peptides having a similar expression response, which means that both peptides regulate the response to the same stress (Chen et al., 2014). These findings arise the question of the possible coapplication of different peptides as an interesting strategy to potentiate Peptide-IR.

6. Costs of Peptide-IR

Activation of plant defenses is commonly associated with fitness cost due to allocation of energy and resources towards the production of defensive compounds (Hulten et al., 2006). As was mentioned above, a beneficial trait of defense priming is the slight fitness cost triggered by the stimulus (Martínez-medina et al., 2016). Since phytocytokine danger signals trigger direct and early defense responses, their perception is expected to be accompanied with a substantial cost on plant growth and development, although, so far, very few studies have measured the possible fitness costs of using peptides as defense inducers.

Corrado and coworkers (2011) reported that overexpression of the Systemin precursor ProSystemin resulted in a constitutive activation of inducible defenses that was costly in tomato, affecting plant growth, development, and reproduction. Note that the cost of a constitutive overexpressing line may differ substantially from that of a punctual treatment. On the contrary, a very recent study reported that ProSystemin overexpressing plants (PS+) did not show fitness reduction; in fact, they increased their growth and productivity (Luna-Martínez et al, 2021) suggesting a role of ProSystemin in the defense-growth balance. Further research is needed to address this issue.

OBJECTIVES

OBJECTIVES

In order to decipher the mechanisms behind Peptide-Induced Resistance in plants against necrotrophic pathogens, the following specific objectives were proposed:

1. Search for heterologous peptides with the ability to induce resistance against the necrotrophic fungi *Plectosphaerella cucumerina* in *Arabidopsis thaliana* plants (**Chapter 1**).

2. Characterization of the Systemin-Induced Resistance mechanisms against *Plectosphaerella cucumerina* in Arabidopsis including early signaling events and downstream metabolomic responses (**Chapter 2**).

3. Decipher the metabolic changes behind Systemin-Induced resistance against *Botrytis cinerea* in its species of origin, tomato, through the combination of different omics technics (**Chapter 3**).

4. Determine the role of MPKs in the early signaling events mediating Systemin-Induced Resistance against *Botrytis cinerea* in tomato plants (**Chapter 4**).

CHAPTER 1

Arabidopsis plants sense non-self peptides to promote resistance against *Plectosphaerella cucumerina*

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Abstract

Peptides are important regulators that participate in the modulation of almost every physiological event in plants, including defence. Recently, many of these peptides have been described as defence elicitors, termed phytocytokines, that are released upon pest or pathogen attack, triggering an amplification of plant defences. However, little is known about peptides sensing and inducing resistance activities in heterologous plants. In the present study, exogenous peptides from solanaceous species, Systemins and HypSys, are sensed and induce resistance to the necrotrophic fungus *Plectosphaerella cucumerina* in the taxonomically distant species *Arabidopsis thaliana*. Surprisingly, other peptides from closer taxonomic clades have very little or no effect on plant protection. *In vitro* bioassays showed that the studied peptides do not have direct antifungal activities, suggesting that they protect the plant through the promotion of the plant immune system. Interestingly, tomato Systemin was able to induce resistance at very low concentrations (0.1 nM and 1 nM) and displays a maximum threshold being ineffective above at higher concentrations, hence following a phytohormonal behaviour. Here, we show evidence of the possible involvement of the JA-signalling pathway in the Systemin-Induced Resistance (Sys-IR) in Arabidopsis. Additionally, Systemin treated plants display enhanced *BAK1* and *BIK1* gene expression following infection as well as increased production of ROS after PAMP treatment suggesting that Systemin sensitizes Arabidopsis perception to pathogens and PAMPs.

Introduction

Plants are constantly challenged by changes in their environment, such as biotic and abiotic stresses. To respond to biotic challenges, such as chewing insects or pathogen attack, plants have developed complex strategies that allow them to mount a proper defence response. Plants can sense pathogens by recognizing the so-called pathogen-associated molecular patterns (PAMPs), which are exogenous molecules that belong to specific classes of microbes, such as flagellin (Flg22) and Elf18 from bacteria or chitin from fungi. PAMPs are recognized by membrane pattern recognition receptors (PRRs), triggering a first layer of inducible plant defence referred to as PAMP-triggered immunity (PTI) that includes reactive oxygen species (ROS) and Ca^{2+} burst, mitogen-activated protein kinases (MAPKs) activation, phytohormones production and transcriptomic and metabolomic reprogramming (Saijo et al., 2018; Hou et al., 2019).

Plants are also able to recognize host-derived molecules that are released from disrupted cells after pest or pathogen attack and bind to PRRs on intact cells, triggering the amplification of immune signalling. These molecules are known as damage-associated molecular patterns (DAMPs) and include, on the one hand, cell wall fragments that are released after cellular damage caused, for example, by herbivores and, on the other hand, peptide molecules that are released and rapidly activated upon pest or pathogen challenge and cause the amplification of immune signalling (Hou et al., 2019).

Although many peptides have been described as DAMPs, recent studies include these peptides in a new classification. Classic DAMPs are cell debris that are passively released after a cellular disruption and are usually components of the cell wall, such as oligogalacturonides (OGs) and xyloglucan oligosaccharides. Nevertheless, peptides are usually actively synthesized, processed and released by cells under a stress situation that does not include cell damage; these peptides are secondary endogenous danger signals, also named phytocytokines due to their similarity to mammalian cytokines (Gust et al., 2017).

Exposure to danger signals, such as PAMPs, DAMPs or phytocytokines, as well as many other stimuli, produces an alarm state in the plant, enhancing defence capacity locally and systemically that protects the plant against future attack (Yu et al., 2017; Gust et al., 2017; Hou et al., 2019). This state is called induced resistance (IR) and can be triggered by pathogenic and non-pathogenic microbes, herbivores and chemicals, leading to systemic acquired resistance (SAR), or by plant beneficial microbes, including plant growth-promoting rhizobacteria and fungi, leading induced systemic resistance (ISR) (Pieterse et al., 2014). The state of induced resistance is characterized by the rapid activation of latent defence mechanisms, for instance, the production of antimicrobial proteins, and confers protection against a broad spectrum of threats (Pieterse et al., 2014).

An increasing number of plant peptides have been described as defence elicitors. These peptides are released upon pest or pathogen attack and usually derived from the processing of larger precursor proteins, secreted into the extracellular space and bind to specific membrane receptors, triggering a cascade of plant defences and causing an amplification of the plant immune response. (Yamaguchi and Huffaker, 2011; Albert, 2013).

Systemin was the first signalling peptide described in plants (Pearce et al., 1991). Systemin is

an 18 aa peptide found in tomato plants that is part of in a 200 aa precursor protein, Prosystemin. Systemin is released upon wounding or herbivory and induces the accumulation of protease inhibitors (PIs) in local and systemic leaves and volatile signalling that attract natural predators of the pest (Corrado et al., 2007). There is also evidence of the role of Systemin in defence against pathogenic fungi (De la Noval et al., 2007; Coppola et al., 2015; Coppola et al., 2019). The hydroxyproline-rich systemins (HypSys) are peptides found in tomato and tobacco that trigger physiological responses that are similar to those triggered by tomato Systemin (Pearce et al., 2001; Pearce and Ryan, 2003). In Arabidopsis, elicitor peptides (Peps) were described as endogenous amplifiers of innate immunity that induce the transcription of defence-related genes, such as defensin *PDF1.2* and *PR1*, and activate the synthesis of reactive oxygen species (ROS; e.g., H2O2) (Huffaker et al., 2006; Klauser et al., 2013). AtPep1 participates in plant resistance against several pathogens, including *Botrytis cinerea*, *Pseudomonas syringae* pv. DC3000 and *Phytophthora infestans* (Huffaker et al., 2006; Yamaguchi et al., 2010; Liu et al., 2013), and contributes to JA-mediated defence against herbivory (Klauser et al., 2015). Another family of peptides, PAMP-induced peptides (PIPs), were identified in Arabidopsis and are induced by pathogens and elicitors. More specifically, when PIP1 and PIP2 are externally applied, they lead to enhanced immune responses and resistance to *Pseudomonas syringae* and *Fusarium oxysporum* (Hou et al., 2014). Likewise, three short peptides from Soybean, GmPep914, GmPep890 and GmSubPep, were found to alkalinize the cellular media and induce pathogen-related genes, such as *Chitinase 1* and *Chalcone Synthas*e, and genes involved in phytoalexin synthesis and production (Pearce et al., 2010; Yamaguchi et al., 2011).

Some peptides that were initially thought to be involved in different physiological events have been later found to have a role in defence responses. The Arabidopsis GRIM RIPER peptide (GRIp) is involved not only in the response to ozone but also in the resistance to bacterial pathogen PstDC3000 (Wrzaczek et al., 2009). Likewise, the IDA-LIKE 6 (IDL6) mature peptide was studied for its role in controlling floral organ abscission and lateral root emergence and was later found to be involved in the mediation of Arabidopsis susceptibility to *Pst* DC3000 (Wang et al., 2017). The peptides from rapid alkalinization factors (RALFs) were shown to positively and negatively regulate plant immunity through the RLK Feronia (FER) receptor (Stegmann et al., 2017). Recently, the plant pentapeptide, phytosulfokine (PSK), was found to enhance auxin-dependent immune responses through cytosolic Ca2+ signalling in tomato (Zang et al., 2018).

Interestingly, some studies have reported peptide sensing and signalling in heterologous plant species. Although a report claims that tobacco cells do not respond to exogenous systemin treatment (Scheer et al., 2003), a later study showed that tobacco calli and suspension cells responded to Systemin by both MAPK activation and weak-medium alkalinization (Malinowski et al., 2009). In addition, it was also reported that constitutive expression of the tomato prosystemin gene in tobacco considerably affected the synthesis of host proteins, several of which are involved in protection against pathogens (Rocco et al., 2008). On the other hand, tobacco cells transformed with the AtPep1 receptor PEPR1 responded to nanomolar concentrations of AtPep1, producing a strong alkalinization of the cell culture medium, suggesting a capacity of tobacco to activate Pep1 signalling (Yamaguchi et al., 2006). More surprisingly, Zang et al., 2017, reported that tomato Systemin was sensed by Arabidopsis plants, leading to an inhibition of seedling root growth and the expression of the plant defensin *PDF1.2*. Moreover, the expression of the tomato prosystemin gene in Arabidopsis conferred resistance to the necrotrophic fungus *Botrytis cinerea* (Zhang et al., 2017). These findings suggest that some plants may be able to sense exogenous peptides and that there could be a common receptor-mediated intracellular signalling pathway in response to peptides.

Small peptides have recently received attention since they are involved in almost all physiological plants processes. The vast agronomical potential of peptides is limited by the studies focused on plant species-self peptides. We tested whether exogenous treatment with peptides produced from different plant species are sensed and able to protect Arabidopsis plants. Hence, the goal of this study was to identify peptides from phylogenetically distant species with plant-resistance inducing activities against necrotrophic fungal pathogens.

Material and methods

Plant material and growth conditions

Seeds of wild type *Arabidopsis thaliana* Col-0 ecotype were sterilised for 30 sec with 70% ethanol, followed by 15 min of a 10% bleach solution, and finally, 4-5 washes with sterile distilled water to remove the sterilization solution. Sterile seeds were sown *in vitro* 24-well plates in medium containing 4.9 g/L basal Murashige and Skoog (MS;1962) salt mixture, 1% sucrose and 6 g/L Agar and 5.7 of pH. The plates were placed in a growth chamber with 9 h

light period at 24ºC and 15 h of darkness at 18ºC; a dark surface was placed beneath the plates. For the mutant screenings, the same procedure was carried out. The mutant *sid2.1* (Nawrath and Métraux 1999) was kindly provided by M. Nishimura (Stanford University, CA, USA), j*ar1* (Matthes et al., 2010) by Jurriaan Ton (University of Sheffield, United Kingdom), and *jin1* (Lorenzo et al., 2004) and *pad4.1* (Nishimura et al., 2003) were provided by Brigitte Mauch-Mani (University of Neuchâtel, Switzerland) and the mutant perp1 was obtained from SALK collection (SALK_059281) and previously described by Flury et al., (2013). Tomato seeds (*Solanum lycopesicum* L. cv. Money Maker) were sterilised by 15 min shaking in a solution of 75% bleach containing 0,1% of Tween, followed by 4-5 washes with sterile distilled water to remove the sterilization solution. The seeds were sown in 100 ml pots containing 30 ml of solid MS medium (described above). The pots were then placed in a growth chamber with 16 h light period at 26ºC and 8 h of darkness at 18ºC; a dark surface was placed beneath the plates.

Peptide treatment, pathogen inoculation and infection quantification by trypan blue staining

The plants were treated 2 weeks after sowing with a range of peptide concentrations from 0.1 to 20 nM (final concentration) by adding the peptides to the medium. Twenty four hours after peptide treatment, plants were challenged with $5x10^3$ spores/ml of *Plectosphaerella cucumerina* by drop inoculation (1µl per leaf). In Arabidopsis plants, BABA was used as a positive control at a concentration of 1 ppm (1mg/L) (Pastor et al., 2013). For the infection quantification, the plants were collected 5 days after infection and dead cells were stained using trypan blue (Ton and Mauch-Mani, 2004). The infection levels were quantified by a disease rating, measured as a percentage of infected leaf surface according to a scale (0 = healthy leaves; 1= leaves with less than 25% of diseased surface; 2= leaves with 25-50%; 3= leaves with 50-75% of diseased surface; 4= leaves with more than 75% diseased surface). A minimum of 6 plants per condition and 4 leaves per plant were analysed. All experiments were repeated a minimum of three times.

Fungal biomass quantification

Infection quantification was also determined by measuring a fungal constitutive gene related to a plant constitutive gene. Arabidopsis tissue of plants treated either with water or 0.1nM systemin was collected for DNA extraction 48h after pathogen infection. For the DNA

extraction, a simple and rapid protocol was followed (Edwards et al 1991). A Quantitative Real-Time PCR (qPCR) was performed with a Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific), using a StepOne instrument (Applied Biosystems). A ratio was calculated of the expression of *PcTUBULIN*, as a constitutive gene of *P. cucumerina*, relative to the expression of *AtUBIQUITIN21,* a constitutive gene of Arabidopsis, following the ΔCt method. Primer sequences are listed in Table S1.

In vitro antifungal assays

Sterile 12-well plates were filled with PDB1/2 medium containing the peptides at the concentration of 20 nM, the highest concentration used in the screenings. A solution with *Plectosphaerella cucumerina* spores was added to each well to a final concentration of 10^4 spores/ml in each well, and the plates were placed in a shaker until the next day. To measure the fungal growth, absorbance at 600 nm was measured 24 h after pathogen inoculation. This method was adapted from Broekaert et al., 1990. A commercial fungicidal was used as a positive control of growth inhibition.

ROS production measurement

H2O2 production after treatments was determined in leaf discs using a luminol-based assay as previously described (Torres et al., 2013). Two different experiments were performed. Firstly, to determine the ROS production in response to Systemin treatments, a group of leaf discs (6 mm diameter; n=8) obtained from 6-week-old plants were stored with 150 ml of water. After 24h the water was replaced by water (blanc) or Systemin at different concentrations (0.1nM, 1nM, 10nM, 100nM and 1000nM) in a 96-well titer plate (one disc/well) with a solution containing luminol (Sigma-Aldrich; 100 μM) and horseradish peroxidase (Sigma-Aldrich; 1 μg mL-1). Secondly, to test whether Systemin treated plants were sensitive to PAMPs, the leaf discs were maintained overnight either with water or with increasing concentrations of systemin (0.1nM, 1nM, 10nM, 100nM and 1000nM). 24h later, H_2O_2 production was triggered by adding 100nM flg22 to the reaction. Plates were analyzed for 1h using a Luminoskan 96 microplate luminometer (Thermofisher) and a signal integration time of 1.5 s. Luminescence was expressed in Relative Luminescence Units.

Targeted HPLC-MS for hormonal analysis

For hormonal analyses, 120 mg of freeze-dried material sampled at 48hpi was powdered in liquid nitrogen and homogenized with 1 ml of MeOH: H2O (0.01%HCOOH) (10:90). Crystal

balls were added to each sample and tubes were placed in shaker during 2.5 min at 30hz. Then, samples were centrifuged and the supernatant was collected into a new tube. A mix of internal standards with salicylic acid-d5 (SA-d5), dehydrojasmonic acid (dhJA), and jasmonate-isoleucine-d6 (JA-Ile-d6) was added to each sample. To quantify precisely, external calibration curves were prepared with each pure compound (quantification, SA-d5 for SA, dhJA for JA and JA-Ile-d6 for JA-Ile). The targeted hormonal analysis was performed in an Acquity ultraperformance liquid chromatography system (UPLC; Waters, Mildford, MA, USA) coupled to a triple quadrupole mass spectrometer (Xevo TQS, Waters Micromass, Manchester, UK). The column used for the LC separation was a UPLC Kinetex 2.6 µm EVO C18 100 Å, 2.1 x 50 mm (Phenomenex). Conditions and solvent gradients used in this chromatographic analysis were the same as described in Sánchez-Bel et al., (2018).

RNA extraction and RT-qPCR analysis

Two days post-inoculation (48 hpi), the leaves were collected, powdered in liquid nitrogen and stored at -80ºC. For the RNA extraction, 1 ml of Trizol was added to 100 mg of grounded leaves. After centrifugation, the supernatant was transferred to a new tube, and 0.22 ml of CHCl3 was added. The samples were centrifuged, and the supernatant was collected in a new tube; 0.35 ml of isopropanol, 0.35 ml of 0.8 M citrate and 1.2 mM NaCl were added and mixed vigorously. After centrifugation, the supernatant was removed, and the pellet was washed twice with 70% EtOH. The pellet was dried and dissolved in nuclease-free water. The synthesis of cDNA was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative Real-Time PCR (qPCR) was performed with a Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific), using a StepOne instrument (Applied Biosystems). The ΔCt method was used to analyse the gene expression data. The housekeeping genes *UBIQUITIN21 (At5g25760)* and *PP2A (At1g13320)* were used to normalize the expression values. The sequences of the primers are shown in Supplementary Table S1.

Peptide extraction

One day after peptide treatment, the seedlings were collected, powdered with liquid nitrogen and stored at -80ºC. Fresh material (250 mg) was homogenized in a tube with 1,5 ml of Phenol/TRIS and saturated (ACROS Organic, ref. 327125000) at pH 8. The suspension was incubated at room temperature for 20 min, crystal balls were added to each sample and the tubes were placed in a shaker for 2.5 min at 30 Hz. The tubes were centrifuged 2 min at

14,000rpm. After centrifugation, the liquid phase was filtered using a hydrophilic PVDF filter with a 25-mm diameter and a pore size of 0.45 µm (FILTER-LAB). After filtration, 6 volumes of pure cold acetone (Scharlau, AC0312, PharmPur ®) were added to each sample, and the samples were stored overnight at − 20°C. The precipitate was recovered the next day and washed twice with cold acetone. The liquid phase was discarded, and the pellet was dried. The final residue was re-suspended in 500 µl of a solution of 0.1% HCOOH in H2O: acetonitrile (9:1, v/v) and injected into the TQS-MS/MS instrument (Xevo TQS, Waters Micromass, Manchester, UK).

Reagents and standards

Supergradient HPLC-grade acetonitrile was purchased from Scharlab (AC 0331). Formic acid was obtained from J.T. Baker (Deventer, Holland, 6037). Methanol (HPLC grade), and trypan blue were purchased from Sigma (www.sigmaaldrich.com). Peptide standards of Systemin, Pep1, HypSysI, HypSysII, HypSysII, PotSysI, PotSysII, PepSys, NishSys, Pep914, Pep890 and Systemin-P13AT17A were purchased from Biomatik (https://www.biomatik.com/).

Optimization of a multi-residue targeted quantitative LC-MS method for small peptide analysis

High-performance liquid chromatography (HPLC) was performed using a Waters Xevo TQ-S. A protocol that was adapted from Pastor et al 2018 was followed. Aliquots of 20 µl were injected into the system through a reversed column Aeris PEPTIDE 3.6 µ XB-C18 (150 × 4.6 mm) from Phenomenex, at a flow rate of 0.3 ml min−1. The peptides were eluted with a gradient of ACN (organic phase) and Milli-Q water containing 0.1% HCOOH (aqueous phase), starting with 5:95 (v/v), linearly increasing to 35:65 (v/v) over 10 min and plateauing at 95:5 (v/v) 1 min later. The gradient was maintained in isocratic conditions for 1 min before the column was left to equilibrate for 3 min in order to reach initial conditions, for a total of 15 min per sample. The effluents originating from the HPLC were introduced into a triple quadrupole mass spectrometer (Xevo TQS, Waters Micromass, Manchester, UK) equipped with T-Wave devices and an ESI interface operated in positive mode. The cone and desolvation gas was nitrogen. The nebulizer gas flow was set to 250 L h−1 and the desolvation gas flow at 1200 L h−1. For operation in tandem MS/MS mode, the collision gas was pure 99.995% argon (Praxair, Madrid, Spain), at a pressure of 4 × 10−3 bar in the collision cell. The

desolvation gas temperature was 650 °C, the source temperature was set to 150 °C, and the capillary voltage was 3.2 kV. The mass spectrometer was set to multiple reaction monitoring (MRM) mode, and the data were acquired and processed using the MassLynx v4.1 software (Waters, Manchester, UK).

For the selection of the precursor and daughter ions of each peptide, peptide standards direct infusion was performed in a Waters Xevo TQ-S instrument, and masses showing the highest signal were selected for fragmentation and daughter ion characterization. Optimal conditions and appropriate cone and collision energies were determined to obtain the characteristic transitions for each peptide (Table S2). Second, the retention time for each peptide was characterized by injecting aliquots of the standard peptides in a range of concentrations to construct calibration curves for each peptide. To quantitatively determine the peptides, an HPLC–MS/MS method was validated regarding the selectivity, linearity, precision, limit of detection (LOD) and quantification (LOQ). The transitions with higher signal intensities were selected as follows: HypSysI (519,8>498,2); HypSysII (595,5>494,6), HypSys III (518,3>394,2); Systemin (503,2>614,3); Potsys I (498,7>816,3); PotSys II (491,7>816,3); PepSys (395,8>392,2) and NishSys (506,3>515,3). All the mentioned parameters are found in supplementary table 2.

Statistical analysis

Statgraphics-plus software for Windows V.5 (Statistical Graphics Corp., Md, USA) was used to determine the statistical analysis by one-way analysis of variance (ANOVA) otherwise indicated in the figure legends. Means are shown with standard errors and their comparative was performed using Fisher's least significant difference (LSD) at 99.5%. Graphs show the averages of one of the experiments. Each experiment contained a minimum of 6 plants per treatment and was repeated at least three times.

Results

Peptides from different plant species are uptaken and induce resistance against *Plectosphaerella cucumerina* in *Arabidopsis thaliana*

Plant peptides are involved in the majority of physiological plant processes. Most peptides that have been studied are peptides involved in plant growth and development. However, although there are some reports related to plant defence and induced resistance triggered by peptides, there remain large unexplored potentials of many peptides that may confer resistance against a wide range of pathogens and insects. In a first attempt, we tested peptides for their potential activities in inducing plant resistance against fungal pathogens. To achieve this goal, we selected peptides from different plant species that were found to be involved in plant defence and performed screening bioassays of induced-resistance in the *Arabidopsis thaliana*-*Plectosphaerella cucumerina* pathosystem.

Pep1 from *Arabidopsis thaliana* (Huffaker et al., 2006; Yamaguchi et al., 2006; Klauser et al., 2015) and systemin from tomato were comparatively tested for induced resistance. As expected, Arabidopsis plants treated with AtPep1, which is known to function as an elicitor of plant defence in response to pathogens, exhibited significantly reduced severity of infection compared with water-treated controls at any of the concentrations tested (Fig. 1; Table 1). Systemin is an 18 aa peptide that has a function similar to that of AtPep1, although this peptide is mostly related to wounding and defence against insects in tomato (Fürstenberg-Hägg et al., 2013). Surprisingly, Systemin at very low concentrations (0.1 and 1 nM) was able to protect the plant against the necrotrophic fungus (Fig. 1). Note that Pep1 and Systemin at the lowest concentrations (0.1 nM) protected plants to an extent similar to the protection conferred by **β**-amino butyric acid (BABA), a well-known inducer of resistance (Pastor et al., 2013). Subsequently, Systemins from other solanaceous species (potato, pepper, nightshade; Fig. S1) (Constabel et al., 1998) were also tested. PepSys, NishSys and PotSysII were able to induce resistance at the same concentration as tomato Systemin (Table 1). Note that all these peptides are produced in species that are taxonomically distant from *Arabidopsis thaliana* (Fig. S1). Moreover, we tested three short peptides from tomato, namely, HypSys I, HypSys II and HypSys III, with functions in the defence against biotic stresses, although with a different sequence from Systemin. Arabidopsis plants were less sensitive to these peptides, although the plants treated with HypSysI and HypSysII at concentrations above 10 nM or with HypSys III at concentrations above 20 nM were also protected (Fig. 2; Table 1). These results suggest that Arabidopsis senses and responds to heterologous peptides.

The previous peptides were shown to function as DAMPs, stimulating the defensive responses following sensing of PAMPs. In addition, there are other peptides involved in defense display direct antimicrobial activity rather than activating signaling cascades. Two antimicrobial peptides (AMPs; AFP1 and AFP2) from radish that were described to be active against a broad spectrum of fungi were also tested for their ability to protect Arabidopsis against *P. cucumerina* (Terras et al., 1992; Fig. S2). AFP-treated plants showed significant levels of protection only at the highest concentration tested (20 nM) (Fig. S3). Finally, two short peptides from Soybean described as defence signals, GmPep914 and GmPep890, were also tested against *P. cucumerina*. These peptides lead to alkalinization of the medium and the activation of defence-related genes (Yamaguchi et al., 2011). None of these peptides succeeded in protecting Arabidopsis plants at any of the concentrations tested (Fig. S3). Interestingly, plants treated with 0.1 and 1nM of GmPep91 are more susceptible to the fungus. This result correlates with the one shown in the antifungal assays (Fig. 3) in which the fungal growth was higher in the presence of GmPep91. It is likely that the fungus is using this peptide as a source of amino acids.

It was previously shown that the T17A and P13AT17A truncated Systemin proteins were not functional at inducing resistance in tomato against fungal pathogens (Pearce et al., 1993; Xu et al., 2018). Furthermore, Sys-P13AT17A also failed to inhibit seedling root growth in Arabidopsis plants (Zhang et al., 2017). However, Sys-P13AT17A induced resistance in Arabidopsis against *P. cucumerina* at the same level as the natural tomato peptide (Fig. S4 A). Alternatively, the functionality of the Arabidopsis peptide Pep1 was tested in tomato against *B. cinerea* and showed no significant protection (Fig. S4 B).

Although it has been shown that some peptides and resistance inducers can produce direct cell death, in our experimental conditions, at all the concentrations used we did not observe any cell death in mock-infected plants following trypan blue staining. Therefore, we can assure that the cell death observed in our experiments is due to the infection.

Few methods for small peptides determination in solanaceous are found along the literature (Mucha et al., 2019). To further confirm the uptake and the presence of the non-self peptides that were able to induce resistance in Arabidopsis we developed a multi-residue analytical method based on the one described in Pastor et al. (2018). In this regard, a fast and accurate quantitative multi-residue method for the simultaneous determination of small peptides was

developed. It was observed that the chromatographic standard peptides in plant complex matrices behaved very similarly to pure standard preparations, making it feasible to identify these peptides in any plant material following root treatments. With this method, we were able to detect and measure them in Arabidopsis plant samples after 24h of the peptides' treatment (Figure S5; table S2).

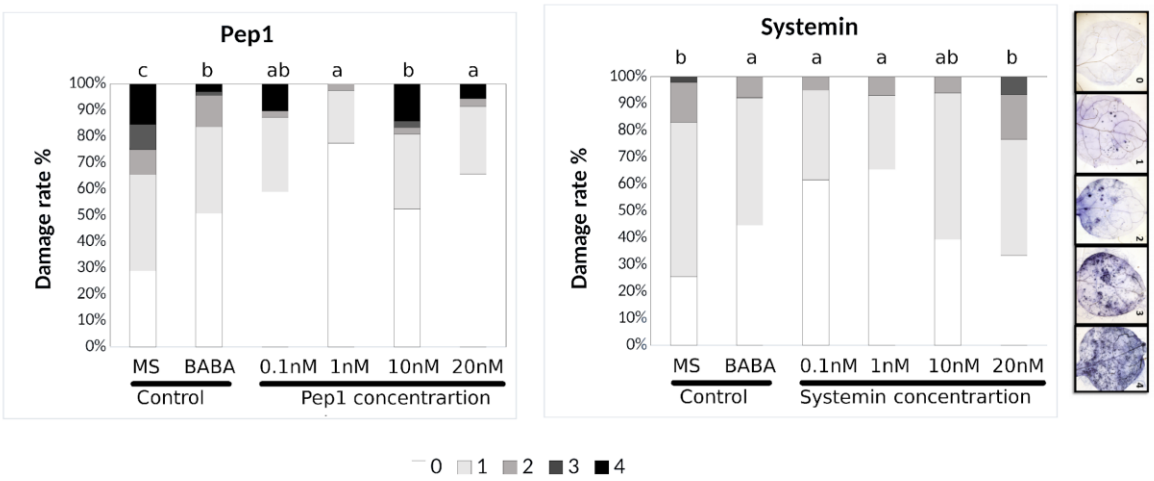

Figure 1. Pep1 and Systemin induced-resistance assays against* Plectosphaerella cucumerina *in Arabidopsis plants. *Infection levels 5 days after inoculation quantified by a disease rating in trypan blue stained leaves, measured as a percentage of the infected leaf surface. Arabidopsis Col-0 plants were treated with increasing concentrations of Pep1 or Systemin (0.1nM, 1nM, 10nM and 20nM) 24h before infection with 1µl droplets of 5x10E3 spores/ml of P.cucumerina BMM. ß-amino butyric acid (BABA) at 1ppm was used as a positive control. Colours mean % of diseased leaves in a scale (0 = healthy leaves; 1= leaves with less than 25% of diseased surface; 2= leaves with 25-50%; 3= leaves with 50-75% of the diseased surface, 4= leaves with more than 75% of the surface diseased). Different letters indicate statistically significant differences (ANOVA, Fisher's Least Significant Difference (LSD) test; P < 0.05 n=24). The experiment had 6 plants per treatment and was repeated at least three times with similar results.*

Table 1. Peptides Induced-Resistance assays summary table. Peptides tested, their species of origin and the results obtained in the induceresistance assays are shown in the table. (+) indicates effective plant protection and (-) indicates control levels of disease.

Peptide	Species of origin	0,1nM	1nM	10nM	20nM
Pep1	Arabidopsis	+	+	+	+
Systemin	Tomato	+	+	-	-
PepSys	Pepper	+	+	-	-
NishSys	Nightshade	-	+	-	-
PotSys I	Potato	-	-	-	-
PotSys II	Potato	+	-	-	-
HypSys I	Tomato	-	-	+	+
HypSys II	Tomato	-	-	+	+
HypSys III	Tomato	-	-	-	+
AFP1	Radish	-	-	-	+
AFP2	Radish	-	-	-	+
Pep914	Soybean	-	-	-	-
Pep890	Soybean	-	-	-	-

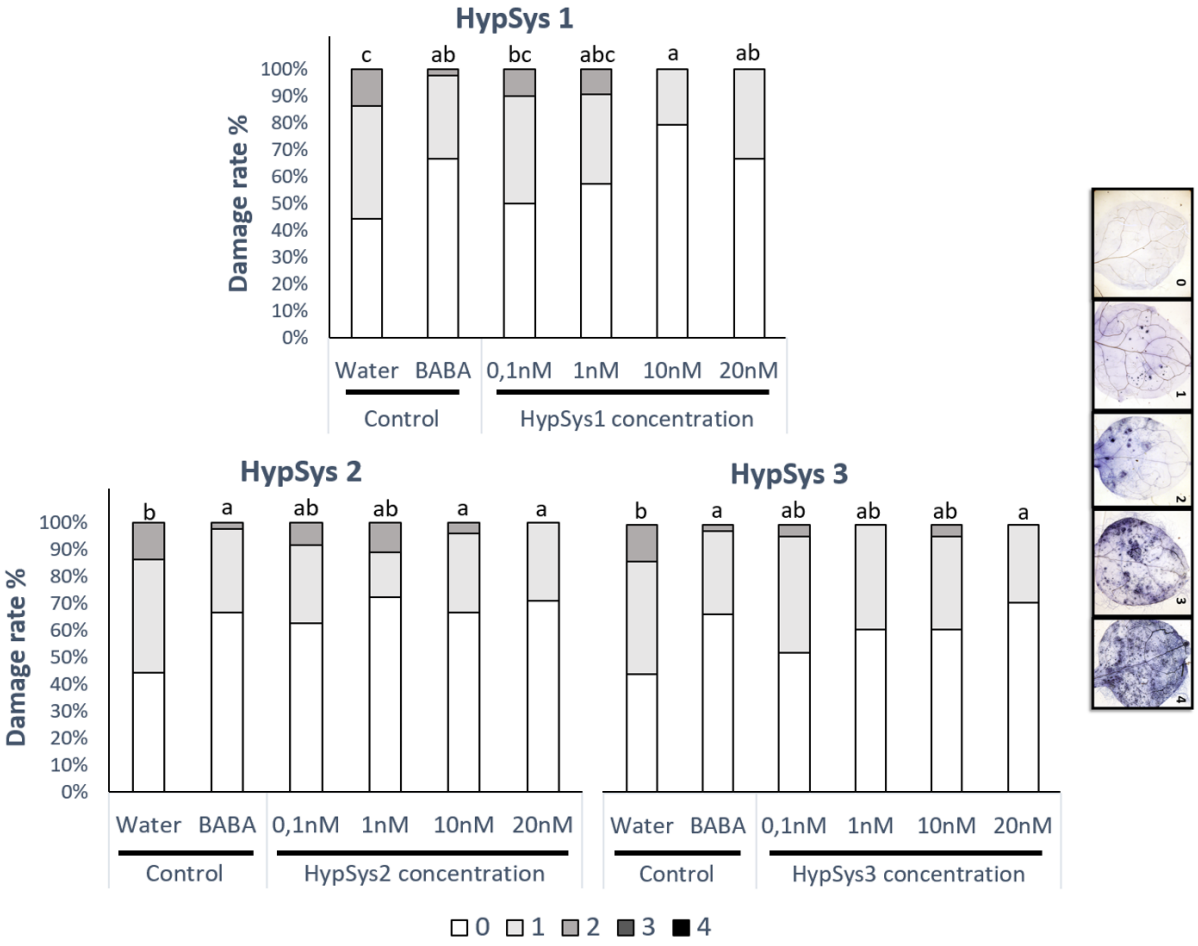

Figure 2. HypSys peptides induced-resistance assays against* Plectosphaerella cucumerina *in Arabidopsis plants. *Infection levels 5 days after inoculation quantified by a disease rating in trypan blue stained leaves, measured as a percentage of the infected leaf surface. Arabidopsis Col-0 plants were treated with increasing concentrations of HypSysI, HypSysII and HypSysII (0.1nM, 1nM, 10nM and 20nM) 24h before infection with 1µl droplets of 5x10E3 spores/ml of P.cucumerina BMM. ß-amino butyric acid (BABA) at 1ppm was used as a positive control. Colours mean % of diseased leaves in a scale (0 = healthy leaves; 1= leaves with less than 25% of diseased surface; 2= leaves with 25-50%; 3= leaves with 50-75% of the diseased surface, 4= leaves with more than 75% of the surface diseased). Different letters indicate statistically significant differences (ANOVA, Fisher's Least Significant Difference (LSD) test; P < 0.05 n=24). The experiment had 6 plants per treatment and was repeated at least three times with similar results.*

The sequence homology of studied peptides is not linked to their IR activity

To determine whether the results in the screening assay of induced resistance could be explained by the phylogenetic proximity to *Arabidopsis thaliana* or sequence identity with the AtPep1, we performed multiple sequence alignment of the amino acid sequences of the peptides tested and built a phylogenetic tree based on the peptide sequences provided by the UniProt database.

By performing a Clustal Omega multiple sequence alignment, we discovered that the different peptides used in the screening have very low or nonexistent sequence homology with AtPep1 or with the other peptides tested (Fig. S6). Interestingly, the species that clade closer to Arabidopsis in the phylogenetic tree are those whose peptides either minimally protected (AFPs from radish) or failed to induce resistance (Peps from Soybean) against the fungus (Fig. S1). By comparison (Fig. S1 and Table 1), a correlation between the phylogenetic distance and effectively induced resistance against *P. cucumerina* in *Arabidopsis* was not observed.
In addition, we analysed if the tested non-self peptides shared common motifs with AtPep1 that would account for their effectiveness in Arabidopsis. Using the Prosite database (http://wwwuser.cnb.csic.es/~pazos/cam97/), we found that Sys, PotSys1, PotSys2, PepSys, HypSys3 and Pep1 showed a serine protein kinase C phosphorylation site (red boxes in Figure S6). Alternatively, AFP1 and AFP2 shared an N-myristoylation site (blue box). All these protein sites are patterns which have a high probability of occurrence, still they could not explain the different results obtained in the resistance induction assays (Figure S6).

The studied peptides do not display any direct antifungal activity against *P. cucumerina*

Because most peptides tested can protect Arabidopsis against the necrotrophic fungus, they likely exert either an induced resistance or a direct antimicrobial effect. To test this possibility, an *in vitro* assay to measure fungal growth in the presence of each peptide was performed. For the assay, we filled sterile 12-well plates with 3 ml of LB medium containing the peptide at the highest concentration (20 nM) to examine the toxic antimicrobial effect. Spores of *P. cucumerina* were added to each well, and fungal growth was measured 24 hpi by assessing the turbidity of the medium at 600 nm. A commercial fungicide (Switch) was used as a positive control (Fig. 3). None of the peptides tested demonstrated antifungal activity against the necrotroph (Fig. 3). Surprisingly, some of the peptides enhanced fungal growth, suggesting that the fungus may use the peptides as a source of amino acids.

These results suggest that the peptides induce resistance through the promotion of the plant immune system.

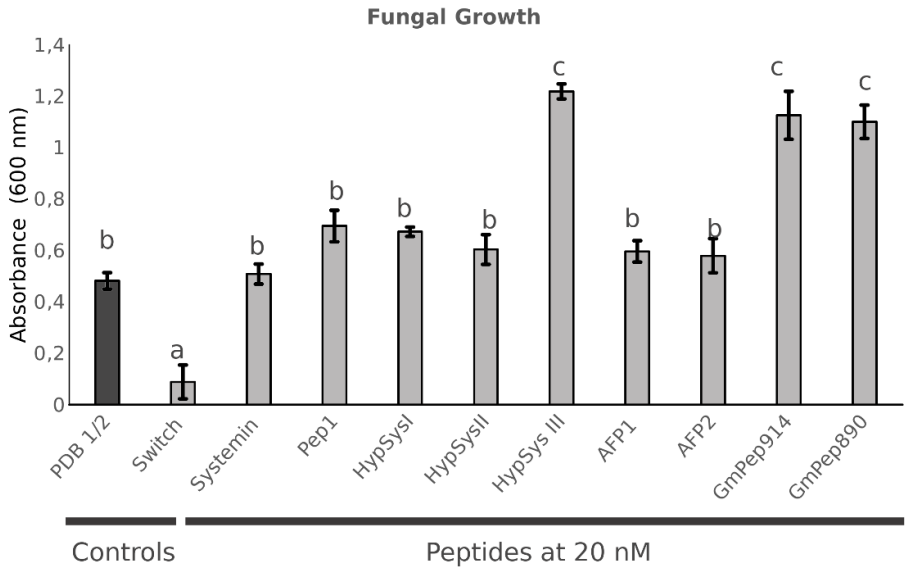

Figure 3. In vitro antifungal assays. Plectosphaerella cucumerina *growth measured after 24h growing in liquid medium containing each peptide at a concentration of 20nM. Fungal growth was measured as the level of turbidity (absorbance 600nM). A commercial fungicidal (Switch) was used as a positive control. Bars represent mean ± standard error (SD), n=3. Different letters indicate statistically significant differences (ANOVA, Fisher's Least Significant Difference (LSD) test; P < 0.05 n=3).*

Alterations in the hormonal imbalance may contribute to Systemin-IR

For subsequent analysis, we focus on the tomato Systemin peptide since it was effective on inducing resistance at very low concentrations (Table 1). To further confirm Sys-IR using a different method for the infection quantification, fungal biomass related to the plant tissue was confirmed that it was significantly lower in plants treated with 0.1nM Systemin (Fig. S7).

In a first approach to understand the likely mechanisms of Systemin-IR in Arabidopsis, SA and JA as the main hormones regulating defence pathways were quantified (Fig. 4A). In tomato, Systemin was shown to accumulate upon herbivory and was linked to JA-dependent responses (Sun et al., 2011; Fürstenberg-Hägg et al., 2013). In Arabidopsis, 0.1nM Systemin treatments triggered an increase in SA, JA and JA-Ile in the absence of infection compared to water-treated plants. In contrast, following infection, the hormonal levels in Arabidopsis plants treated with Systemin remained similar to the levels before the infection. These observations suggest that

SA- and JA-dependent pathways may contribute to Systemin-IR; however, the hormonal changes triggered by Systemin take place independently of the infection.

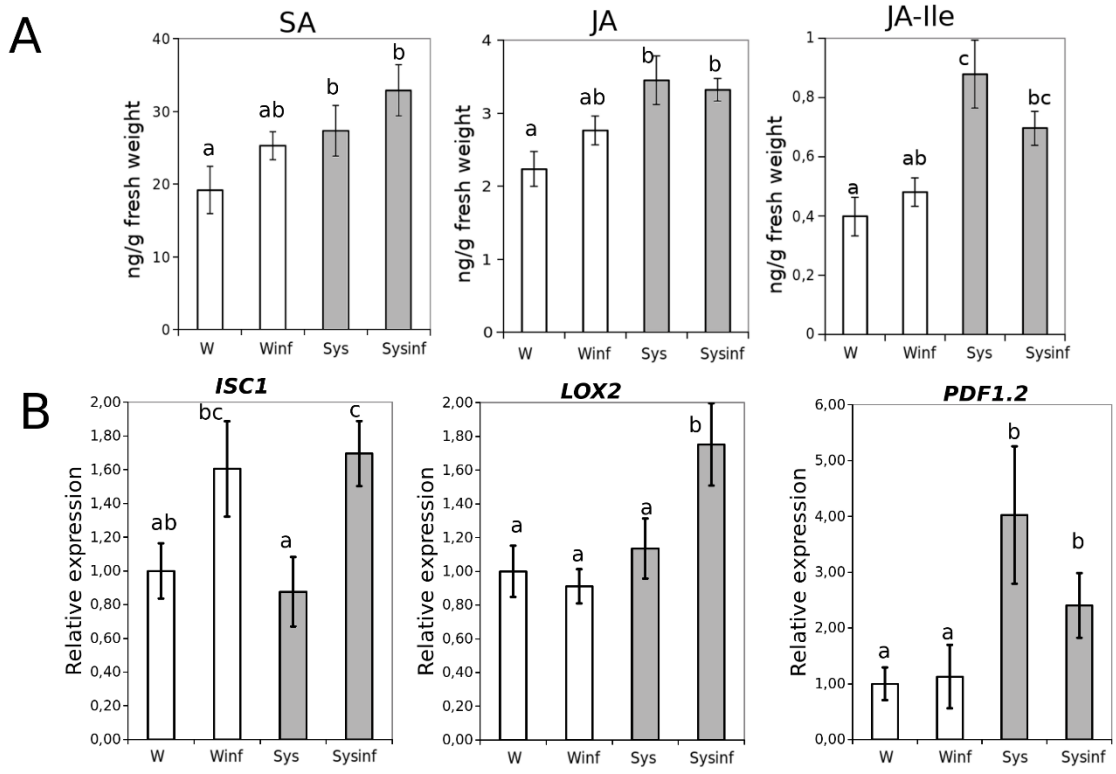

Figure 4. Systemin treatment impact in hormonal profiles. *(A) Salicylic acid (SA), Jasmonic acid (JA) and JA-isoleucine (JA-ile) hormone quantitative levels (ng/g fresh weight) measured in Arabidopsis seedlings 48h after* P.cucumerina *infection in control (W) control infected (W inf) 24h Systemin-pretreated (Sys) and 24h Systemin-pretreated infected (Sys inf) plants by targeted HPLC-MS analysis. (B) Quantitative reverse transcription-polymerase chain reaction analysis of ICS1, LOX2 and PDF1.2 in seedlings 48h after* P.cucumerina *infection in Water plants "W", water infected plants "W inf", 0.1nM Systemin treated plants "Sys" and Sys infected plants "Sys inf". Bars represent mean ± standard error (SD), n=6. Different letters represent statistically significant differences. (ANOVA, Fisher's Least Significant Difference (LSD) test; P < 0.05 n=6)*

To complement the previous observations on the hormonal imbalances, we performed an analysis of *ICS1*, *LOX2* and *PDF1.2* gene expression (Fig. 4B). The JA-biosynthesis gene *LOX2* was boosted by systemin in the presence of infection displaying a priming profile (Mauch-Mani et al., 2017), whereas PDF1.2 gene expression was triggered by the treatment

independently of the infection. *ICS1* expression levels increased due to the infection being significantly higher only in plants treated with Systemin.

To be more confident about the role of both hormonal pathways, mutants impaired in the SA and JA-related pathways were treated and infected (Fig 5). Interestingly, only those mutants altered in the JA responses were impaired in the Systemin-IR, while the SA-related *pad 4.1* and *sid2.1* mutants were protected by the peptide.

Based on these results, although SA is induced by Systemin treatments, the gene expression and the mutant analysis suggest that, like in tomato, JA-dependent responses may regulate Systemin-IR in Arabidopsis. However, JA functions in Systemin-IR may likely happen coordinately with other yet unknown mechanisms to contribute to the observed induced resistance phenotype.

Systemin enhances PTI responses in Arabidopsis

To gain knowledge on the perception and signalling of tomato Systemin in Arabidopsis we analysed some well-known PTI responses. On the one hand, we measured the expression of the *BAK1* and *BIK1* membrane receptors as PTI markers in Arabidopsis plants treated with systemin and challenged with spores of *P.cucumerina* (Fig. 6). None of the tested genes were directly induced by systemin treatments. However, both PTI markers were strongly upregulated in treated plants after infection (Fig 6), showing a typical priming profile.

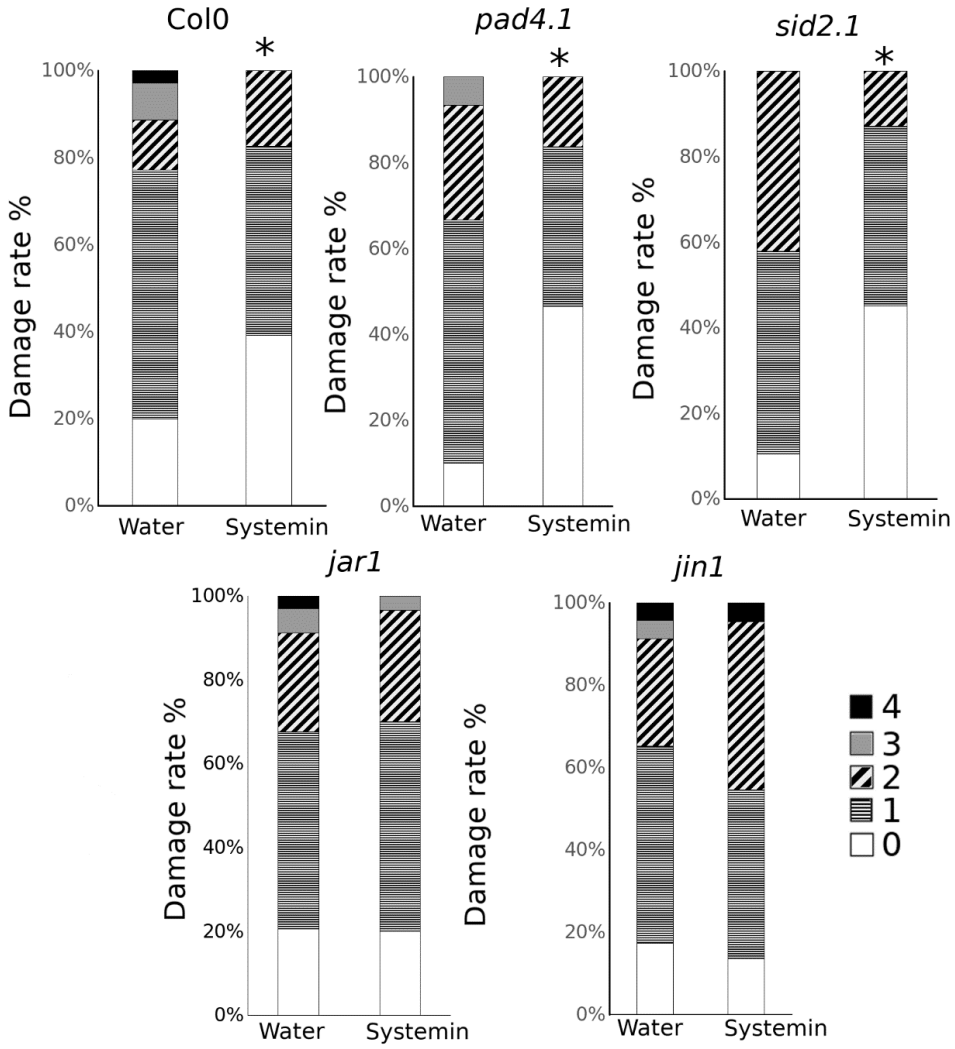

Figure 5. Sys-IR assays in mutants impaired in the SA and JA-related pathways. *Col-0,* pad4.1, sid2.1, jar1 *and jin1 plants were challenged with 1µl droplets of 5x10E3 spores/ml of* P.cucumerina *BMM 24h after treatment with 0.1nM Systemin. Infection levels were quantified 5 days after inoculation by a disease rating in trypan blue stained leaves, measured as a percentage of the infected leaf surface. Colours mean % of diseased leaves in a scale (0 = healthy leaves; 1= leaves with less than 25% of diseased surface; 2= leaves with 25-50%; 3= leaves with 50-75% of the diseased surface, 4= leaves with more than 75% of the surface diseased). Asteriscs mean statistial significant differences; T-test; P < 0.05 n=12). The experiment had 12 plants per treatment and was repeated at least three times with similar results.*

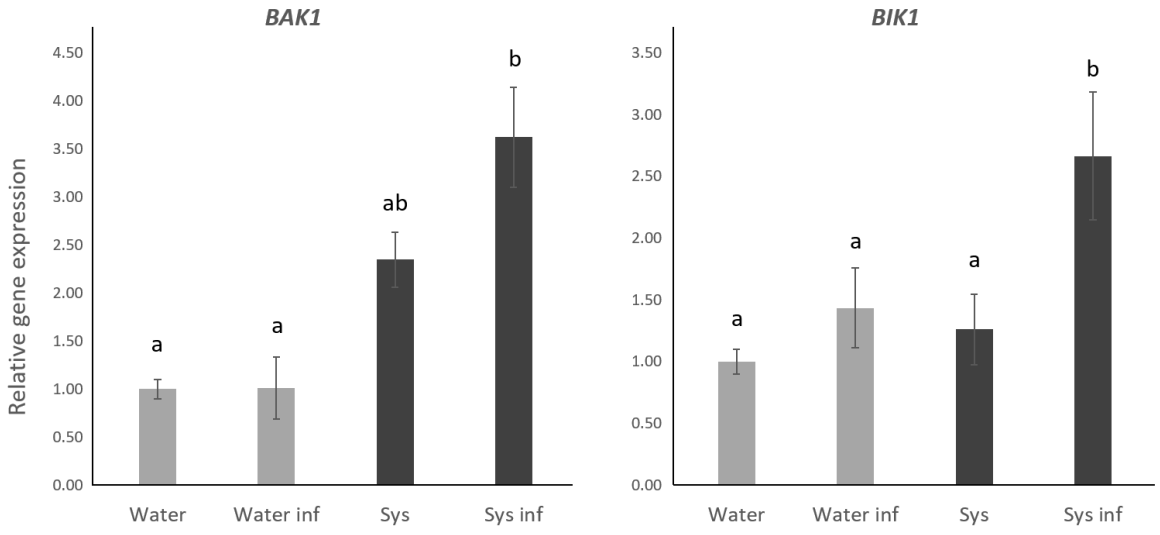

Figure 6. Systemin treatment impact in* BAK1 *and* BIK1 *gene expression. *Quantitative reverse transcription-polymerase chain reaction (qPCR)analysis of* BAK1 *and* BIK1 *in seedlings 48h after* P. cucumerina *infection in normal water plants "W", water infected plants "W inf", 0.1nM Systemin treated "Sys" and Sys infected plants "Sys inf" plants. Bars represent mean ± standard error (SD), n=6. Different letters represent statistically significant differences. (ANOVA, Fisher's Least Significant Difference (LSD) test; P < 0.05 n=6).*

On the other hand, we measured ROS production induced by Systemin and a PAMP challenge after 24h systemin treatment (Fig. 7). A wide range of Systemin concentrations was used (0.1nM, 1nM, 10nM, 100nM and 1000nM). Systemin treatments in the absence of a PAMP did not induce the production of H2O2 (Fig 7 and Fig S8) but ROS production was significantly induced when plants that were treated with Systemin 24h before and challenged with flg22 (Fig. 7). The induction was higher with increasing concentrations of Systemin showing a maximum threshold (100nM). When Systemin was applied at higher concentrations the ROS accumulation decayed to levels similar to 0.1nM os Systemin. This result shows a dose-threshold response of Arabidopsis to Systemin, resembling the protection pattern that we observed in the IR assays (Fig. 1 and Table 1). The results commented above suggest that Arabidopsis perceives tomato Systemin but in a non-canonnical perception unlike classical DAMPs such as Pep1. To further study this hypothesis we confirmed that the mutant pepr1 displays a wild-type phenotype of Sys-IR (Fig. S9), hence this reinfoced a PEPR1-independent function of systemin.

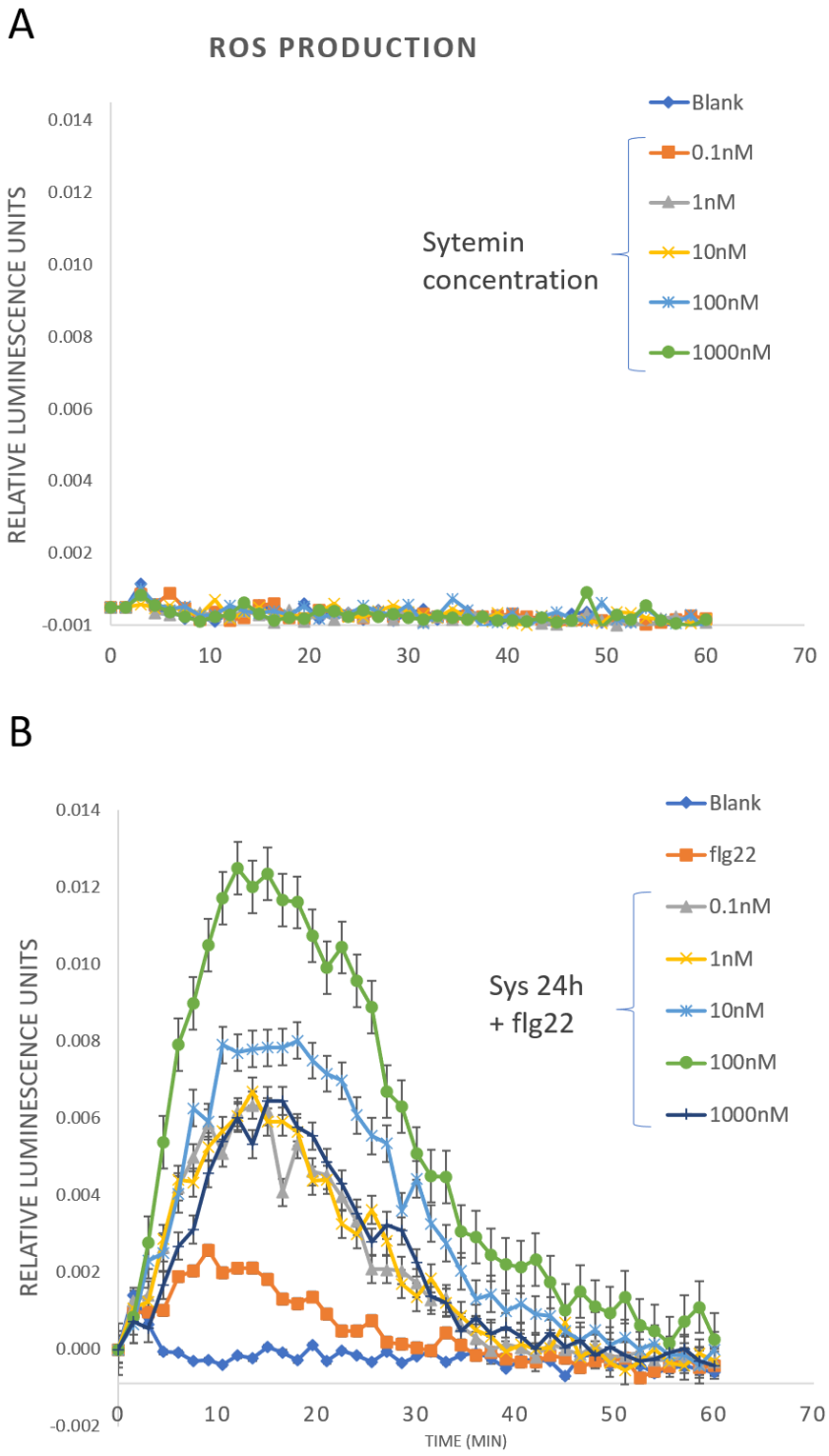

Figure 7. ROS production in response to Systemin and PAMP challenge. *H2O2 production was measured during 1h in leaf disks after elicitation with (A) Systemin at different concentrations and (B) 100nM flg22 in leaf disks that were pre-treated for 24h with different concentrations of Systemin. Luminescence was expressed in Relative Luminescence Units. Slopes represent the means of each time-point ± standard error (SD), n=8.*

Discussion

The understanding of small peptides as signalling molecules in plants has grown significantly in the last few years. In the present study, the role of Arabidopsis self and non-self peptides in inducing resistance against *P. cucumerina* has been analysed. Reasonably, self-peptides are active in protecting Arabidopsis, but surprisingly, other heterologous peptides, such as Systemins from Solanum species, protect Arabidopsis in the nanomolar range. Besides, other peptides from phylogenetically distant plant species are also active in defence, although to a different extent.

Alternatively, most knowledge of small peptides functioning throughout the plant physiology has been generated by studying the gene expression of their respective propeptides. However, the posttranslational processing of these propeptides is tightly regulated, which makes the analytical characterization and quantification of the active peptides essential. For this reason, we have generated a multi-residue UPLC coupled to mass spectrometry method for the simultaneous analysis of small plant peptides (15-20 amino acids).

Small peptides were shown to participate in plant defence as amplifiers of PAMP sensing; therefore, they were suggested to function as DAMPs, which are also known as phytocytokines (Gust et al., 2017). For instance, PAMP-induced peptides (PIPs) from Arabidopsis were shown to amplify flg22 responses and resistance to *PstDC3000* (Hou et al., 2014), and similarly, elf18 responses increased upon co-treatment with RALF17 (Stegmann et al., 2017). Previous studies described the functionality of the Arabidopsis endogenous peptide Pep1 in the defence against fungal pathogens, such as *B. cinerea* (Liu et al., 2013). In the current study, Pep1 exogenously applied in a range from 0.1 to 20 nM was found to protect plants against *P. cucumerina*. Pep1, at the concentrations tested, was as functional as the well-known priming agent $\boldsymbol{\beta}$-amino butyric acid (BABA). In parallel, a screening of non-self peptides for induced resistance against the necrotroph was performed. The screening included peptides from other Brassicaceae, such as AFP1 and 2 (Terras et al., 1992), Solanaceae, such as Systemin, PepSys, NishSys, PotSysI and II (Constabel et al., 1998), HypSys I, II and III (Pearce and Ryan., 2003), and Fabaceae, such as Pep914 and 890 (Yamaguchi et al., 2011). Unexpectedly, the solanum peptides were the most effective in protecting Arabidopsis. Systemin-induced resistance from tomato and pepper and PEP1-IR were as strong as that induced by BABA-IR and Pep1-IR at the very low concentrations of 0.1 and 1 nM. In contrast, a Systemin from potato (PotSysI) and peptides from soybean (Pep914 and 890) did not induce resistance at the concentrations studied. HypSys

I, II and III as well as AFP1 and 2 demonstrated protection only at the highest concentrations. These observations suggest that either Arabidopsis has specific receptor(s) for heterologous plant peptides, which is rather unlikely, or that other yet unknown receptors may bind nonspecifically other small peptides. Further research is needed to clarify this hypothesis.

Because induced resistance was observed, a double analysis of the peptides was performed. The likely link between phylogenetic proximity of the plant species that produce the peptides and the effectiveness inducing resistance was studied. The phylogenetic distance of radish is closer to Arabidopsis compared with tomato, pepper or soybean, although systemins from tomato and pepper were the most effective. Hence, the protection conferred by the tested peptides may not be related to the phylogenetic proximity of the plant species. Second, the sequence homology and the motifs contained in the peptides were also studied. Any of these biochemical properties were linked to higher efficiency in protection. In fact, Pep1 from Arabidopsis shares higher sequence homology with AFPs and Pep from soybean, while Systemin, PepSys and PotSysI and II share very high sequence homology. Note that Systemin and PepSys treatments induced strongly Arabidopsis resistance against the fungus, while PotSysI treatment was ineffective. Alternatively, the only motif shared by these small peptides was a phosphorylation site that was present in Systemin, PepSys, Pep1, PotSys1, PotSys2 and HypSys3. Therefore, neither a conserved sequence nor specific motifs can explain the differential function in Arabidopsis protection.

To fully exclude the possibility that these peptides protect Arabidopsis by inhibiting *P. cucumerina* growth or germination, the *in vitro* antimicrobial effect of all peptides at the highest concentration was tested. None of the small peptides inhibited fungal growth, although surprisingly some of them promoted mycelium expansion, such as HypSys III from tomato and Pep914 and 890 from soybean. These peptides may function as additional nutritional sources for the fungus, which would explain its enhanced growth. Especially surprising was the absence of an antimicrobial effect of the antifungal peptides AFP1 and 2, since their inhibitory properties against several fungi, including the necrotroph *B. cinerea,* have been previously shown, although at concentrations higher than those used in our tests (Terras et al., 1992*;* De Lucca et al., 1999; Thevissen et al., 2012*)*. Regarding the remaining peptides, any of them either promoted or reduced fungal growth, which suggest they protect Arabidopsis through activation of the plant immunity.

Under our experimental conditions, Pep1 treatments protected Arabidopsis plant at any of the

concentrations tested (0.1 - 20 nM). Nevertheless, Systemin treatments significantly protected Arabidopsis at the very low doses of 0.1 and 1 nM, but it was not active at the higher concentrations.

This mode of action has been previosly reported for some well-known resistance inducers and phytohormones. BABA shows a threshold of protection againts *Phytophthora infestans* between 1-10 mM while 0.1 and 20 mM are less effective (Floryszak-Wieczorek, et al 2015). Morevover, BABA-induced callose accumulation in response to PAMPs has also a maximum in the range of 1-5 ppm, while decays at higher concentrations (Pastor et al., 2013). Similarly, BTH was shown to protect better a low doses triggering PAL and inducing coumarin accumulation (Katz et al., 1998). Regarding phytohormones, as an example, brassinosteroid showed maximum threshold on promoting root elongation, while they trigger root elongation at low doses (0.05-0.1 nM) they fail above 1 nM (Müssig et al 2003). Therefore, we can assume that Systemin-IR in arabidopsis acts in a dose-threshold manner, whats was also confirmed by the ROS assays.

There are reports of enhanced resistance of transgenic Arabidopsis plants overexpressing the Prosystemin gene (Zhang et al., 2017). The overexpression of Prosystemin has a strong impact on the Arabidopsis transcriptome with upregulation of stress-related genes. Prosystemin is a 200 amino acid peptide that is processed in tomato by phytaspases. Subsequently, leucine aminopeptidase A removes the terminal Leu, releasing the active form of systemin (Beloshistov et al., 2017). Despite the functionality of overexpression of prosystemin in Arabidopsis, it is still unknown whether the propeptide is active by itself or whether other Arabidopsis phytaspases and a LapA-like protein can process Prosystemin. In the present experiments, it was shown that not only Systemin but also its truncated form Sys-P13AT17A (Pearce et al., 1993) are sensed by Arabidopsis. This result suggests that a core of amino acids in the peptide may be responsible for the non-specific perception and downstream signalling in Arabidopsis since the truncated forms are entirely impaired in inducing resistance in tomato (Pearce et al., 1993; Xu et al., 2018).

Conversely, Pep1 treatments did not protect tomato plants against *B. cinerea*. Thus, it appears that tomato very specifically senses Systemin but not Pep1, while Arabidopsis can sense Pep1 though its known receptors (PEPR1 and 2) and Systemin through an unknown mechanism. In this regard, not only Systemin but also several other tested peptides, such as PepSys, NighSys, HypSys I, II and III, can induce resistance in Arabidopsis, although at higher concentrations.

This finding reinforces the hypothesis that Arabidopsis may have alternative non-specific receptors for non-self peptides. It is tempting to hypothesize that extracellular peptides, as it has been shown for DNA or ATP, may function as danger signals, although not all peptides exert the same activity.

As a first approach to decipher mechanisms underlying Sys-IR, a hormonal analysis showed that SA- and JA-related signalling could be involved. Despite their antagonism, both SA and JA increased following Systemin treatments in Arabidopsis. The active hormone JA-Ile was also triggered following Systemin treatments. Accordingly, several hormone-related genes, such as *LOX2* and *PDF1.2* from the JA-dependent pathway, were also induced by Systemin treatments. The hormone induction and the gene expression have consistent behaviour in the activation of both pathways in Systemin-treated plants upon infection, indicating that a more complex regulation of defences may occur following Systemin sensing that indeed has an impact on hormonal signalling. Note that the PEPR pathway co-activates SA- and JA/ET-mediated immune branches in Arabidopsis (Ross et al., 2014). Despite the induction of SA levels after Systemin treatments, the mutant analysis showed that SA-impaired mutants were fully protected suggesting that JA-dependent responses are behind Sys-IR in Arabidopsis. Similarly, Systemin treatments have been shown to trigger JA-dependent responses in tomato (Ryan 2000; Sun et al., 2011; Fürstenberg-Hägg et al., 2013) and involve the upstream oxylipin pathway following herbivory. Thus, the JA induction following Systemin treatments appears to be a conserved molecular response in Arabidopsis and tomato.

To understand Systemin perception in Arabidopsis we analysed both *BAK1* and *BIK1* gene expression and the generation of ROS. Following Systemin treatment any of the studied markers were directly induced. However, following *P. cucumerina* infection both transcripts increased significantly and additionally flg22 application in Systemin-treated plants induced strong increases in ROS production. To strengthen these observations, we confirmed that Sys-IR is functional in the mutant *pepr1*, hence PEPR1-independent. Note that it was reported previously that systemin effects on root architecture in Arabidopsis is also PEPR1-independent (Zhang et al., 2017). This suggests that Arabidopsis senses Systemin although it is inducing a non-canonical function compared with endogenous peptidic DAMPs such as Pep1/2 that directly induce responses. Although Systemin clearly amplifies PAMP/pathogen response, it is likely that the low doses used do not trigger direct responses resembling priming defence as

it has been previously suggested for other priming stimuli (Mauch-Mani et al 2017; Wilkinson et al 2019)

Much of the understanding of the function of peptides in plant immunity has been based on propeptide gene expression. In very few cases, the processing of these propeptides, the final receptors and signalling cascades have only been recently discovered (Wang et al., 2018; Xu et al., 2018; Hou et al., 2014; Yamaguchi et al., 2006). Following the propeptide translation, proteolytic processing is involved in the cleavage and release of the active peptide from a larger precursor. Non-self peptides should not be specifically processed in Arabidopsis, since they are not naturally present, although it could be possible that they can be processed by other non-specific phytaspases or peptidases that are ubiquitous among plants. Using a multi-residue chromatographic method, we have confirmed the uptake and systemic transport of the heterologous peptides in Arabidopsis.

In conclusion, Systemin and other related peptides that are not produced in Arabidopsis can induce resistance against *P. cucumerina,* triggering protection at very low doses and to a comparable extent as the protection provided by BABA, which indicated that Arabidopsis can sense non-self peptides from phylogenetically distant plant species that are not related in structure or sequence. Furthermore, we show evidence that the JA-dependent signalling mediates Systemin-Induced Resistance that amplifies PAMP receptor expression and ROS production in the presence of a challenge. Pre-challenge induction may prepare the plant for subsequent exposure. These findings open future research to decipher the mechanisms underlying Sys-IR in Arabidopsis.

Acknowledgements

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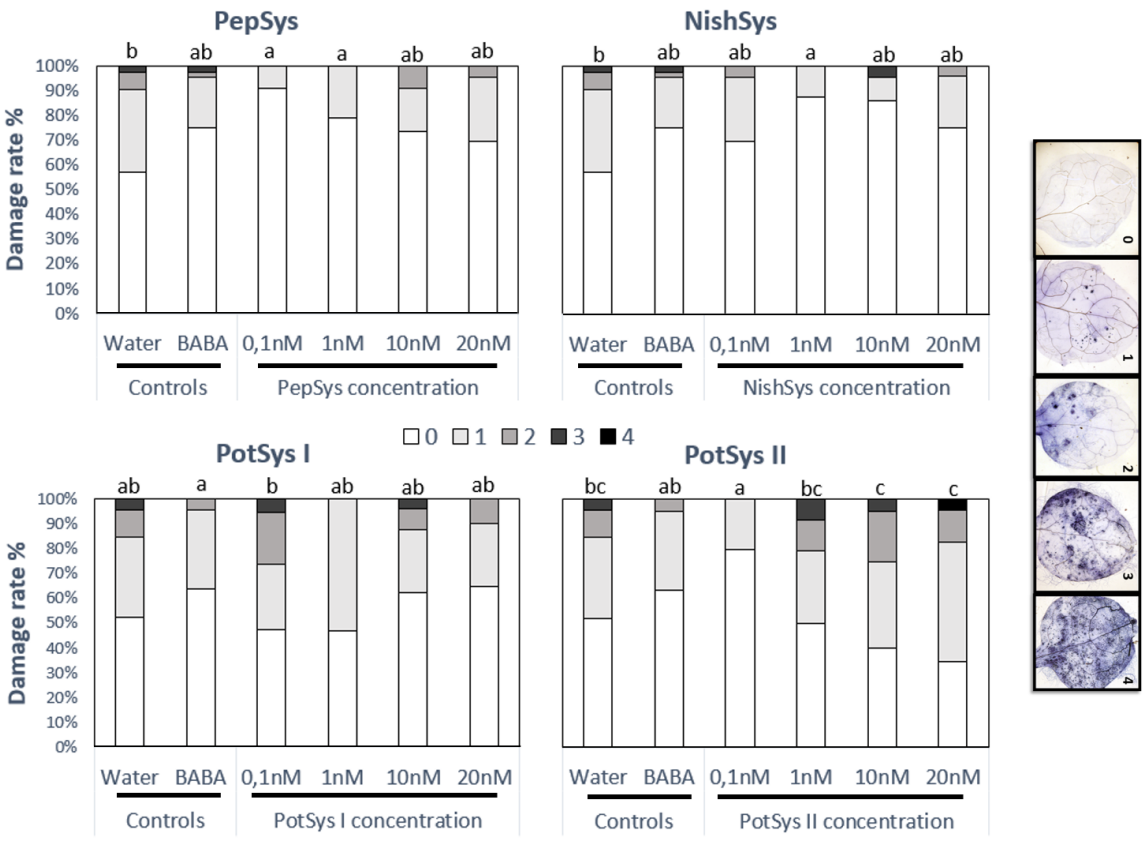
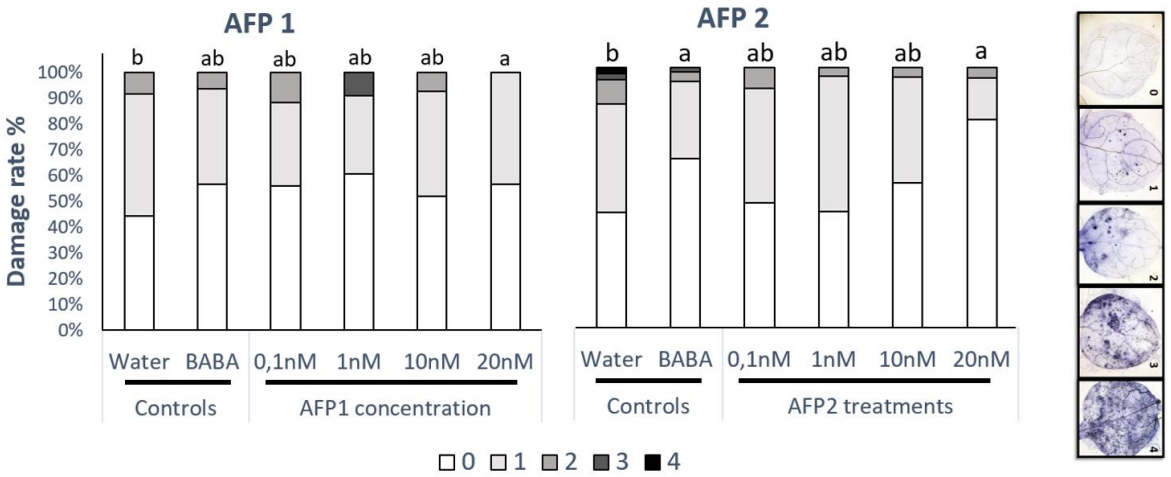
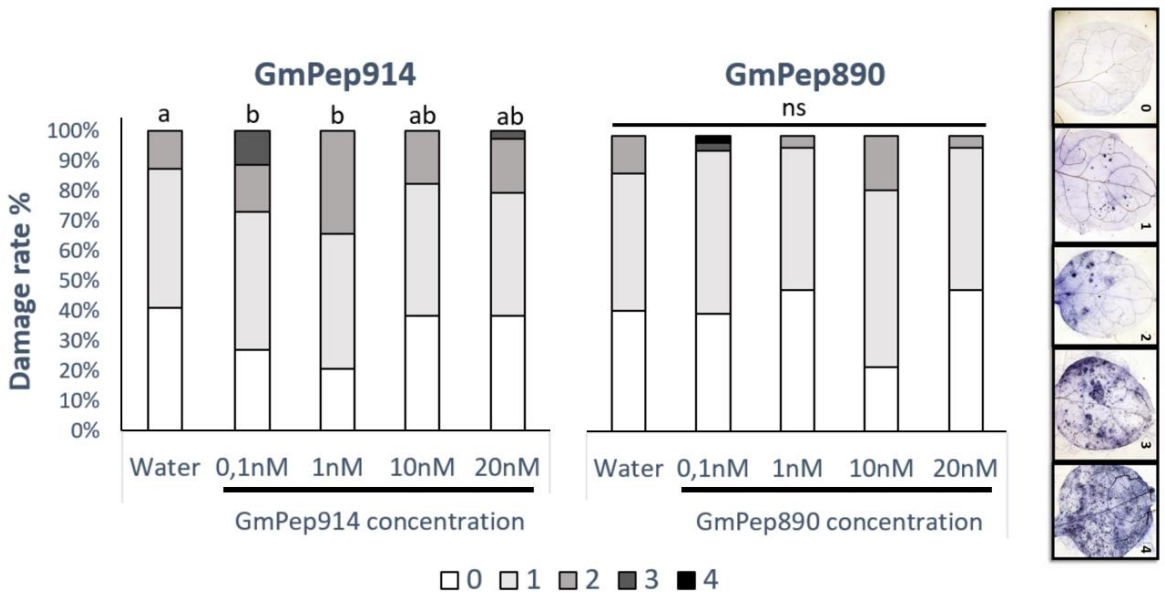
Supplementary information



Supplementary figure 1. Systemins from Solanaceous species induced-resistance assays against* Plectosphaerella cucumerina *in Arabidopsis plants. *Infection levels 5 days after inoculation quantified by a disease rating in trypan blue stained leaves, measured as a percentage of the infected leaf surface. Arabidopsis Col-0 plants were treated with increasing concentrations of PotSys (potato systemin), PepSys (pepper systemin), NishSys (nightshade systemin) (0.1nM, 1nM, 10nM and 20nM) 24h before infection with 1µl droplets of 5x10E3 spores/ml of* P.cucumerina *BMM. ß-amino butyric acid (BABA) at 1ppm was used as a positive control. Colours mean % of diseased leaves in a scale (0 = healthy leaves; 1= leaves with less than 25% of diseased surface; 2= leaves with 25-50%; 3= leaves with 50-75% of the diseased surface, 4= leaves with more than 75% of the surface diseased). Different letters indicate statistically significant differences (ANOVA, Fisher's Least Significant Difference (LSD) test; P < 0.05 n=6). The experiment had 6 plants per treatment and was repeated at least three times with similar results.*

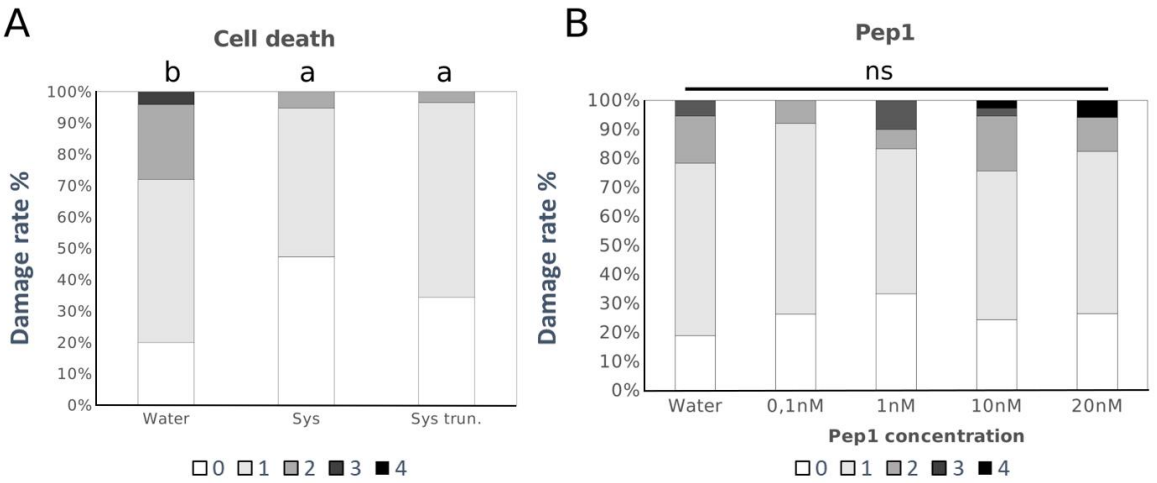


Supplementary figure 2. Antimicrobial peptides from radish induced-resistance assays against Plectosphaerella cucumerina in Arabidopsis plants. *Infection levels 5 days after inoculation quantified by a disease rating in trypan blue stained leaves, measured as a percentage of the infected leaf surface. Arabidopsis Col-0 plants were treated with increasing concentrations of AFP1 and AFP2 (0.1nM, 1nM, 10nM and 20nM) 24h before infection with 6ul droplets of 5x10E3 spores/ml of* P. cucumerina *BMM. ß-amino butyric acid (BABA) at 1ppm was used as a positive control. Colours mean % of diseased leaves in a scale (0 = healthy leaves; 1= leaves with less than 25% of diseased surface; 2= leaves with 25-50%; 3= leaves with 50-75% of the diseased surface, 4= leaves with more than 75% of the surface diseased). Different letters indicate statistically significant differences (ANOVA, Fisher's Least Significant Difference (LSD) test; P < 0.05 n=6). The experiment had 6 plants per treatment and was repeated at least three times with similar results.*

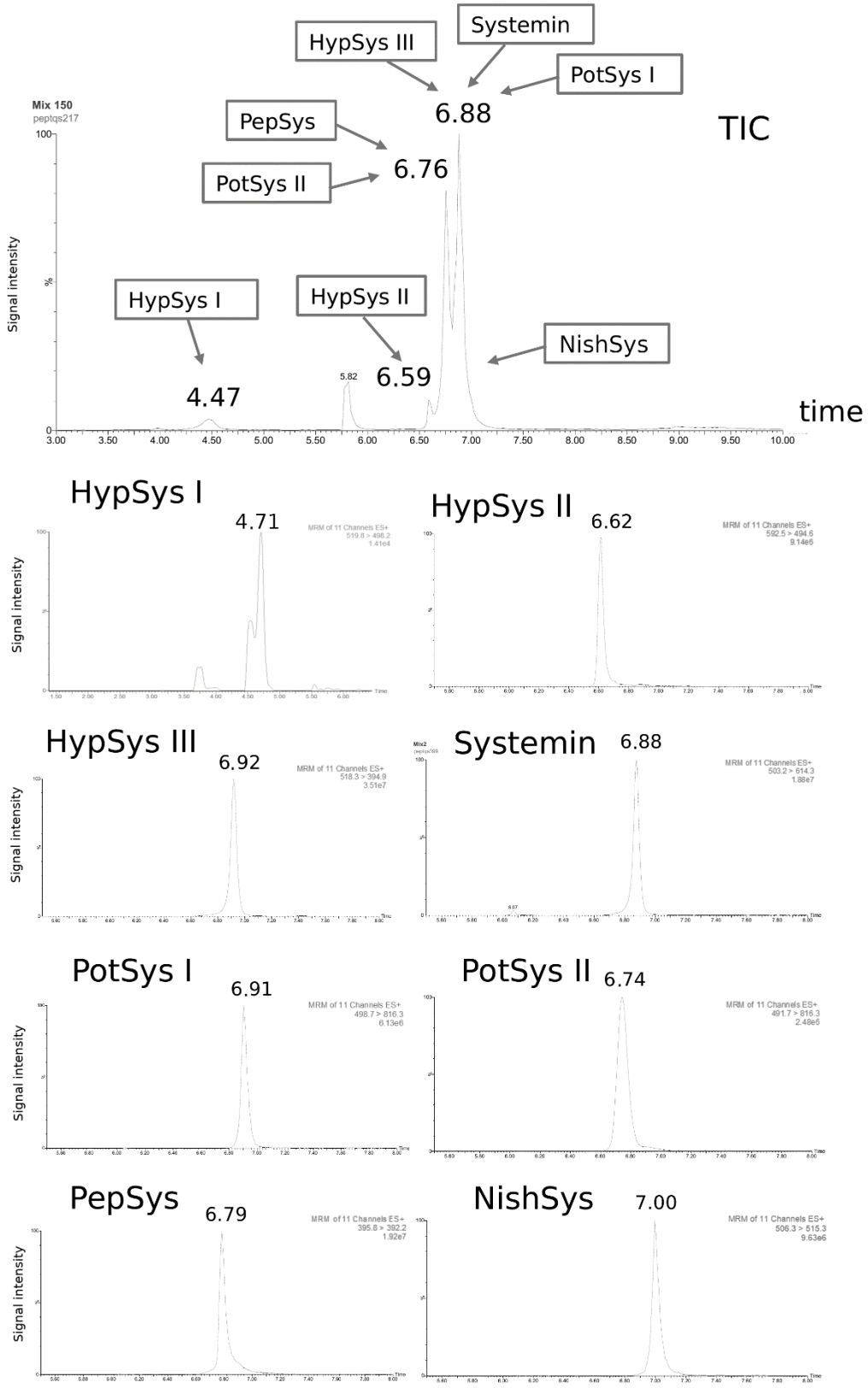


Supplementary figure 3. Soybean peptides induced-resistance assays against Plectosphaerella cucumerina in Arabidopsis plants. *Infection levels 5 days after inoculation quantified by a disease*

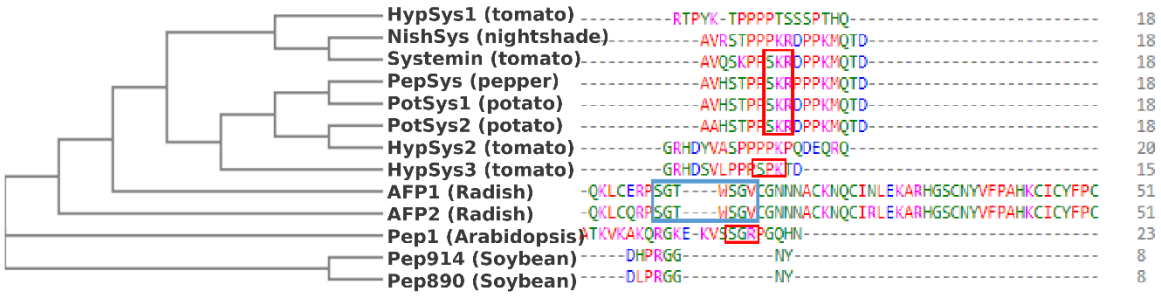
rating in trypan blue stained leaves, measured as a percentage of the infected leaf surface. Arabidopsis Col-0 plants were treated with increasing concentrations of GmPep914 and GmPep890 (0.1nM, 1nM, 10nM and 20nM) 24h before infection with 1µl droplets of 5xs10^3 pores/ml of P.cucumerina *BMM. Colours mean % of diseased leaves in a scale (0 = healthy leaves; 1= leaves with less than 25% of diseased surface; 2= leaves with 25-50%; 3= leaves with 50-75% of the diseased surface, 4= leaves with more than 75% of the surface diseased). Different letters indicate statistically significant differences (ANOVA, Fisher's Least Significant Difference (LSD) test; P < 0.05 n=6). The experiment had 6 plants per treatment and was repeated at least three times with similar results.*



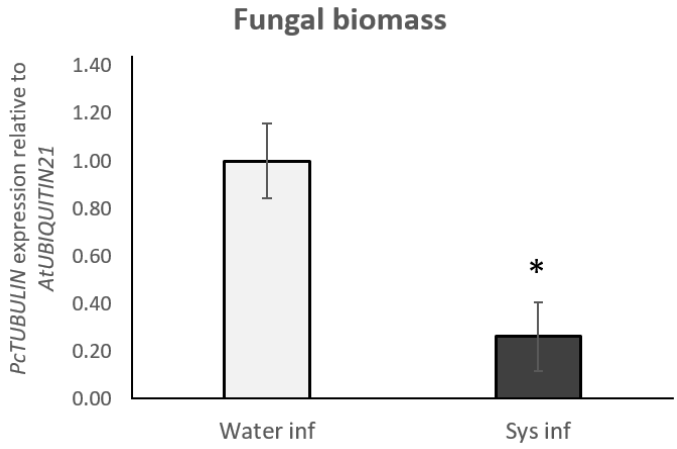
Supplementary figure 4. Induced- Resistance assays of Sys-P13AT17A in Arabidopsis and AtPep1 in tomato. *Infection levels of Arabidopsis Col-0 plants treated with 0.1nM of truncated Systemin (Sys-P13AT17A) (A) and tomato wildtype plants treated with increasing concentrations of AtPep1 (0.1nM, 1nM, 10nM and 20nM) (B) 24h before infection. Infection was quantified 5 days after inoculation with 1µl droplets of 5x10^3 spores/ml of* P.cucumerina *BMM by a disease rating in trypan blue stained leaves, measured as a percentage of the infected leaf surface. Colours mean % of diseased leaves in a scale (0 = healthy leaves; 1= leaves with less than 25% of diseased surface; 2= leaves with 25-50%; 3= leaves with 50-75% of the diseased surface, 4= leaves with more than 75% of the surface diseased). Different letters indicate statistically significant differences (ANOVA, Fisher's Least Significant Difference (LSD) test; P < 0.05 n=6). The experiment had 6 plants per treatment and was repeated at least three times with similar results.*



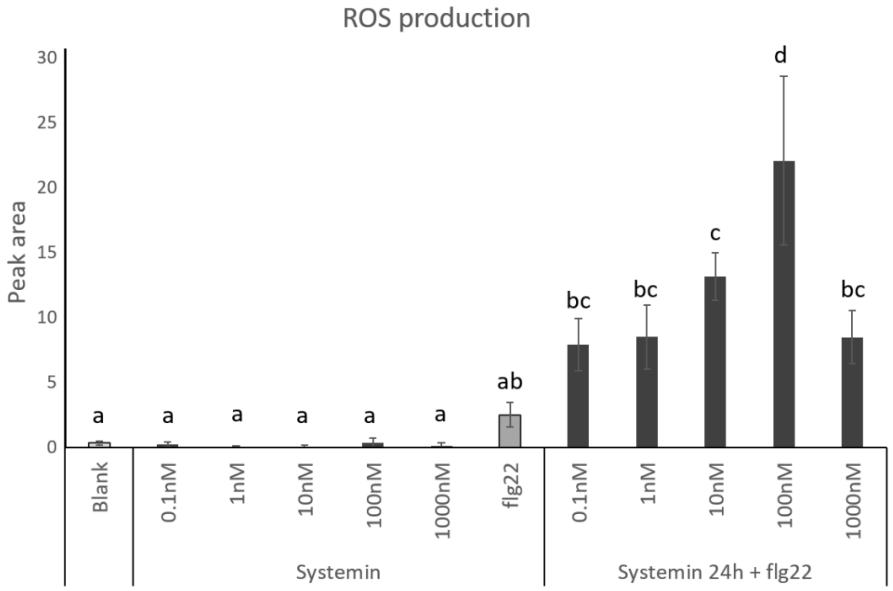
Supplementary figure 5. Peptides measured by HPLC-MS in planta. *A) total ion current (TIC) in ESI (+) of a mix of peptide standards and (B) HPLC–MS/MS chromatograms of specific transitions for each peptide of study detected in Arabidopsis plants 24h after peptide treatment.*



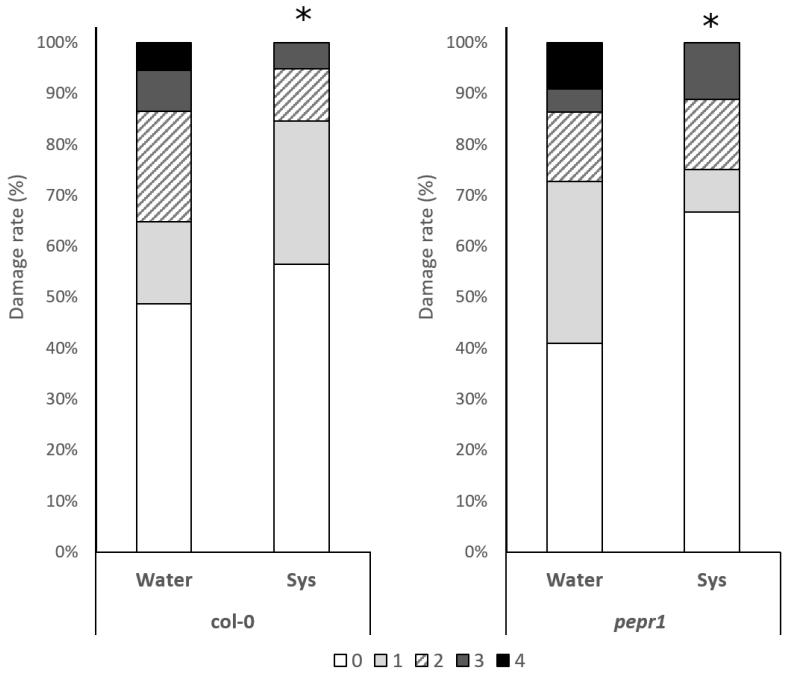
Supplementary figure 6. Peptides phylogenetic tree and multiple alignment based on their amino acid sequence. *Phylogenetic tree and multiple alignment were performed using the Clustal Omega multiple alignment of the EMBL-EBI online tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) using the peptides amino acid sequence provided by the Uniprot database. Numbers on the right indicate peptides' length (number of aminoacids). Highlighted in boxes are the motifs found in each peptide using the Prosite Database (http://wwwuser.cnb.csic.es/~pazos/cam97/). Red boxes indicate the Serine Protein Kinase C phosphorylation sites, blue box indicate N-myristoylation sites.*



Supplementary figure 7. P. cucumerina Infection quantification by measuring fungal biomass. *A ratio of PcTUBULIN relative to AtUBIQUITIN21 was calculated after performing a qPCR from gDNA of Arabidopsis infected plant samples 48h after pathogen inoculation in watered plants and plants treated with 0.1nM systemin 24h before inoculation of P.cucumerina. Bars represent mean ± standard error (SD), n=6. Different letters represent statistically significant differences. (ANOVA, Fisher's Least Significant Difference (LSD) test; $P < 0.05$ n=6)*



Supplementary figure 8. ROS production areas in response to Systemin and PAMP challenge. *H_2O_2 production was measured during 1h in leaf disks after elicitation with Systemin at different concentrations and with 100nM flg22 in leaf disks that were pre-treated for 24h with different concentrations of Systemin. Luminescence was expressed in Relative Luminescence Units. Bars represent means of peak areas ± standard error (SD), n=8. Different letters represent statistically significant differences. (ANOVA, Fisher's Least Significant Difference (LSD) test; $P < 0.05$ n=8).*



Supplementary figure 9. Sys-IR assays in the pepr1 mutant. *Col-0 and pepr1 plants were challenged with 1μl droplets of 5x10E3 spores/ml of* P.cucumerina *BMM 24h after treatment with 0.1nM Systemin. Infection levels were quantified 5 days after inoculation by a disease rating in trypan blue stained*

leaves, measured as a percentage of the infected leaf surface. Colours mean % of diseased leaves in a scale (0 = healthy leaves; 1= leaves with less than 25% of diseased surface; 2= leaves with 25-50%; 3= leaves with 50-75% of the diseased surface, 4= leaves with more than 75% of the surface diseased). Asteriscs mean statistial significant differences; T-test; P < 0.05 n=12). The experiment had 12 plants per treatment and was repeated at least three times with similar results.

Supplementary Table 1. *Primers used for the qPCR analysis of gene expression and* Plectosphaerella cucumerina *quantification.*

Accession number	**Primer name**	**Primer Sequence 5'-3'**
AT1G74710	ICS1	Fw - GCGTCGTTCGGTTACAGG
		Rv - ACAGCGAGGCTGAATCTCAT
AT3G45140	LOX2	Fw - TGATATCCGCGGCAGATCA
		Rv - CTACCGTAACCGCTGGTCAGT
AT5G44420	PDF1.2	Fw - TTCTCTTTGCTGCTTTCGACG
		Rv - GCATGCATTACTGTTTCCGCA
AT5G25760	UBIQUITIN21	Fw - GCTCTTATCAAAGGACCTTCGG
		Rv - CGAACTTGAGGAGGTTGCAAAG
AT1G13320	PP2A	Fw - TAACGTGGCCAAAATGATGC
		Rv - GTTCTCCACAACCGCTTGGT
AT4G33430	BAK1	Fw - TGACGGAATTGGTGAGCTTG
		Rv- TGTTATTAAGACGCAAGAAACGGAG
AT2G39660	BIK1	Fw - CTGGTAAGCGAGCGTTGGAT
		Rv - TGTCTAGCCGATTGTCCACG
MK164271	PcTUBULIN	Fw - CAAGTATGTTCCCCGAGCCGT
		Rv - GGTCCCTTCGGTCAGCTCTTC

Supplementary table 2. HPLC-MS method parameters to measure multiple peptides.

Compound	Sequence	MRM (m/z)	Cone voltage (V)	Collision energy (eV)	Calibration equation	R^2	Precision intraday	Precision interday	LOD (µg/L)	LOQ (µg/L)
HypSys I	RTOYKTOOOOTSSSOTHQ	519,8>498,2	20	15	665,508x - 4665,36	0.999606	0.51	3.52	1	10
HypSys II	GRHDYVASOOOOKPQDEQRQ	592,5>494,6	20	10	438,597x - 8603,13	0.991632	8.10	8.37	1	25
HypSys III	GRHDSVLOOSOKTD	518,3>394,9	20	15	6503,06x - 3585,99	0.994665	7.73	9.77	1	50
Systemin	AVQSKPPSKRDPPKMQTD	503,2>614,3	35	14	2209,26x - 71105,8	0.99275	4.24	6.87	1	50
PotSys I	AVHSTPPSKRDPPKMQTD	498,7>816,3	20	15	687,871x - 34622,1	0.9958	1.66	2.95	10	50
PotSys II	AAHSTPPSKRDPPKMQTD	491,7>816,3	20	20	175,734x - 6098,75	0.992069	0.70	0.95	10	50
PepSys	AVRSTPPPKRDPPKMQTD	395,8>392,2	20	10	3555,2x - 21017,3	0.991091	4.70	4.56	1	70
NishSys	AVHSTPPSKRPPPKMQTD	506,3>515,3	20	20	1125,93x - 65264,3	0.994275	5.85	7.68	10	50

CHAPTER 2

Tomato Systemin induces resistance against *Plectosphaerella cucumerina* in Arabidopsis through the induction of phenolic compounds and priming of tryptophan derivatives

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Abstract

Phytocytokines are endogenous danger peptides that are actively released after a pest or pathogen attack, triggering an amplification of plant immune responses. Here, we found that Systemin, a peptide from tomato, has a substantial impact at the molecular level in Arabidopsis plants that leads to induced resistance against *Plectosphaerella cucumerina*. Using transcriptional and metabolomics approaches, and loss-of-function mutants to analyse the molecular mechanisms underlying induced resistance against the necrotroph, we decipher the enhanced molecular responses in Systemin-treated plants following infection. Some protein complexes involved in the response to other danger signals, including the BAK1-BIK1 protein complex and heterotrimeric G proteins, as well as MPK activation, were among the early signalling events triggered by Systemin in Arabidopsis upon infection. Non-targeted analysis of the late responses underlying Systemin-Induced Resistance (Sys-IR) showed that phenolic and indolic compounds were the most representative groups in the Systemin metabolic fingerprint. Lack of flavonoids resulted in the impairment of Sys-IR. On the other hand, some indolic compounds showed a priming profile and were also essential for functional Sys-IR. Evidence presented here shows that plants can sense heterologous peptides from other species as danger signals driving the participation of common protein cascades activated in the PTI and promoting enhanced resistance against necrotrophic fungus.

Keywords: Arabidopsis, indolic compounds**,** induced resistance, phytocytokines, *Plectosphaerella cucumerina,* Systemin.

Introduction

Plants have evolved complex strategies to cope with biotic challenges, including physical and chemical barriers and a sophisticated immune system. Plants perceive pathogen attacks by recognizing non-self molecules that belong to specific microbes, called pathogen-associated molecular patterns (PAMPs), via pattern recognition receptors (PRRs) (Yu et al., 2017). The recognition of PAMPs leads to the PRRs associating or disassociating with their partner proteins to initiate the first line of inducible defence signalling, called pattern-triggered immunity (PTI) (Jones & Dangl, 2006). This first line

of defence often includes reactive oxygen species (ROS) burst, an increase in intracellular calcium and the activation of calcium-dependent and mitogen-associated protein kinases (CDPKs and MPKs) (Hou et al., 2019).

In addition to recognizing PAMPs, plants can also recognize self-derived molecules that are passively or actively released upon pathogen or pest invasion; these molecules are called damage-associated molecular patterns (DAMPs), and they trigger amplification of the immune signalling initiated by PAMPs to more rapidly counteract biotic challenges (Hou et al., 2019). Some of these self-derived molecules are peptides actively produced by damaged cells and bind to cell membrane receptors to amplify the immune response (Albert, 2013). They are considered secondary danger signals and are known as phytocytokines (Gust et al., 2017). The recognition of DAMPs and the initiation of immune responses often involve common protein complexes that recognize and activate immunity upon PAMP challenge since PRR signalling shares common molecular principles in response to a variety of elicitors (Saijo et al., 2018). The bacterial peptide flg22 is recognized by the Arabidopsis membrane leucine-rich repeat receptor-like kinase (LRR-RLK) FLS2 (Gómez-Gómez & Boller, 2000; Zipfel et al., 2004). FLS2 is coupled to the LRR-RLK BAK1, which acts as a coreceptor (Sun et al., 2013) and in turn forms a complex with the receptor-like cytoplasmic kinase (RLCK) BIK1 (Lu et al., 2010). Similarly, Arabidopsis plant elicitor peptide 1 (Pep1) is a phytocytokine that binds to membrane PEP RECEPTOR 1 (PEPR1) (Yamaguchi et al., 2006), which, like FLS2, is also coupled to the BAK1-BIK1 complex to initiate downstream signalling (Z. Liu et al., 2013).

The perception of both ligands (PAMP and DAMP) by the PRR-BAK1 complex leads to the activation of BAK1 and the phosphorylation of BIK1, which dissociates to initiate intracellular signalling (Lu et al., 2010). BIK1 interacts with and phosphorylates the NADPH oxidase RBOHD, leading to its activation and the production of a reactive oxygen species (ROS) burst (Kadota et al., 2014; Z. Liu et al., 2013). BIK1 promotes RBOHD association with heterotrimeric G-proteins, ensuring RBOHD activation upon flg22 perception by FLS2 (Liang et al., 2016).

Heterotrimeric G proteins are a group of GTP-hydrolysing proteins that in plants form a heterotrimer composed of 3 different subunits, Gα (encoded by GPA1), Gβ (encoded by AGB1) and Gγ (encoded by AGG1, AGG2 and AGG3), and they play a role in a variety of plant events, including plant defence against different pathogens (Liang et al., 2016;

Trusov & Botella, 2016). Upon perceiving specific ligands, the heterotrimer dissociates into two functional elements, the Gα subunit and the Gβγ dimer, which initiate downstream events (Pandey & Vijayakumar, 2018). Several studies have demonstrated the involvement of G proteins in downstream PRR signalling in immune responses, including the mediation of ROS production (Aranda-Sicilia et al., 2015; Bender & Zipfel, 2018; Liang et al., 2016; J. Liu et al., 2013). Some G proteins directly interact with the RLK receptors BAK1, CERK1 and BR1 (Aranda-Sicilia et al., 2015).

G proteins are also key elements in Arabidopsis resistance against necrotrophic pathogens such as *Plectosphaerella cucumerina*, *Alternaria brassicicola* and *Fusarium oxysporum* (Delgado-Cerezo et al., 2012; Llorente et al., 2005). The *agb1.1* mutant, which is defective in the Gβ subunit, shows increased susceptibility and impaired callose accumulation upon infection with *P. cucumerina,* whereas the *gpa1* mutant, which is defective in the Gα subunit, shows enhanced resistance to the pathogen (Llorente et al., 2005).On the other hand, the *agg1agg2* double mutant, which is defective in the Gγ subunit, is as susceptible to *P. cucumerina* as is *agb1.1* (Delgado-Cerezo et al., 2012).

As described above, endogenous DAMPs amplify and increase damage signals promoting cellular resistance (Gust et al., 2017). Despite these cellular defenses, the promotion of defense is sometimes too slow and weak to mount an efficient resistance. A few studies suggest the potential of non-self DAMPs to promote resistance in heterologous systems. Exogenous treatments with of OGs as primary DAMPs induce enhanced resistance responses (de Azevedo Souza et al., 2017), and seemingly a few studies suggest that exogenous DNA induces resistance and may trigger a enhanced defense status so called priming (Quintana-Rodríguez et al., 2018; Walters & Heil, 2007; Samuel W. Wilkinson et al., 2019). Using an adequate stimulus plants display super activation of specific defences (primed state of the plant) (Mauch-Mani et al., 2017), resulting in a clear benefit for plant fitness following a pathogenic or insect challenge (Martínez-Medina et al., 2016). The priming activity of phytocytokines as DAMPs with potential to induce resistance against fungal diseases is poorly understood and much less is known about the perception and signaling mechanisms of priming induced by phytoctokines.

However, the resistance of Arabidopsis to necrotrophic fungi is complex and involves not only early signalling events but also products of secondary metabolism. Tryptophan-derived metabolites, including indole-glucosinolates (IGs), indole carboxylic acids (ICAs) and the phytoalexin camalexin, are among the most important secondary

metabolites required for Arabidopsis resistance against *P. cucumerina* (Jordi Gamir et al., 2018; Kosaka et al., 2021; Sanchez-Vallet et al., 2010). Mutants in both the *CYP79B2* and *CYP79B3* genes, which are defective in the production of indolic-derived metabolites, show increased susceptibility to adapted and non-adapted *P. cucumerina* isolates (Sanchez-Vallet et al., 2010). Indole-3-carboxylic acid (I3CA) induces resistance against the same pathogen by priming callose accumulation, whereas it does not display antimicrobial effects (Jordi Gamir et al., 2018). CYP79B2 and CYP79B3 redundantly convert tryptophan to indole-3-acetaldoxime (IAOx), and from this point, the pathway branches through IG production, camalexin biosynthesis and the pathway leading to ICAs (Frerigmann et al., 2016). Recent studies suggest that two different enzymes separately contribute to the branching from IAOx to camalexin and ICAs: whereas CYP71A12 has been characterised as the enzyme required mainly for the biosynthesis of ICAs (Pastorczyk et al., 2020), CYP71A13 is involved in the camalexin biosynthetic pathways but not in ICAs production (Kosaka et al., 2021).

In a previous study, we demonstrated that the tomato peptide Systemin induces resistance against the necrotrophic fungus *P. cucumerina* in phylogenetically distant species such as *Arabidopsis thaliana* (Julia Pastor-Fernández et al., 2020). This result was consistent with a previous report showing that Arabidopsis overexpressing Systemin shows strong proteomic changes (Zhang et al., 2018). However, the signalling and molecular mechanisms that are triggered by Systemin in Arabidopsis remain unknown. In tomato, Systemin mediates plant defence against herbivores activating the production of protease inhibitors (PIs) (and & Pearce, 2003; Pearce et al., 1991) and the induction of volatile compounds that attract natural enemies (Corrado et al., 2007). Moreover, the overexpression of the Systemin precursor Prosystemin also confers resistance against pathogens through the activation of the JA pathway (Bubici et al., 2017; Coppola et al., 2015). Two LRR-RKs, SYR1 and SYR2, were recently described as Systemin receptors in tomato (Wang et al., 2018).

A recent study demonstrated that Prosystemin, the Systemin precursor, is an intrinsically disordered protein (IDP), especially in the Systemin region (Buonanno et al., 2018). IDPs are proteins that lack a stable and ordered three-dimensional structure and that may fold into different conformations, which allows them to interact promiscuously with many different partners (Dunker et al., 2008). Hence, the Systemin IDP may bind to nonspecific

receptors in different plant species, which may occur in Arabidopsis (Zhang et al 2018; Pastor-Fernández et a., 2020) and tobacco (Malinowski et al., 2009).

In the present study, we analysed the signalling components of Systemin-Induced Resistance (Sys-IR) at early stages, following the perception of Systemin in Arabidopsis after challenge with a necrotrophic fungus, analysing the implications of BAK1-BIK1 coreceptors, heterotrimeric G protein participation and kinase cascades following Systemin treatment and infection. In addition, we further studied the later events occurring at the metabolomic and transcriptional levels in response to peptide treatment in the absence and presence of pathogen challenge. The results indicate relevant roles for the flavonoid pathways and indolic compound derivatives for functional Sys-IR.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana Col-0 was used as the wild-type ecotype for all the experiments described. The following mutants in the *Col-0* background were utilised in this work: *tt4.2* and *tt4.11* (kindly provided by Gloria K. Muday; (Buer & Muday, 2004)), *agb1-1* (Llorente et al., 2005), *agg1agg2* (Trusov et al., 2006), *rbohd* (Torres et al., 2002), *mpk3* and *mpk6* (kindly provided by Uwe Conrath, Aachen University; (Beckers et al., 2009)), *cyp71B2cyp71B3, cyp71A13 and pad2.1* (kindly provided by Félix Mauch, University of Fribourg), *cyp71A12* (Pastorczyk et al., 2020), *pad3* (kindly provided Jurriaan Ton, University of Sheffield), *cyp71B6 (*SAIL_726_H11 and SALK_115336) *and aao1* (SALK_079567; (J. Pastor-Fernández et al., 2019)).

Arabidopsis seeds were sterilised with 70% ethanol for 30 seconds, followed by a 10% bleach solution for 15 min, and then washed 4-5 times with sterile distilled water to remove the sterilization solution. Sterile seeds were sown *in vitro* in 24-well plates in a medium containing 4.9 g/L Murashige and Skoog (MS; (Murashige & Skoog, 1962)) basal medium (Sigma), 1% sucrose and 6 g/L agar at pH 5.7. The seedlings were grown in a growth chamber with a 9 h light period at 24 °C and 15 h of darkness at 18 °C; a dark surface was placed beneath the plates.

Systemin and flagellin (flg22) treatment

Two-week-old plants were treated with a final concentration of 0.1 nM Systemin in a multi-well plate by adding the peptide, which had been dissolved in distilled water, to the agar medium. Control plants were treated with the same amount of sterilised distilled water.

For the MPK analysis via immunoblotting, we performed an overnight treatment with 100 nM Systemin as described above. The next day, the plants were sprayed with a solution of 1 ppm flg22. Then, the plants were sampled in a 60-min time course: at 0 h, 15', 30' and 60' after the PAMP challenge.

2.3. Pathogen inoculation and disease quantification

Twenty-four hours after the Systemin treatment, 4 leaves per plant were inoculated with $5x10^3$ spores/ml of *Plectosphaerella cucumerina BMM* via drop inoculation. Five days after infection, the plant leaves were sampled, and dead cells were stained using trypan blue as previously described (Flors et al., 2008). The infection levels were quantified using a disease rating, assigning numbers to different percentages of infected leaf surface (0 = healthy leaves; 1= leaves with less than 25% of diseased surface; 2= leaves with a diseased surface between 25-50%; 3= leaves with a diseased surface between 50-75%; 4= leaves with more than 75% diseased surface), according to previous reports (Agut et al., 2014; Slaughter et al., 2012; S. W. Wilkinson et al., 2018).

Protein extraction and Western blot analysis

Proteins were extracted from ground fresh plant tissue with a protein extraction buffer containing TBS, Triton, PMSF 1 mM, DTT 10 mM and a protease and phosphatase inhibitor cocktail (Sigma–Aldrich) that was added in a 1:3 ratio (100 mg:300 µl). Samples were vortexed for 15" and incubated on ice for 10 min. Then, the supernatant was collected after centrifugation (10 min at maximum speed). Laemmli sample buffer was added to the protein extract, and the mix was boiled for 5 min. The protein concentration was determined via the Bradford method using BSA as a standard.

A protein gel was prepared containing 4.5 ml of 12% resolving gel and 1 ml of 5% stacking gel. The gel was run for 1 h 30' at 100-150 V with an adjusted amount of 20 µg of protein in each well. One gel was stained with colloidal Coomassie stain (Bio-Rad) following the manufacturer's instructions to check the quality of the protein extract. Then,

another gel prepared under the same conditions was used for protein transfer to a nitrocellulose membrane and immunoblot analysis. Phospho-p42/p44 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Werfen) was used for immunodetection. Peroxidase-conjugated goat anti-rabbit IG (Sigma–Aldrich) secondary antibody was used for blotting, and the membranes were revealed using ECL Prime Western blotting Detection Reagents (Cytiva). The image analysis software GIMP 2.10.8 was used to compare band intensities.

RNA extraction and RT–qPCR analysis

Plant leaves were collected two days postinoculation (48 hpi), ground in liquid nitrogen and stored at -80 °C. For RNA extraction, 1 ml of TRIzol was added to 90 mg of powdered leaves. The supernatant was collected and transferred to a new tube after centrifugation, and 0.22 ml of chloroform ($CHCl_3$) was added. The samples were centrifuged again, and the supernatant was transferred into a new tube; 0.35 ml of isopropanol and 0.35 ml of 0.8 M citrate/1.2 mM NaCl were added and gently mixed. The supernatant was removed after centrifugation, and the pellet was washed twice with 70% EtOH. Finally, the pellets were dried and dissolved in nuclease-free water.

The RNA was cleaned using a DNAse kit (DNAse I, RNAse-free, Fisher Scientific). Then, cDNA was synthesized using a retrotranscription kit (PrimeScript RT reagent, Takara). Quantitative real-time PCR (qPCR) was performed using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific) and a StepOne instrument (Applied Biosystems). The ΔCt method was used to analyse the gene expression data. The housekeeping genes *UBIQUITIN21 (At5g25760)* and *PP2A (At1g13320*) were used to normalize the expression values. The primer sequences of measured genes are shown in Supplementary Table 1.

Non-targeted metabolomic analysis. LC-ESI full-scan mass spectrometry.

Two hundred milligrams of fresh ground leaf material was extracted at 4 °C with 1 ml of MeOH: H2O (30:90) containing 0.01% HCOOH. The supernatant was collected after centrifugation at full speed at 4 °C for 15 min and filtered through 0.2 μm cellulose filters (Regenerated Cellulose Filter, 0.20 μm, 13 mm D. pk/100; Phenomenex). Metabolomic analysis was performed by injecting 20 μl into an Acquity UPLC system (Waters, Milford, MA, USA) interfaced with a hybrid quadrupole time-of-flight instrument (QTOF MS Premier).

Subsequently, a second fragmentation function was introduced into the TOF analyser to identify the signals detected. This function was programmed in a t-wave ranging from 5 to 45 eV to obtain a fragmentation spectrum of each analyte (Agut et al., 2014; Jordi Gamir et al., 2014). Positive and negative electrospray signals were analysed independently to obtain a global view of the data. To elute the analytes, a gradient of methanol and water containing 0.01% HCOOH was used. Six independent biological replicates per sample were randomly injected. LC separation was performed using a 2.1 × 50 mm Kinetex C18 analytical column, 1.7 µm particle size, (Phenomenex). Chromatographic conditions and solvent gradients were set as described by (Agut et al., 2014; Jordi Gamir et al., 2014).

To accurately identify the metabolites, an internal library of plant metabolites was created with chemical standards. The specific retention time, exact mass, and spectrum fragmentation of the compounds in the library were characterized according to Schymanski *et al.* (Schymanski et al., 2014). Eighty-four compounds were prepared at a final concentration of 100 ppb in the same solution (Rivero et al., 2015). The standard solution was injected through the UPLC in positive and negative ion mode for electrospray ionization (ESI) while applying the same conditions used with the plant samples. For those compounds that were not represented in the library, the signals obtained in the non-targeted metabolomic analysis were assured by comparing the fragmentation spectra in the Massbank or Metlin databases (www.massbank.jp; www.masspec.scripps.edu).

Full-scan data analysis and bioinformatic processing.

Supervised principal component analysis (sPLSDA) and heat map analysis were performed using MetaboAnalyst software (https://www.metaboanalyst.ca/). Data acquired with Masslynx 4.1 software (Masslynx 4.1, Waters) in the raw format were transformed into .cdf files using the Databridge tool. Chromatographic signals were processed with R software (http://www.r-project.org/) for statistical purposes. Signals from positive and negative electrospray ionization (ESI+; ESI−) signals were analysed independently. The peak, grouping, and signal corrections were obtained using the XCMS algorithm (www.bioconductor.org) (Smith et al., 2006). The amounts of the metabolites were analysed based on the normalized peak area units relative to the dry weight. A nonparametric Kruskal–Wallis test (*$p<0.05$) was performed to test differences between treatments. Adduct, isotope correction, and clustering were executed with the Mar-Vis

Suit 2.0 software packages MarVis Filter and MarVis Cluster. To identify specific phenolic compounds, we searched in our self-built library of chemical standards and matched retention time, exact mass and fragmentation spectra with the data obtained in the metabolomic analysis.

Targeted HPLC–MS for indolic compound analysis

To analyse indoles, a targeted method modified from Sánchez-Bel (2018) (Sánchez-Bel et al., 2018) was implemented. Thirty milligrams of powdered freeze-dried material was homogenized with MeOH:H_2O (30:70) extraction buffer containing 0.01% HCOOH and 10 µg.l-1 indole acetic acid-d5 (IAA-d5). Each standard had been previously injected into the mass spectrometer to optimize the chemical-physical parameters (Supplementary Table 2). External calibration curves of each compound in a range from 1 to 150 µg.l-1 were injected under the same chromatographic conditions as the plant samples. The targeted analysis was performed on an Acquity ultra-performance liquid chromatography system (UPLC; Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK). The column used for LC separation was a UPLC Kinetex 2.6 µm EVO C18 100 Å, 2.1 x 50 mm (Phenomenex). The conditions and solvent gradients used in this chromatographic analysis were the same as those described in Sánchez-Bel *et al.* (2018) (Sánchez-Bel et al., 2018).

Statistical analysis

Bioassays of the mutants included 12 plants per treatment, 4 infected leaves per plant and were repeated at least three times. Graphs show the results of a mix of the three experiments. Statgraphics-plus software for Windows V.5 (Statistical Graphics Corp., MD, USA) was used to determine the statistical analysis via a Kruskal-Wallis test analyses (*$p<0.05$).

Gene expression and metabolomic experiments included 6 biological replicates per treatment and each replicate was a pool of 48 seedlings. Means are shown using bar graphs and are represented with standard error and their comparator. Statgraphics-plus software for Windows V.5 (Statistical Graphics Corp., MD, USA) was used to determine the statistical analysis via one-way analysis of variance (ANOVA, Fisher's least significant difference (LSD) test; $P < 0.05$, n=6). Different letters indicate statistically significant differences.

Results

BAK1 and BIK1, together with G proteins, are involved in Systemin signal transduction triggering Sys-IR.

The phytocytokine Systemin, which is produced by solanaceous plants, is perceived by and induces molecular changes in *Arabidopsis thaliana* that lead to enhanced resistance against pathogens (Julia Pastor-Fernández et al., 2020; Zhang et al., 2018). However, the early signalling events triggered upon Systemin perception remain elusive.

The gene expression of the correceptor *BAK1* and the receptor-like cytoplasmic kinase *BIK1* are induced by Systemin upon *P. cucumerina* infection (Julia Pastor-Fernández et al., 2020). To further confirm their relevance in Systemin-IR we compared both *bak1* and *bik1* knockout mutants with wild-type plants, which exhibited impaired Systemin-triggered protection (Figure 1), indicating the need for an intact BAK1 and BIK1 functioning in Sys-IR in Arabidopsis. This observation prompted us to characterize additional elements in the signalling cascade following the perception of Systemin.

Heterotrimeric G proteins are involved in PRR signalling in response to different stimuli (Liang et al., 2016) and interact with the BAK1 correceptor (Aranda-Sicilia et al., 2015). These proteins are key elements in defence against necrotrophic pathogens such as *Plectosphaerella cucumerina* (Delgado-Cerezo et al., 2012; Llorente et al., 2005). We tested whether they are relevant in the cellular early events of Sys-IR. Thus, gene expression and loss-of-function mutants of *AGB1*, *AGG1* and *AGG2* were analysed to determine whether the Gβγ dimer plays a role in Sys-IR against *P. cucumerina* in Arabidopsis (Delgado-Cerezo et al., 2012).

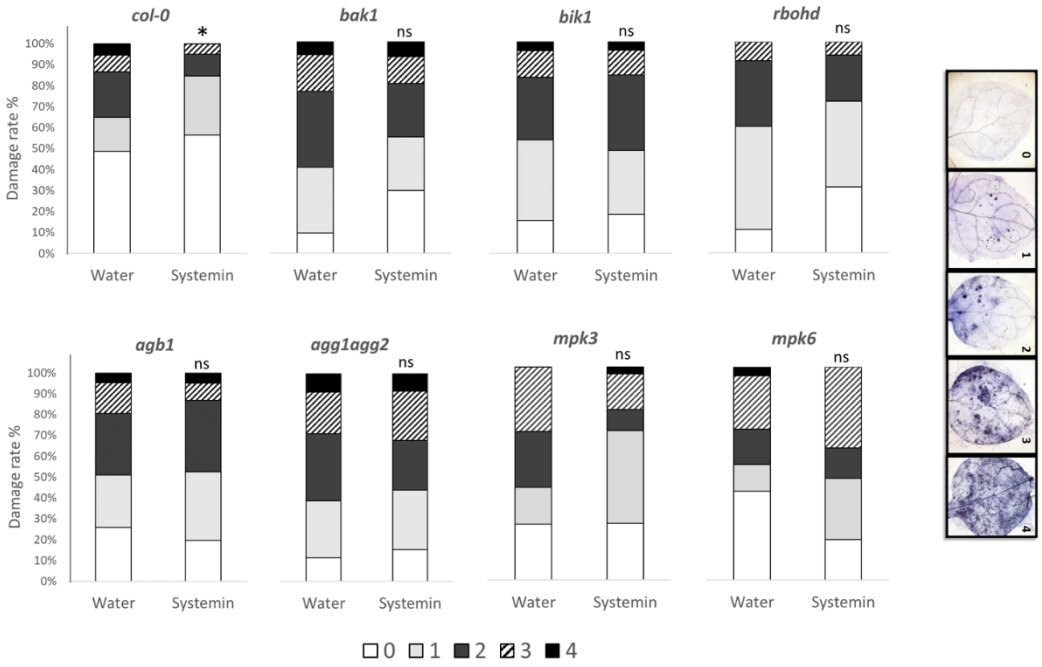

Figure 1. Screening of mutants in perception and PTI signalling elements in Sys-IR assays upon P. cucumerina infection*. Infection levels of normally watered (Water) and Systemin-treated plants (Systemin) quantified 5 days after infection (5x10^3 spores/ml) using a disease rating and trypan blue staining. Infection levels were measured using a scale based on the percentage of infected leaf surface (0 = 0%; 1= less than 25%; 2= 25-50%; 3= 50-75%, 4= more than 75%). Graphs represent averages of three experiments each including 12 plants and 4 infected leaves per plant. Asterisks indicate statistically significant differences performing Kruskal-wallis test (*P<0.05, n=36); "ns" indicates no statistically significant difference.*

AGG2 gene expression was significantly induced in Systemin-treated plants upon infection (Figure 2a), showing a priming profile. In addition, the expression level of *AGB1* in Systemin-treated plants after infection was significantly higher compared with the water-infected plants, whereas no significant differences were found in the AGG1 gene expression (Figure 2a). Both the *agb1* and then double *agg1agg2* knockout mutants were completely impaired in Sys-IR, showing the same levels of infection as the untreated controls (Figure 1). These results indicate that G proteins, and more specifically the Gβγ dimer, are essential for the initiation of defence signalling along with Systemin-IR.

Liang et al. (Liang et al., 2016) showed that G proteins are involved in RBOHD activation for ROS production. Notably, during signalling events following pathogen perception, BIK1 can directly phosphorylate and thus activate RBOHD (Z. Liu et al., 2013). We observed that G proteins and the BAK1, BIK1 are involved in Sys-IR in Arabidopsis and

that Systemin treatments prime ROS production following flg22 challenge (Julia Pastor-Fernández et al., 2020), collectively, we tested whether *RBOHD* affects Sys-IR against *P. cucumerina*. Systemin treatments significantly induced *RBOHD* gene expression following infection (Figure 2a). Accordingly, the *rbohD* mutant could not perceive Sys-IR against *P. cucumerina* (Figure 1) suggesting that RBOHD-mediated ROS production is a component in the early signaling in the Sys-IR.

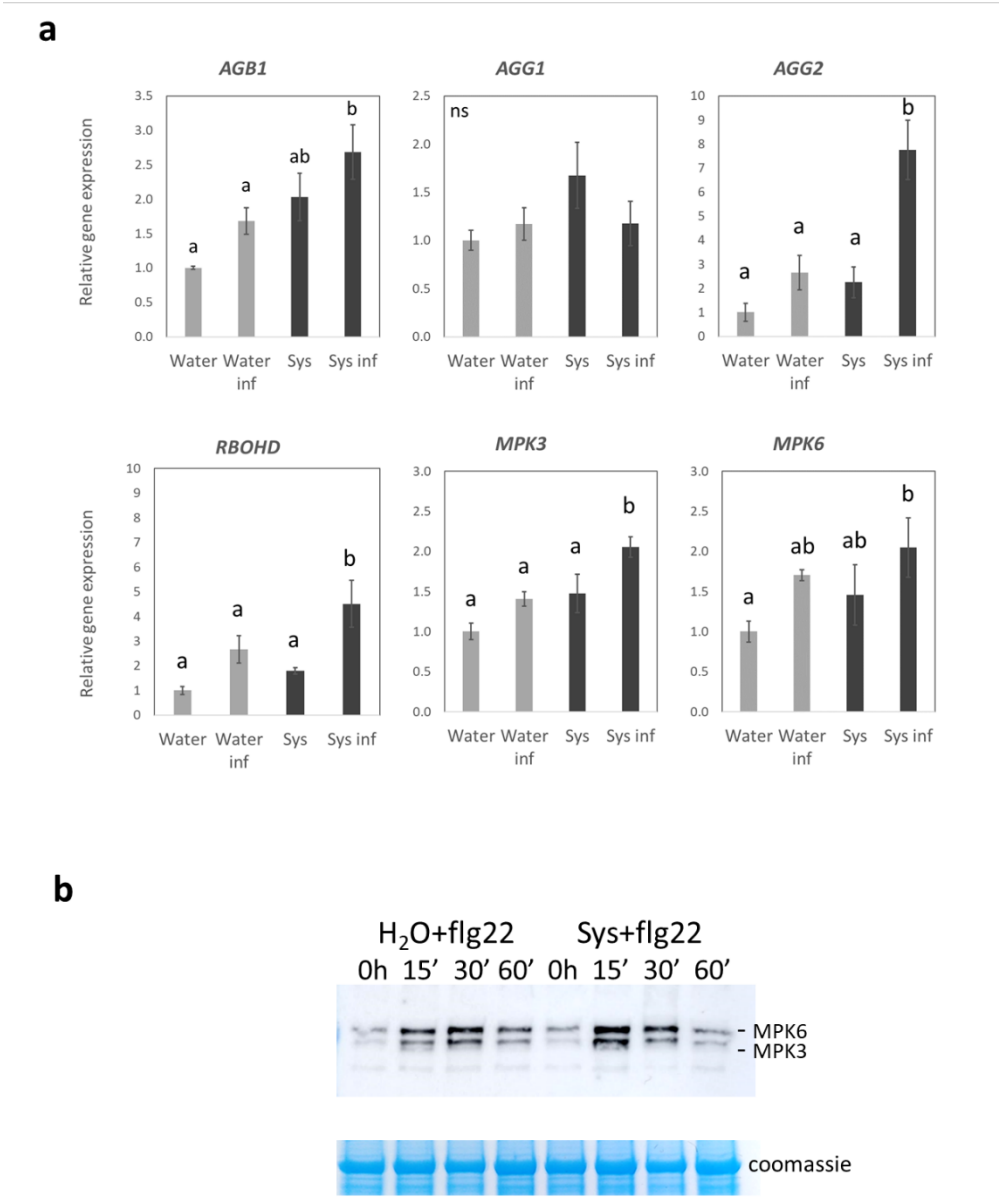

Figure 2. Gene expression analysis and protein quantification of perception and early signalling elements in Sys-IR assays. *(a) RT-qPCR analysis of AGB1, AGG1, AGG2, RBOHD, MPK3 and MPK6 gene expression in seedlings 24h after P. cucumerina infection with 5x10*^{3}

spores/ml (inf) either in normally watered (Water) or Systemin-treated (Sys) plants. Different letters indicate statistically significant differences (ANOVA, Fisher's least significant difference (LSD) test; P < 0.05 n=6). (b) WB of phosphorylated MPKs using the p42/44 antibody in Arabidopsis seedlings at 0 h, 15', 30' and 60' after challenge with 1 ppm flg22 solution 24h after water or treatment with 100 nM Systemin.

Systemin activates MAPKs upon *P. cucumerina* infection

MAPK cascades are a common signalling event that is activated during PTI in response to a variety of danger signals (Hou et al., 2019; Yu et al., 2017). Increase in the MPKs phosphorylation is part of the signalling elements triggered by Systemin after it binds its membrane receptor in tomato plants (Fürstenberg-Hägg et al., 2013; Xu et al., 2018). In accordance with this hypothesis, we tested whether MPK3 and MPK6 were implicated in Sys-IR, since both mediate defence priming responses in Arabidopsis (Beckers et al., 2009).

To determine the possible role of MPKs in Sys-IR in Arabidopsis, we conducted an experiment in which the *mpk3* and *mpk6* mutants were studied in Sys-IR bioassays against *P. cucumerina*. Both mutants were completely impaired in Systemin-IR (Figure 1). Accordingly, an analysis of gene expression patterns revealed that genes encoding both MPKs were induced in Systemin-treated infected plants, note that MPK3 showed a priming profile in Sys-treated plants upon infection (Figure 2a).

Finally, we analysed the levels of phosphorylated MPK3 and MPK6 proteins after a PAMP challenge in Systemin-treated and control plants by using a phospho-p42/p44 antibody. Plants treated with 100 nM Systemin were challenged with flg22 24 hpt in a 60-min time-course experiment. In the immunoblotting assay, the amount of phosphorylated protein was higher in Systemin-treated plants at 15' after the PAMP challenge for both MPK3 and MPK6 bands whereas non-treated plants showed a delay in the MPKs phosphorylation (Figure 2b and Figure S1). This result suggests that the Systemin treatment triggers stronger activation of MPKs, thus enhancing the PTI response. Collectively, the above-mentioned results indicate that MPK3 and MPK6 are relevant in Systemin-IR in Arabidopsis.

Systemin treatment induces a metabolomic rearrangement in Arabidopsis plants

To obtain a broader picture of the impact of Systemin in Arabidopsis plants, we performed a non-targeted metabolomic analysis of treated Arabidopsis plants 24 h after *P. cucumerina* infection by using a UPLC-QTOF MS instrument.

By performing sPLSDA using MetaboAnalyst_5.0 online software, we observed that Systemin itself had a substantial impact on the overall metabolomic profile, as its replicates grouped together and according to the second main component explaining variability, were separated from the water-treated plants (Figure 3a). After infection, the water- and Systemin-treated plant profiles grouped closer, although differences between the two treatments could be observed.

An ANOVA-LSD ($p<0.05$; MetaboAnalyst_5.0) study showed 429 signals statistically different. We built a heat-map analysis with these signals (Figure 3b). Firstly, the hierarchical clustering indicated that the infection was the main grouping criteria, however within each cluster there were strong differences in the accumulation of metabolites between water and Systemin-treated plants. Secondly, we selected a cluster compounds overaccumulated in plants treated with Systemin. A pathway classification of these compounds was performed based on Marvis tentative pathways assignment after dismissing the isotopes or adducts and based on the exact mass (Table 1). Using Masslynx 4.1, the presence of the compounds in the chromatogram and their differential accumulation was confirmed. This classification showed that phenylpropanoid pathway, flavonoid biosynthesis and tryptophan metabolism had the highest number of compounds. These pathways were previously linked to plant defence (Zaynab et al., 2018). The boxplots representing the relative amounts among the different treatments are shown in the Figure S2 for compounds ionized in positive ESI mode and in the Figure S3 in negative ESI mode.

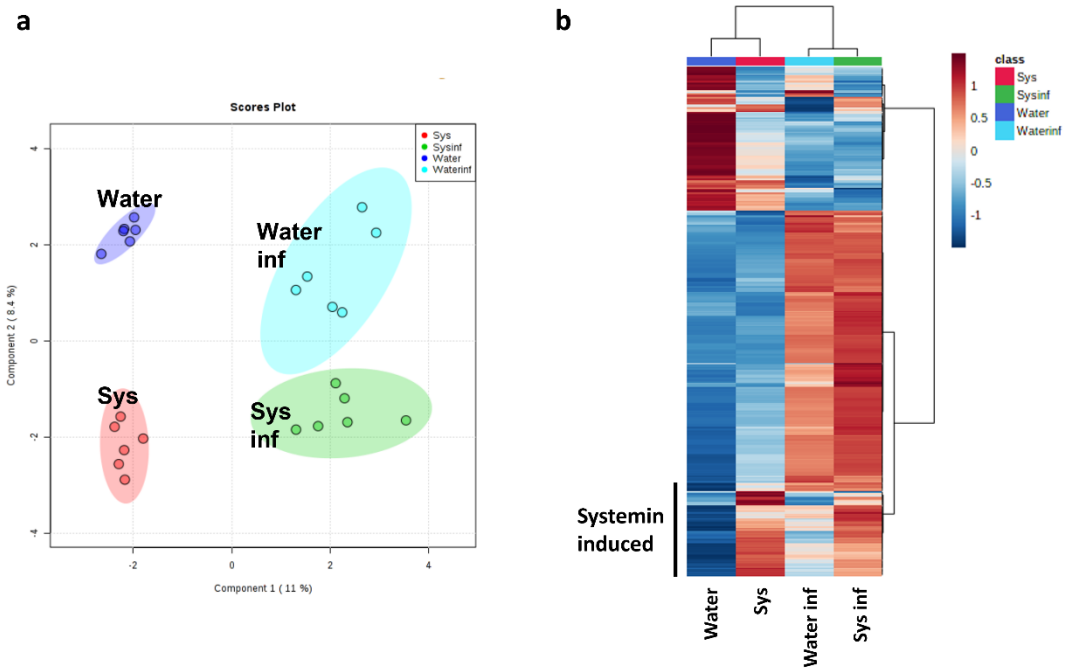

Figure 3. Metabolomic fingerprint of Systemin-treated plants upon P. cucumerina infection*. (b) Sparse partial least squares discriminant analysis (sPLSDA) and (b) heat map of metabolic compounds in Arabidopsis plants under four different conditions: normally watered plants (Water) and Systemin-treated plants (Sys) in either the absence or presence of infection (inf) with* $5x10^3$ *spores/ml P. cucumerina. The red colour in the heat map indicates over-accumulated compounds, and the blue colour indicates under-accumulated compounds. The black line indicates a selected cluster of compounds.*

Since phenylpropanoid pathway, flavonoid biosynthesis and tryptophan metabolism were the pathways with highest number of hits, we built dedicated internal libraries containing commercial standards of these pathways in order to get an absolute identification (Table S3 and S4). We could precisely identify the hydroxy-cinnamic acids ferulic acid and sinapic acid and the conjugate chlorogenic acid (Figure 4). Interestingly, all them showed increased accumulation in Systemin treated plants following infection. In fact, ferulic acid showed a priming profile, whereas the other two acids were already induced by the Systemin treatments. In addition, we also identified two flavonols, ampelopsin and catechin and the flavonoid glucoside naringin. All three compounds were induced by Systemin treatments suggesting a participation in the induced resistance (Figure 4). To further confirm this hypothesis, we performed a bioassay with the mutant *tt4* impaired in the chalcone synthase, an upstream step in the flavonoid biosynthesis (Buer and Muday, 2004). As expected, the mutant did not perceive Sys-IR (Figure 5).

Table 1. Neutral masses and tentative pathways of compounds belonging to a cluster with Systemin-induced profile in the metabolomic analysis.

Marker ID	Neutral mass	ion mode	Tentative pathway
66	214.0908	ESI+	Phenylalanine metabolism
126	338.1115	ESI+	Phenylpropanoid biosynthesis
117	208.0391	ESI-	Phenylpropanoid biosynthesis
218	631.2347	ESI-	Phenylpropanoid biosynthesis
242	610.1786	ESI+	Flavonoid biosynthesis
108	302.0148	ESI+	Flavonoid biosynthesis
36	354.1049	ESI-	Flavonoid biosynthesis
469	320.1377	ESI-	Flavonoid biosynthesis
569	290.1037	ESI-	Flavonoid biosynthesis
38	450.1178	ESI-	Flavonoid biosynthesis
12	340.1010	ESI-	Flavonoid biosynthesis
219	592.1546	ESI+	Flavone and flavonol biosynthesis
85	218.1300	ESI+	Tryptophan metabolism
191	221.1127	ESI+	Tryptophan metabolism
54	158.0594	ESI+	Tryptophan metabolism
555	179.0821	ESI+	Tryptophan metabolism
215	190.0859	ESI-	Tryptophan metabolism
21	189.1108	ESI-	Tryptophan metabolism
240	276.1752	ESI+	Biosynthesis of aminoacids
339	202.0920	ESI+	2-Oxocarboxylic acid metabolism
171	234.0763	ESI+	2-Oxocarboxylic acid metabolism
421	294.1824	ESI+	alpha linolenic acid metabolism
192	518.1374	ESI+	Antocyanin biosynthesis
361	712.1691	ESI+	Antocyanin biosynthesis
10	152.0500	ESI-	Alkaloid biosynthesis
13	480.250-5	ESI-	Alkaloid biosynthesis

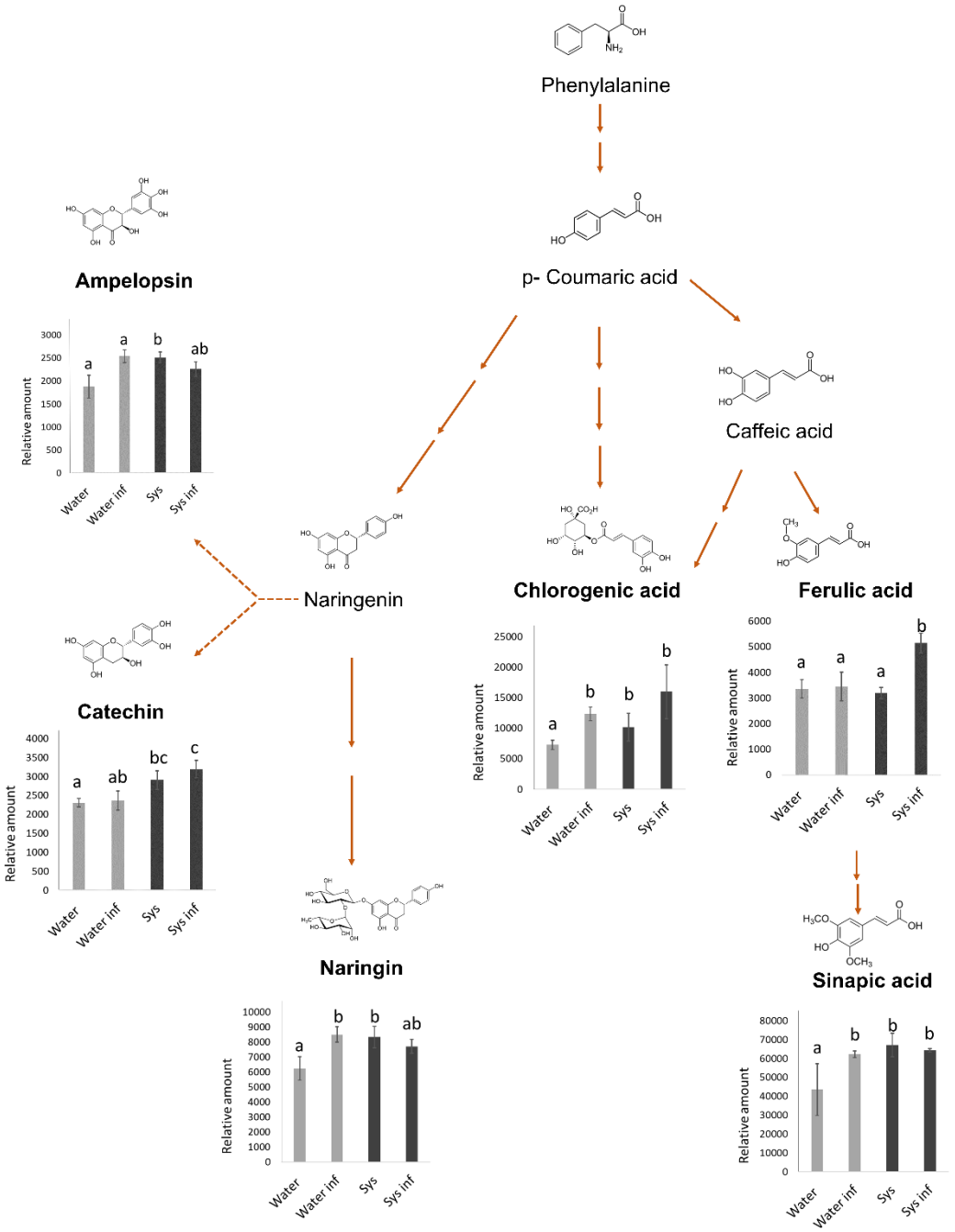

Figure 4. Profiling of phenolic compounds induced by the Systemin treatment. *Relative amounts of phenolic compounds that were induced by the Systemin treatment identified from the metabolomic analysis using internal libraries. Pathway representation was based on KEGG online database. Dotted arrows indicate multiple metabolic steps; solid arrows indicate single steps. Different letters indicate statistically significant differences (ANOVA, Fisher's least significant difference (LSD) test; $P < 0.05$ n=6).*

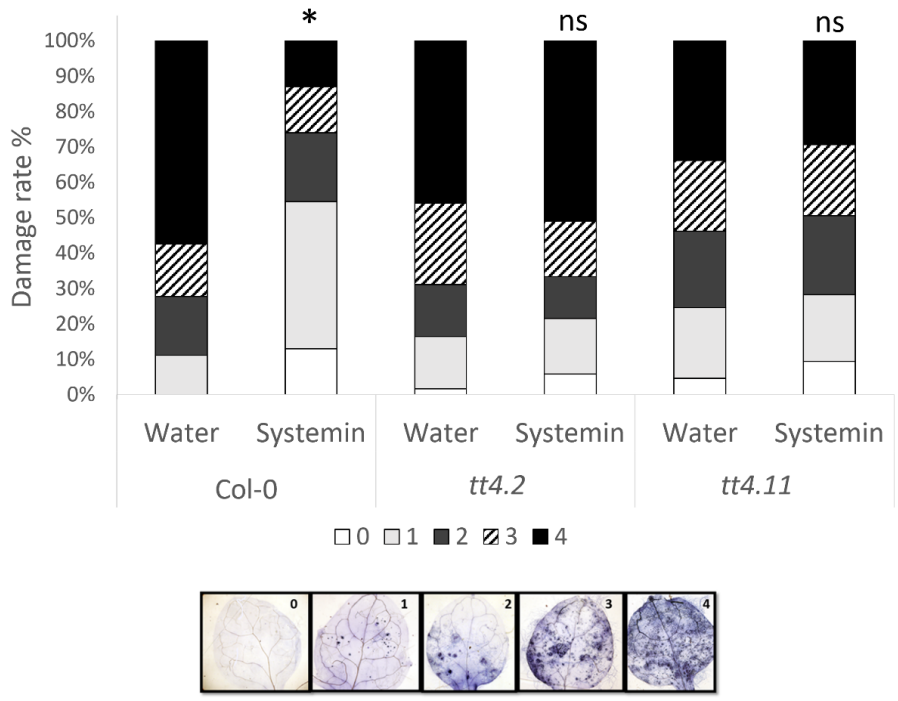

Figure 5. Sys-IR is not functional when flavonoids' biosynthesis is impaired*. Phenotyping of two alleles of tt4 knockout mutants imapired in flavonoids' biosynthesis.Infection levels of normally watered (Water) and Systemin-treated plants (Systemin) quantified 5 days after infection (5x10^3 spores/ml) by a disease rating using trypan blue staining. Infection levels were measured as a percentage of infected leaf surface using a scale (0 = 0%; 1= less than 25%; 2= 25-50%; 3= 50-75%, 4= more than 75%). Graphs represent averages of three experiments each including 12 plants and 4 infected leaves per plant. Asterisks indicate statistically significant differences performing a Kruskal-wallis test (*p<0.05, n=36).*

Following a similar procedure, we combined the precise identification from the metabolome using internal libraries (Table S4) and we also generated a targeted method for indole-3-acetonitrile (IAN), indole-3-acetamide (IAM), camalexin, indole acetic acid (IAA) and indole-3-carboxylic acid (I3CA) measurement (Table S2). We found that, from all the identified compounds, IAM, IAN, I3CA, I3CA methyl ester and indole-3-acetic acid-L-aspartic, were overaccumulated in Systemin treated plants following infection indicating their likely relevance in Sys-IR (Figure 6). Alternatively, to determine the relevance of the indole-glucosinolate branch we performed a search of several glucosinolates from the metabolome by using exact mass and spectral databases

(metlin and pubchem). Two indole-glucosinolates were identified (Table S5), but neither indole-3-ymethyl glucosinolate (I3M) nor 1-methoxy-I3M showed significant changes regarding Systemin treatment (Figure S4). To further understand the relevance of the Trp derivatives in Sys-IR we analysed the gene expression and performed a mutant screening of relevant genes in the biosynthesis of the above-mentioned Trp derivatives. Systemin primed *CYP71A13*, *CYP71B6* and *AAO1* gene expression following infection by *P. cucumerina* (Figure 6), however we did not observe significant changes in *CYP79B2*, *CYP79B3*, *CYP71A12* or *PAD3*. Mutant screening confirmed the relevance of *CYP71A13, CYP71B6* and *AAO1* in Sys-IR since none of them showed enhanced resistance with the treatments (Figure S5). Despite the lack of significance in the gene expression, the mutants *cyp79B2/3*, *cyp71A12* and *pad3* were also impaired in Sys-IR (Figure S5) revealing that an intact indolic derivatives pathway is needed to display protection induced by the peptide.

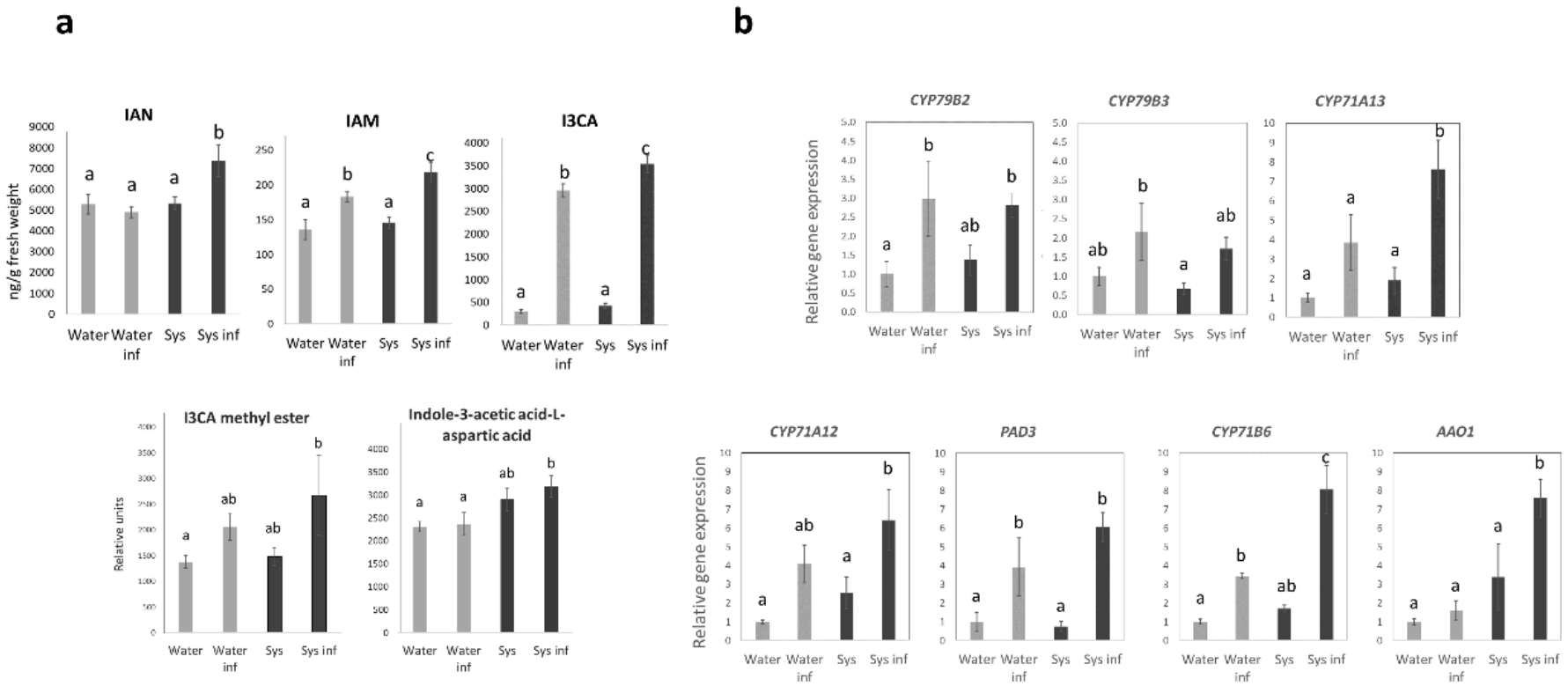

Figure 6. The priming effect of Systemin on the tryptophan-derived pathway against necrotrophic fungal infection. *Bar graphs showing (a) the quantification of indolic compounds and (b) tryptophan-derived compounds-related genes expression profiles of normally watered (Water) and Systemin-treated plants (Systemin) in either the absence or presence of infection(inf) with* $5x10^3$ *spores/ml of P. cucumerina (inf). Different letters indicate statistically significant differences (ANOVA, Fisher's least significant difference (LSD) test;* $P < 0.05$ *n=6).*

Discussion

Previously, we reported that the phytocytokine Systemin produced by *Solanum lycopersicum* is perceived and it induces resistance in Arabidopsis against the necrotrophic fungus *P. cucumerina* (Pastor-Fernández et al., 2020). Furthermore, treatments with this peptide induced the priming of H_2O_2 after challenge with the PAMP flg22. This result clearly indicates that Arabidopsis perceives heterologous peptides; however, the perception, signalling cascades and downstream mechanisms of peptide-induced resistance remains unexplored. In the present study, we show that early signalling of Sys-IR in Arabidopsis is mediated by the co-receptor kinases BAK1 and BIK1 and heterotrimeric G proteins AGB1 and AGG2. MPK3 and MPK6 are also relevant elements in the downstream responses mediating Sys-IR; they are part of the second layer of signalling following Systemin perception. Systemin induces a metabolomic reorganization, and it induces several hydroxycinnamic acids, a flavonoid and two flavonols, and primes some specific Trp derivatives. Collectively, we have identified several elements in the Systemin signalling cascade and downstream responses mediating induced-resistance to a necrotrophic pathogen. Thus, this phytocytokine, when treated in advance, magnifies defensive responses following pathogen perception in Arabidopsis.

In the present research, we observed that Systemin perception is assisted by the BAK1-BIK1 complex since loss-of-function mutants are impaired in Sys-IR. The expression of BAK1 and BIK1 was previously shown to be primed by the peptide against *P. cucumerina* (Julia Pastor-Fernández et al., 2020). Since the Systemin sequence has an inherently disordered character, it may be able to adapt its conformation to bind multiple partners (Buonanno et al., 2018). In fact, in tomato, in addition to its two physiological receptors, SYR1 and SYR2 (Wang *et al.*, 2018), other Systemin interactors have been described, such as SR160 (Scheer et al., 2003) and PORK1 (Xu et al., 2018). Despite being specifically produced by tomato, Arabidopsis (Pastor-Fernández et al., 2020; Zhang et al., 2018) and tobacco (Malinowski et al., 2009) sense Systemin when treated exogenously. Based on previous observations, we speculate that a specific Systemin receptor in other plant species is unlikely.

In the present study, we observed that in addition to the BAK1a and BIK1, Systemin-IR is also assisted by other components of early defence signalling, such as ROS and MAPKs, when combined with PAMP treatments. Similarly, Arabidopsis Peps, PIPs, and

the recently described SCOOP12 have been shown to induce rapid ROS bursts in a BAK1-dependent manner (Gully et al., 2019; Hou et al., 2014; Rhodes et al., 2021; Tang et al., 2015). PIPs were also shown to enhance flagellin-triggered immunity (Hou et al., 2014).These observations suggest that protein complexes involved in perception and signalling, as well as interactions, may respond unespecifically to different phytocytokines even those non-produced by Arabidopsis .

The present study shows that the G protein subunits Gβ1, Gγ1 and Gγ2 mediate Sys-IR in Arabidopsis. G proteins participate in plant defence responses against different challenges and function as a convergence point between RLKs and downstream signalling. In fact, the Gγ1 and Gγ2 subunits directly interact with BAK1 and CERK1, among other receptors (Aranda-Sicilia et al., 2015). Heterotrimeric G proteins are required for PRR-mediated responses and RBOHD activation for ROS production upon flg22, elf18 and chitin challenge (Ishikawa, 2009; Liang et al., 2016; J. Liu et al., 2013). Regarding the pathogen response, Gβ1 and both Gγ1 and 2 are essential for resistance against necrotrophic fungi, such as *A. brassicicola*, *F. oxysporum* and *P. cucumerina*, since their loss-of-function mutants show increased susceptibility to these pathogens (Delgado-Cerezo et al., 2012; Llorente et al., 2005). However, to our knowledge, there are no previous reports on G proteins mediating peptide-induced defence signalling.

Although it is still unclear whether there is a direct interaction between heterotrimeric proteins and MPKs in plant immunity, an interaction between the Gβγ protein AGB1 and MPK3 and MPK6 was reported in zygote development (Yuan et al., 2017). We did not research such a direct interaction, but both events are equally essential to express Sys-IR since *agb1* or *agg1agg2* and *mpk6* or *mpk3* KO plants are impaired in induced resistance against *P. cucumerina*. Furthermore, we observed an early phosphorylation of MKP3/6 when Systemin treatment was followed by a PAMP treatment.

To further understand downstream defence responses triggered by Systemin in Arabidopsis, we performed a non-targeted analysis of the Systemin-responsive metabolome in the absence and presence of infection. This analysis showed that Systemin treatment has a substantial impact on the plant metabolomic profile, suggesting that it does not function mainly through defence priming in Arabidopsis but rather produces direct changes following treatment (Mauch-Mani et al., 2017). Among the selected cluster of metabolites changed under our experimental conditions, Systemin treatment triggered a modification in the levels of hydroxyphenolic acids and their derivatives in the absence

of infection. Phenylpropanoids, including hydroxycinnamic acids and flavonoids, are among the main groups of secondary metabolites involved in plant defences (Zaynab et al., 2018). This group includes many compounds with different modes of action, including compounds with antifungal activity or phytoalexins against herbivores (Zaynab et al., 2018). In Arabidopsis, phenylpropanoids have been shown to participate in defence against the necrotrophic fungus *Botrytis cinerea* via ethylene-mediated immune responses (Lloyd et al., 2011). Notably, many hydroxycinnamic acids are precursors of flavonoid and flavonol phytoalexins. Ferulic acid, caffeic acid and sinapic acid, were induced by Systemin treatment, and consistent with this observation, we also found that the flavonols ampelopsin and catechin and the flavonoid glycoside naringin were over accumulated following treatment, whereas the tt4 mutant, impaired in this pathway did not show Systemin-IR. Attending to these observations, Systemin-triggered accumulation of hydroxycinnamic acids and flavonoids may prepare the plant for a future attack by accumulating antifungal compounds that may be detrimental to fungal infection.

The second pathway with a highest number of hits overaccumulated following treatment with Systemin was the Trp metabolism. The cytochrome P450s participate in the conversion of Trp in indole-3-acetaldoxime that is further processed generating other indolic compounds. These indole-derivatives play an essential role in defence against necrotrophic fungi (Delgado-Cerezo et al., 2012; Jordi Gamir et al., 2018; Pastorczyk et al., 2020). The I3CA methyl ester, indole-3-acetic acid-L-aspartic acid, indole-3-acetonitrile (IAN), indole-3-acetamide (IAM), camalexin and indole-3-carboxylic acid (I3CA) indolic compounds were found in the untargeted analysis and were further confirmed using pure chemical standards. These compounds were overaccumulated in Systemin-treated plants following infection. In a previous study, the indolic compound I3CA was found in the priming fingerprint signals against *P. cucumerina* in Arabidopsis using different priming stimuli (Gamir et al., 2012; Gamir et al., 2014). A later study revealed that I3CA itself could induce resistance against *P. cucumerina* by priming callose (Gamir et al., 2018). Transcription analyses of the *CYP79B2* and *CYP79B3* genes and mutant bioassays, demonstrated that the intact functioning of these genes is needed to express Sys-IR. These genes redundantly participate upstream of the indolic pathway. Alternatively, Systemin treatments primed the expression of the downstream genes *CYP71A13, CYP71B6* and *AAO1*, and all the mutants in genes downstream of IAOx and IAN were impaired in Sys-IR, indicating that a nearly intact pathway is needed to display

Systemin-IR. Note that we cannot exclude that a fraction of I3CA comes from the decomposition of indole-3-carbonyl nitriles during the extraction procedure in a water:methanol buffer (Rajniak et al., 2015). In addition, the phytoalexin camalexin, that displays known antimicrobial activity (Glawischnig, 2007; Schlaeppi et al., 2008; Schlaeppi & Mauch, 2010) contributes to the Systemin-IR since, the mutant *pad3* impaired in camalexin biosynthesis is insensitive to Systemin protection.

Although herein we demonstrate that Systemin enhances components of both PRR-mediated defence and downstream defence responses (secondary metabolites), thus far, there is no evidence of a link between the two layers of defence. It has been suggested that they function independently. For example, G protein-mediated defences are not dependent on other common mechanisms against necrotrophic fungi, such as hormones or tryptophan-derived metabolites (Delgado-Cerezo et al., 2012). On the other hand, a very recent study reported that indolic activation is BAK1 independent since the accumulation of these compounds was not impaired in the *bak1* mutant (Kosaka et al., 2021). Notably, in some cases, these studies were performed in a *pen2* background to generate plants susceptible to *Alternaria brassicicola*. In our experimental conditions *bak1* and mutant *bik1* were both impaired in Sys-IR. Conversely, BIK1 is a convergence point for multiple PRR pathways, and in addition to BAK1, it interacts with CERK1 and phosphorylates RBOHD (Couto & Zipfel, 2016), indicating that alternative signalling cascades to BAK1 may be also relevant for Sys-IR.

These results suggest that despite a specific receptor being unlikely, Systemin boosts the mechanisms of defence in Arabidopsis against necrotrophic fungi, inducing a subset of metabolic responses to effectively cope with adapted *P. cucumerina* infection. Collectively, we provide evidence on some aspects of the early signalling of Systemin in Arabidopsis, as well as downstream induced defence responses upon *P. cucumerina* infection (Figure 7). When the plant is treated with Systemin, Systemin is perceived by one or more unknown receptor(s) that may be coupled to heterotrimeric G proteins and the BAK1-BIK1 complex. These protein complexes are activated upon pathogen challenge to mount a first layer of defence (Figure 7). On one hand, BIK1 likely dissociates to phosphorylate RBOHD and trigger a rapid ROS burst (Julia Pastor-Fernández et al., 2020) . On the other hand, MAPKs activation is enhanced by Systemin upon infection. Subsequently, the plant initiates downstream signalling to mount a second layer of defence that includes defence gene activation and metabolomic rearrangement.

Phenolic compounds accumulate upon Systemin treatment before challenge, which may contribute to enhanced resistance (Figure 7). However, several hydroxycinnamic acids, the flavonols apigenin and catechin together with the flavonoid glycoside naringin and the indolic derivatives camalexin, IAN, IAM, and I3CA participate in Sys-IR (Figure 7), resulting in a chemical active defence response and contributing to a more resistant phenotype against the necrotrophic pathogen.

Concluding, although more research is needed, we have shown that a heterologous peptide can be sensed by Arabidopsis; it enhances defence responses and amplifies signalling cascades and secondary metabolism-dependent defences. The research of perception of phytocytokines by heterologous species may be extended beyond model plants to wide knowledge in the field of more sustainable crop protection.

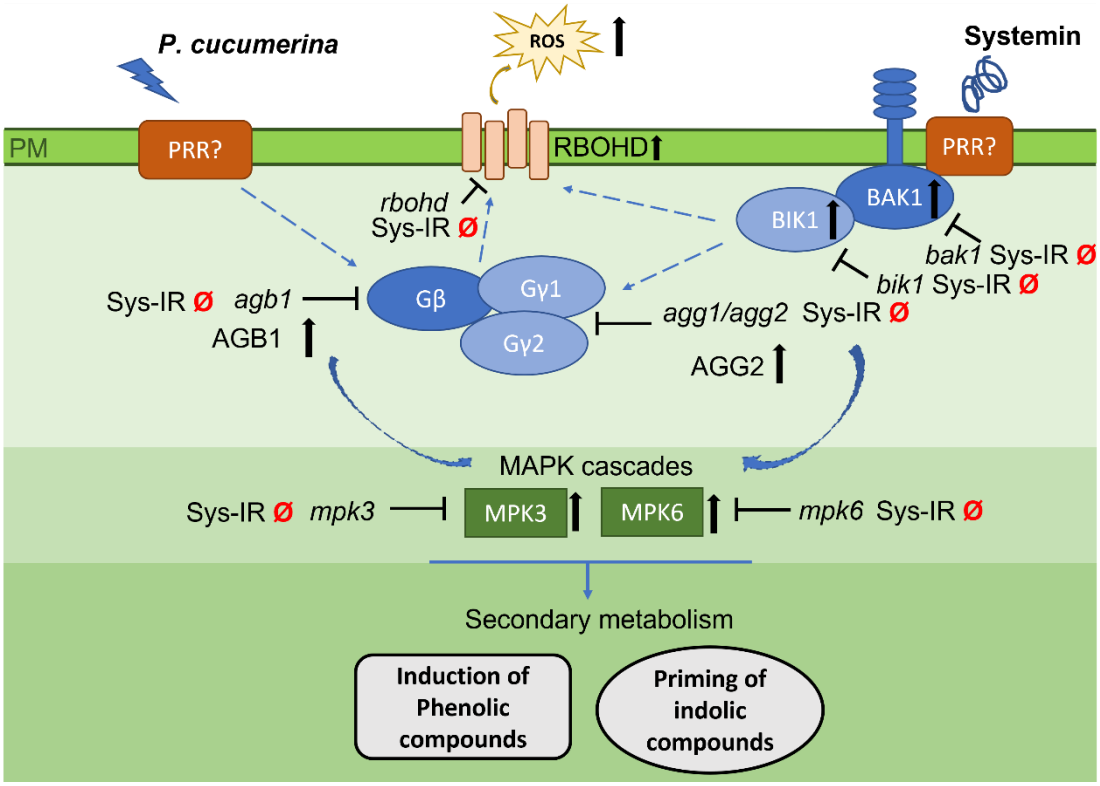

Figure 7. Systemin acts as an amplifier of defence responses against P. cucumerina in Arabidopsis plants. *Systemin is perceived by one or more unknown receptor(s) that may be coupled to heterotrimeric G proteins and, via either the BAK1-BIK1 complex or BIK-independent signalling, can be activated upon pathogen challenge, leading to protein dissociation to mount the first layer of defence. On the one hand, BIK1 very likely dissociates to phosphorylate RBOHD and trigger a rapid ROS burst; on the other hand, the Gβγ dimer may dissociate from the Gα subunit to initiate downstream signalling, which may also include ROS production. Moreover, the activation of MAPKs, especially MPK3 and MPK6, is also enhanced by Systemin upon infection. At the same time, the plant initiates downstream signalling to mount a second layer of defence that includes defence gene activation and metabolomic rearrangement. Phenolic compounds accumulate upon Systemin treatment before challenge. Indolic derivatives, which usually increase in basal defence against P. cucumerina, are primed by Systemin treatment. All these events result in a faster and more-effective defence response that makes the plant more resistant to necrotrophic pathogens. "Sys-IR Ø" indicates KOs that were impaired in Sys-IR against P. cucumerina, black arrows indicate genes that were induced in Systemin-treated plants upon infection, and dotted blue arrows indicate likely interactions between signalling elements.*

Acknowledgements

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Supplementary information

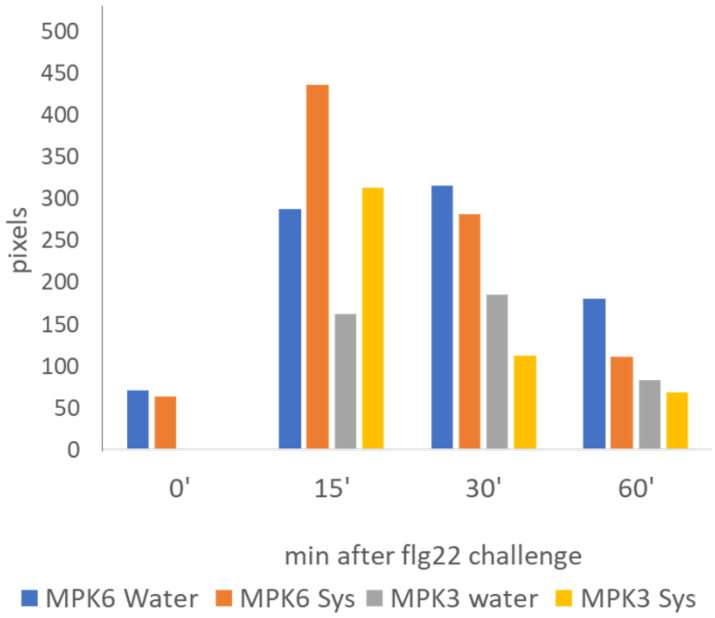

Figure S1. Quantification of phosphorylated levels of MPK3 and MPK6. *Western Blot band intensities of phosphirylated levels of MPK3 and MPK6 in plants treated with water or Systemin during 24h and then challenged with flg22 were quantified using GIMP 2.10.8 software.*

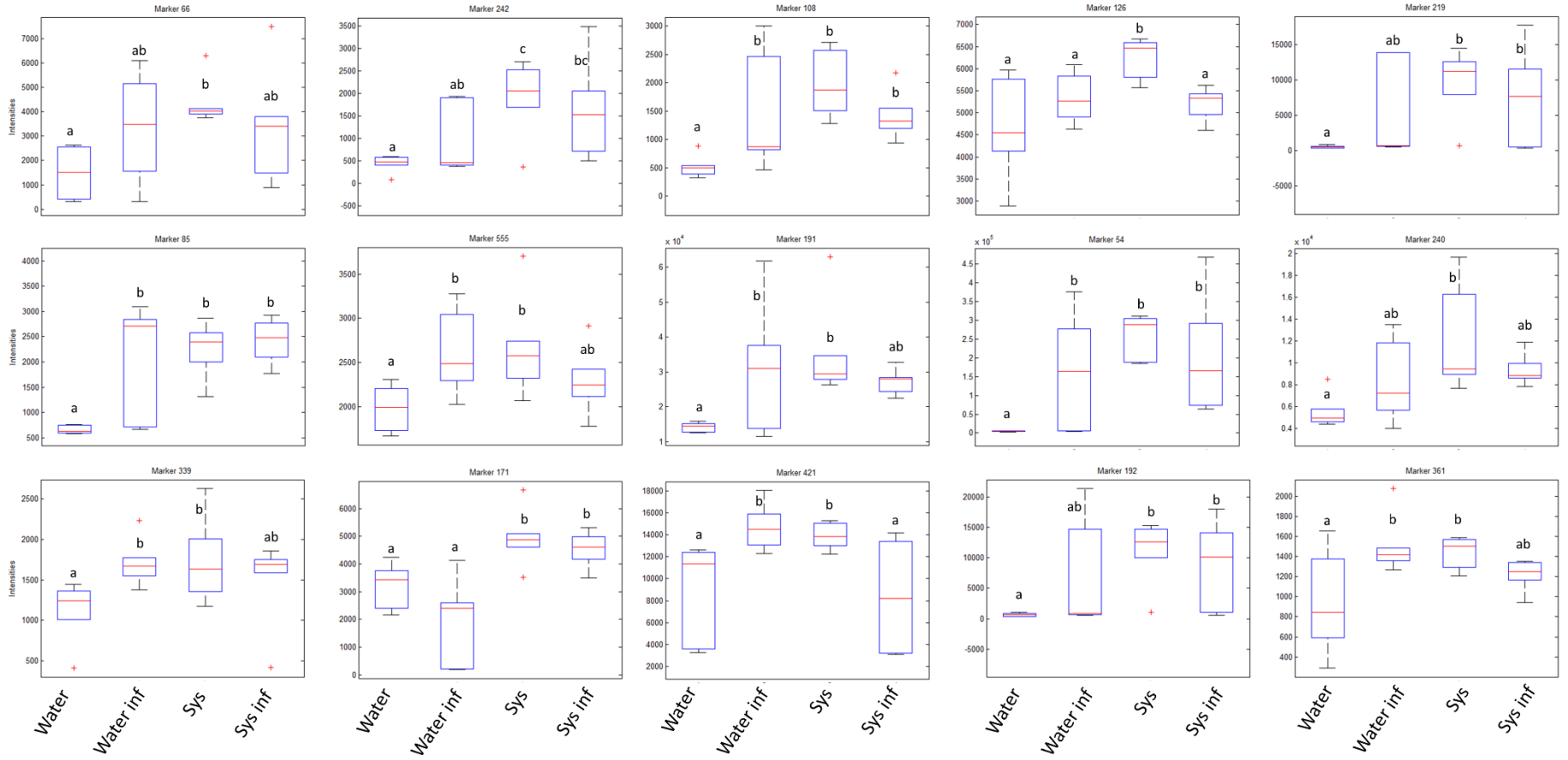

***Figure S2**. **Boxplots of exact masses of compounds showing a Systemin-induced profile in ESI+ mode.** Markers IDs correspond to those shown table 1. Different letters indicate statistically significant differences (ANOVA, Fisher's least significant difference (LSD) test; $p<0.05$; $n=6$).*

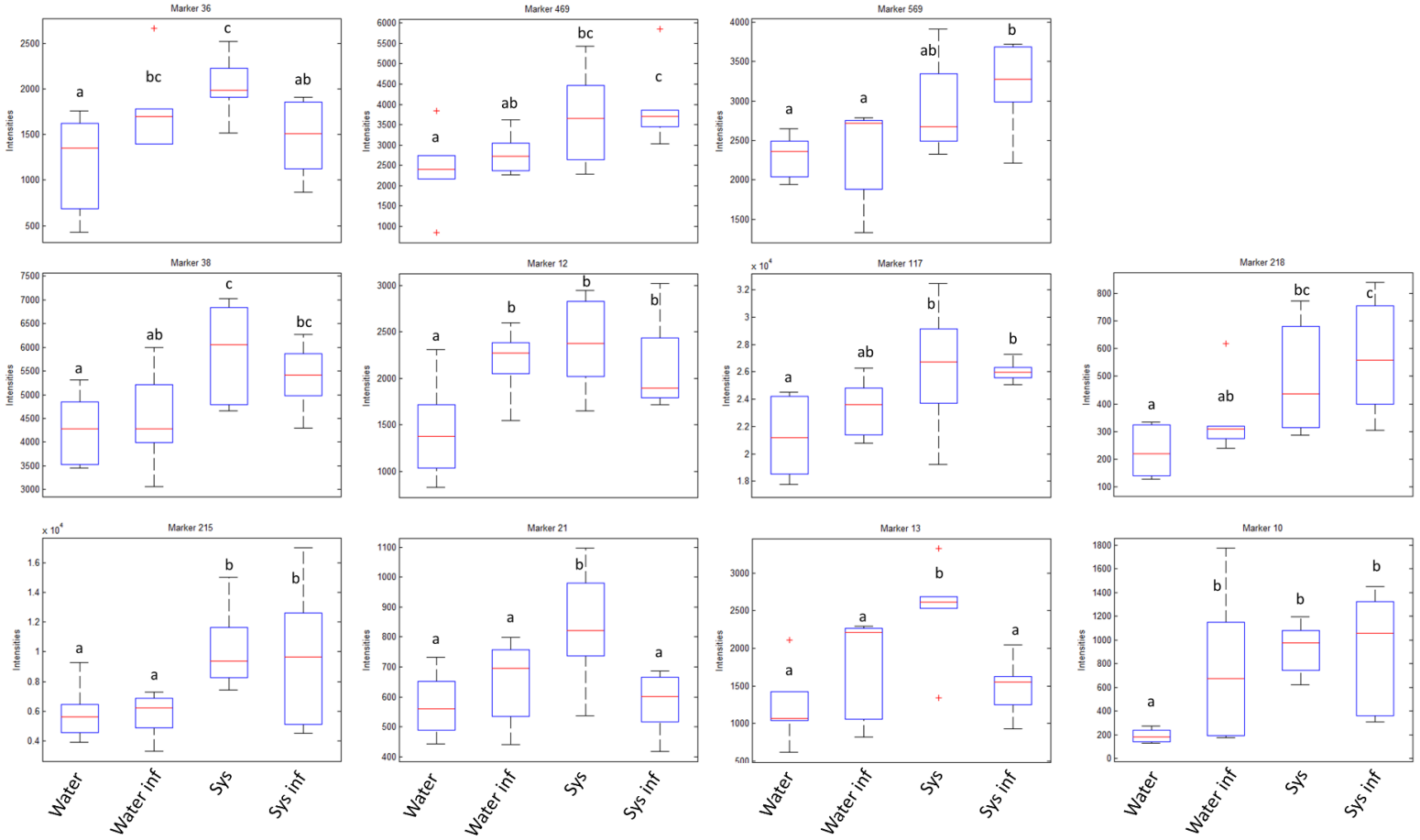

Figure S3. Boxplots of exact masses of compounds showing a Systemin-induced profile in ESI- mode. *Markers IDs correspond to those shown table 1. Different letters indicate statistically significant differences (ANOVA, Fisher's least significant difference (LSD) test; $p<0.05$; $n=6$).*

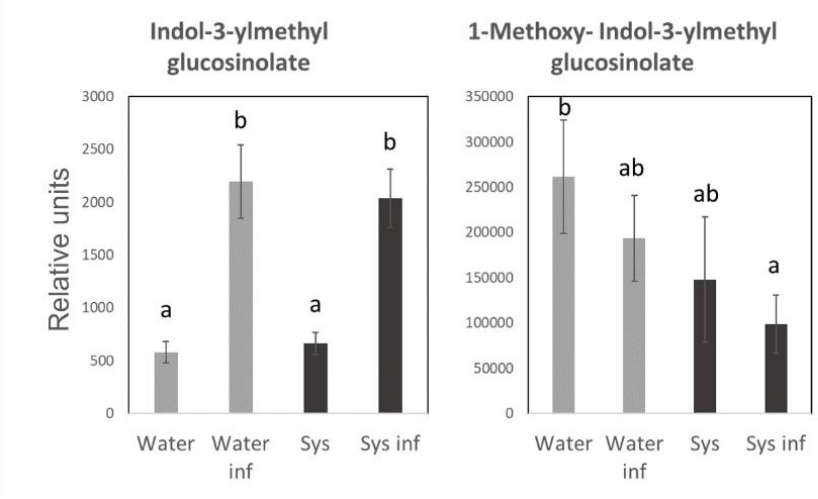

Figure S4. Identified Indole-glucosinolates profiles. *Indole-3-ymethyl glucosinolate (I3M) and 1-Methoxy- I3M were identified from the metabolomic analysis by matching exact mass based on online databases. Different letters indicate statistically significant differences (ANOVA, Fisher's least significant difference (LSD) test; $p<0.05$; n=6).*

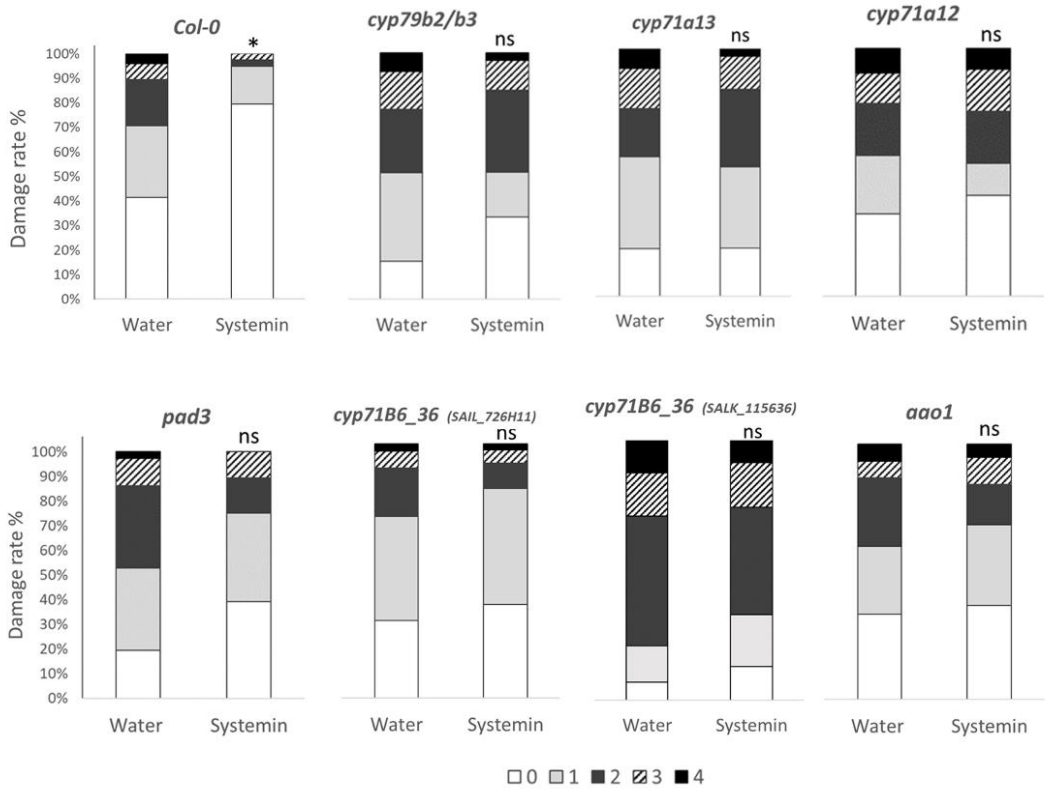

Figure S5. ***Phenotyping of knock out mutants of indolic compounds in Sys-IR assays***. *Infection levels of normally watered (Water) and Systemin-treated plants (Systemin) quantified 5 days after infection ($5x10^3$ spores/ml) by a disease rating using trypan blue staining. Infection levels were measured as a percentage of infected leaf surface using a scale (0 = 0%; 1= less than 25%; 2=*

*25-50%; 3= 50-75%, 4= more than 75%). Graphs represent averages of three experiments each including 12 plants and 4 infected leaves per plant. Asterisks indicate statistically significant differences performing a Kruskal-wallis test (*p<0.05, n=36).*

Supplementary table 1. List of primers used for RT qPCR analysis of gene expression.

Accession number	Primer name	Primer Sequence 5'-3'
AT5G25760	UBIQUITIN21	Fw - GCTCTTATCAAAGGACCTTCGG
		Rv - CGAACTTGAGGAGGTTGCAAAG
AT1G13320	PP2A	Fw - TAACGTGGCCAAAATGATGC
		Rv - GTTCTCCACAACCGCTTGGT
AT4G34460	AGB1	Fw - AACTCCTTCAACACGCTACTCA
		Rv - GCGTTTTATCGTCTGCGTTCA
AT3G63420	AGG1	Fw - TTGAAGGAGGTCGAGAACAC
		Rv - TTAGTGGCAACAGAGGATCG
AT3G22942	AGG2	Fw – AGAGGCAAACACAGGATTCA
		Rv - TTTCGGGAAGAAGAGGATCG
AT3G22942	RBOHD	Fw - AGGACACCTATGAGCCGATG
		Rv - CAACCATCACAATGCCAG
AT5G47910	MPK3	Fw – TGACGTTTGACCCCAACAGA
		Rv – CTGTTCCTCATCCAGAGGCTG
AT2G43790	MPK6	Fw - CCGACAGTGCATCCTTTAGCT
		Rv - TGGGCCAATGCGTCTAAAAC
AT1G08980	AMI1	Fw - CAGAACATCCGACGAGAAGA
		Rv - CCCGAGCAAAGTTGAAAGAG
AT4G39950	CYP79B2	Fw - GCCGACCCACTTTGCTTTAAA
		Rv - GCACAACCTCTTTTCCCGGTA
AT4G39950	CYP79B3	Fw - ATGCTAGCGAGGCTTTTGCA
		Rv - CCAACACCAAAGGCTTCGAA
AT2G30750	CYP71A12	Fw - GGCTTTGGCATGGATAGACA
		Rv - TCGCTAAACCCTTGACTTACTT
AT2G30750	CYP71A13	Fw - TAAAGAGGTGCTTCGGTTGC
		Rv -TATCGCAGTGTCTCGTTGGA
AT2G30770	CYP71B6	Fw - GGTCACACATACTACACAACCAA
		Rv - GGTGGACCTGGTGGAAGATT
AT5G20960	AAO1	Fw - ATGCCACACAATGCGGTTTC
		Rv - TACAAGCATCCACAAGCGGT
AT3G26830	PAD3	Fw - CAACAACTCCACTCTTGCTCCC
		Rv - CGACCCATCGCATAAACGTT
AT2G44490	PEN2	Fw - GGACGGTGCTCCAAGTATGT
		Rv - CGGATCATATGGCTCGTACC
AT3G44300	NITII	Fw - CTTGACCGTAAATGAGCACC
		Rv - GAACTGACGAATCACAACCG

Supplementary table 2. Transitions of the LC-MS targeted method for indolic compounds measurement.

Compound name	ESI mode	Cone energy (V)	Collision energy (eV)	Retention time (min)	Mass transition
Indole-3-acetamide	-	25	12	2.1	173.2>129.9
Indole-3-acetonitrile	-	20	5	2.8	154.9>154.9
IAA	+	25	15	2.8	176>130
d5IAA	+	25	15	2.8	180.8>133.9
Camalexin	+	25	30	3.25	201>59.1
Indole-3-carboxylic acid	-	25	15	2.7	160>116

Supplementary table 3. Internal libraries of phenolic compounds.

Compound	Exact mass (Da)	Theoretical Ion mass (Da)	Ionization mode	RT (min)	Experimental fragments mass (Da)
Kaempferol	286.0477	285.0403	ESI-	4.99	289.5019>285.0403>258.52>90.2879
		287.0832	ESI+	4.99	178.0209>147.0335>105.0416>93.0052>91.0563
Apigenin	270.0528	271.0764	ESI+	6.06	271.0764>227.0226>159.0134>117.0411
		269.0515	ESI-	6.06	269.0517>196.528>178.0027>141.0176>136.0505

Neohesperedin	610.1898	609.1816	ESI-	4.85	609.1816>579.157>489.1453>324.0904>323.0561
Chlorogenic Acid	354.0951	353.0949	ESI-	3.14	351.7326>345.4958>311.6933>289.5353>245.0340
(-) Epicatechin	290.079	291.086	ESI+	2.91	147.0789
		289.74	ESI-	9.6	289.074>245.0774>205.0086>203.0544>125.0331
Coumarine	146.0368	147.059	ESI+	3.94	147.0579>103.0703>91.0665
Quercitrin	448.1006	447.093	ESI-	4.94	447.0922>344.5011>302.0369>300.0330>271.0234
(-)-Epigallocatechin	458.0849	307.0893	ESI+	2.96	307.0504>193.4857>186.3156>139.0691
		305.0679	ESI-		305.0534>289.0748>165.0147>144.4718>125.0207
Shikimic acid	174.0528	173.045	ESI-	0.48	137.025>93.0515>73.0510
Naringin	580.1792	579.1694	ESI-	4.45	579.1715>461.1854>460.1287>459.1172>293.0524
Quercetin	302.0427	303.248	ESI+	7.69	
Coniferyl alcohol	180.0786	181.0672	ESI+	8.51	163.0698>149.0447>129.0809>93.0643
Ferulic Acid	194.0579	193.0669	ESI-	3.83	177.0735>139.9852>117.0542>98.9603>89.0517
Caffeic Acid	180.0423	179.023	ESI-	2.89	163.06>145.0111>135.0599>117.0407>89.0564
3-Dehidroshikimate	172.0372	171.0303	ESI-	0.66	127.0589>109.0424>81.0761
4-Coumaryl alcohol	150.0681	149.0609	ESI-	2.95	135.049>131.044>128.861>122.949
capsaicin	305.1991	306.2155	ESI+	6.51	137.0796>122.0477>94.078

Ampelopsin	320.0532	319.0454	ESI-	2.11	245.0786>205.0786
Taxifolin	304.0583	303.0476	ESI-	5.01	285.0204>259.0641
3-Dihydroxybenzoic acid	154.0266	153.0188	ESI-	1.02	108.0368
Skimmianine	259.0845	260.099	ESI+	5.76	245.0862>227.0907
(+) - Catechin	290.079	289.0723	ESI-	3.97	289.089>109.0299
Sinapic Acid	224.0685	223.0606	ESI-	3.95	208.043>164.045

Supplemetary table 4. Internal libraries of indolic compounds.

Compound	**Exact mass**	**Theoretical Ion mass (Da)**	**Ionization mode**	**RT (min)**	**Experimental fragments mass (Da)**
Indole-3-acetamide	174.079	175.08	ESI+	2.84	175.0978>130.0844
Indole-3-acetyl-L-alanine	246.1004	247.1083	ESI+	4.23	247.1279>130.077
Indole-3-acetyl-Isoleucine	288.1474	289.1552	ESI+	5.96	289,1587>243.1655>86.1135
Indole-3-acetyl-L-phenylalanine	322.13	323.139	ESI+	6.23	323,156>304.3021>166.0975>120.0911
Indole-3-carboxaldehyde	145.0528	1,440,449	ESI-	3.76	144.0449>104.444
		146.0606	ESI+		146.1255>118.0784>91.0793
Indole-3-acetonitrile	156.06	157.0766	ESI+	5.63	157.0794>130.0668>103.05
Indole-3-acetic acid-L-aspartic acid	290.09	289.0824	ESI-	4.27	289,0842>144,0435>132.0575>130.0783>88.0476

Indole-3-pyruvic acid	203.0582	202.0504	ESI-	6.35	202.0468> 174.9545>115.0579
I3CA methyl ester	175.06	176.0712	ESI+	5.17	175,1073>130,0763>103.719
Indole-3-acetyl-L-tryptophan	361.142	3,601,348	ESI-	6.02	360,1425>203,0822>130,111
		362.1505	ESI+		243.1606>188.0812
Indole fragment	-	116.07	ESI-	-	-

Supplementary table 5. Chemical parameters of identified Indole-glucosinolates.

Compound	**ion**	**Molecular formula**	**RT (min)**	**Theoretical m/z**	**Experimental m/z**	**m/z Deviation**
I3M	$[M-H]^-$	$C_{16}H_{19}N_2O_9S_2^-$	7.24	447.05	447.069	0.019
1-methoxy-I3M	$[M-H]^-$	$C_{17}H_{21}N_2O_{10}S_2^-$	4.48	477.06	477.065	0.005

CHAPTER 3

Deciphering the mechanisms behind Sys-IR against *B. cinerea* in tomato plants: A holistic approach.

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Abstract

Plant defense peptides are paramount endogenous danger signals secreted after a challenge that intensifies the plant's immune response. Systemin was the first plant peptidic hormone characterized in plants in 1991. It has emerged as a versatile resistance inducer in several plant-pathosystems though the mechanisms behind Sys-IR when exogenously applied are still elusive. In the present study, we characterized the molecular mechanisms behind Sys-IR in tomato plants against the necrotrophic pathogen *Botrytis cinerea* through a holistic approach. We performed proteomic, metabolomic, and enzymatic studies to decipher the Systemin-induced changes before and after the challenge at several metabolic levels. Systemin treatment produced massive rearrangement in the proteomic profile by itself but the number of proteins differentially affected after the infection was scarce although very targeted to pathogen resistance, danger perception, and amplification of the signaling. At the metabolomic level, the impact of the systemin treatment showed the opposite behaviour following a general priming profile. The proteomic and enzymatic results revealed that Systemin conditioned the primary metabolism towards the production of available sugar monomers and carbon structures that, once the challenge was present, could be fuelling the priming of callose deposition found in Systemin-treated samples as well as the biosynthesis of defense-related metabolites found in the metabolomic analysis such as phenolic compounds and alkaloids. Finally, although Systemin induced the over-accumulation of many proteins and metabolites by itself in the absence of infection, we did not find associated fitness costs in the physiological parameters measured between Systemin-treated and control plants.

Introduction

In nature, plants evolved a variety of strategies in order to strengthen their immune system and effectively fight against invading attackers. Plant defense can be constitutive or inducible. Exposure to specific stimuli can trigger a state of danger alert that leads to enhanced resistance against future attackers called Induced-Resistance (IR) which can occur both locally and in systemic tissues. Plants under the IR state show augmented defense responses at several metabolic levels and lower susceptibility to different challenges (Mauch-Mani et al., 2017; De Kessel et al., 2021). The stimuli that lead to the

IR state can be of different nature, including those from biological and chemical origin. Biological stimuli including beneficial microbes such as growth promoting bacteria (PGPRs) or symbiotic fungi lead to a systemic induced resistance called Induced Systemic Resistance (ISR; Pieterse et al., 2014). On the other hand, local necrotizing pathogen attack leads to enhanced systemic resistance called Systemic Acquired Resistance (SAR; Durrant et al 2004; Spoel et al., 2012). In addition to microorganisms, non-biological stimuli such as chemicals, non-proteinaceous amino acids, physical damage, or volatile organic compounds (VOCs) are also able to induce resistance in plants (Mauch-Mani et al., 2017; De Kessel et al., 2021). Sometimes the physiological output in response to the IR stimulus is not instantaneous but occurs when a challenge is present leading to a faster and stronger response. This enhanced resistance state is called "defense priming" (Martínez-Medina et al., 2016; Mauch-Mani et al., 2017). Recently the IR definition was revisited and considered to be a sum of both direct and primed defense activation (Kesel et al., 2021).

In the past few years there has been an emerging number of reports on peptides acting as endogenous danger signals that function as amplifiers of the plant immune responses upon pest attack (Yamaguchi and Huffaker, 2011; Marmiroli and Maestri 2014; Chen et al., 2020). These peptides are known as secondary danger signals or phytocytokines (Gust et al., 2017). They are released actively by the attacked cell after a challenge, often involving processing from a larger precursor, and perceived by membrane receptors of intact adjacent cells to spread the warning alarm (Albert et al., 2013; Gust et al., 2017). Although their natural function is being studied, their potential as resistance inducers needs further research. In Arabidopsis an increasing number of phytocytokines have been discovered and studied. Regarding its function as resistance inducers, it was shown that application of either Pep3, PIP1 or SCOOP12 leads to Arabidopsis resistance to *Pseudomonas syringae DC3000* (Ma et al., 2013; Hou et al., 2014; Gully et al 2019). Similarly, maize peptide ZmPep1 treatment led to enhanced resistance against necrotrophic pathogens and ZmPep3 application produced a reduction in *Spodoptera exigua* larval growth in maize plants (Huffaker et al., 2011; 2013). In tomato, CAPE1 induces resistance to the herbivore *Spodoptera litura* as well as to the biotrophic pathogen *P. syringae DC3000* (Chen et al., 2014), whereas tomato phytosulfokine (PSK) treatment enhances resistance to the necrotrophic fungi *Botrytis cinerea* (Zhang et al., 2018).

Systemin is a 18aa peptic hormone found in most Solanaceous species that acts as a phytocytokine. It was first discovered in tomato plants and considered as the first known peptide regulating systemic signaling in plants. It induces the accumulation of protease inhibitors (PI) in response to wounding and herbivory not only locally but also in systemic unwounded tissue (Pearce *et al.*, 1991). Systemin is released by proteolytic processing from a larger precursor of 200aa called Prosystemin (PROSYS) (Ryan and Pierce 1998; McGurl et al., 1992), however, the molecular mechanism by which Systemin is released from PROSYS is still unclear. A recent study reported that two plant phytaspases from the subtilase family hydrolyses PROSYS at two aspartate residues producing L-Systemin and subsequently the action of a Leu aminopeptidase is necessary to remove de Leu residue and release the mature peptide (Beloshistov *et al.*, 2018). After its release, Systemin binds to a LRR-RLK membrane receptor that was recently described as SYSTEMIN RECEPTOR 1 in tomato and other Solanaceae (SYR1; Wang *et al*., 2018). SYR1 is important for defense against pests in tomato plants. Interestingly, the tomato PEPR1/2 ORTHOLOG RECEPTOR-LIKE KINASE 1 (PORK1) was also found to regulate responses to wounding and Systemin treatment (Xu *et al*., 2018). In fact, PORK1 is essential for Systemin-mediated defense signalling acting very likely in collaboration with SYR1, since PORK1 RNAi lines with intact SYR1 lack Systemin signalling responses (Xu *et al.*, 2018).

Systemin binding to its receptor(s) triggers cascade of intracellular defense signalling responses which include the accumulation of reactive oxygen species (ROS), depolarization of the plasma membrane, opening of ion channels, an increase of the intracellular calcium (Ca^{2+}) and the activation of mitogen-activated protein kinases (MPKs) (Ryan 2000; Zhang et al., 2020). These early signalling events lead to the release of linolenic acid and the biosynthesis of jasmonic acid (JA) (Sun et al, 2011; Fürstenberg-Hägg *et al.*, 2013). Among the long-distance transported signals, Jasmonates, ProSys mRNA and Systemin itself are proposed as the mobile signals of Systemin-mediated systemic signalling (Li *et al*., 2002 check; Sun 2011; Zhang and Hu, 2018). Moreover, volatile organic compounds (VOCs) have been reported to be involved in the Systemin-mediated regulation of systemic defense in distal tissue and in neighboring plants (Corrado *et al*., 2007; Coppola *et al*., 2017).

Several studies have demonstrated how changes in the endogenous levels of Prosystemin, the Systemin precursor, affects plant resistance against pests. Silencing of Prosystemin (PS) gene in tomato reduced resistance against *Manduca sexta* (Orozco-Cadenas *et al.*, 1993). Tomato *PS+* plants showed enhanced resistance against the aphid *Macrosiphum euphorbiae* and *Spodoptera litoralis* larvae (Coppola *et al.*, 2015). Interestingly, these plants were also more resistant to the necrotrophic fungi *Botrytis cinerea* and *Alternaria alternata,* suggesting that Systemin involvement in plant defense is not restricted to pests but also effective against pathogens. In fact, PS overexpressing plants resulted in a reduced susceptibility to *Cucumber mosaic virus* in tomato (Bubici *et al*., 2017). On the other hand, PS overexpression in heterologous plants also affects the plant defense response. PS gene overexpression in Arabidopsis conferred resistance against necrotrophic fungi inducing an upregulation of defense-related genes (Zhang *et al.,* 2017).

There are few reports studying protection by exogenous treatments with Systemin since most approaches were done in PS+ plants. After it was discovered, Pearce et al., 1991, observed that Systemin was able to induce protease inhibitors proteins both locally and systemically when supplied to tomato plants. In consonance, exogenously applied Systemin imapired laval growth of *Spodoptera littoralis* as well as reduced leaf colonization by the pathogen *B. cinerea* through the induction of direct and indirect plant defenses including defense-related genes and VOCs respectively (Coppola et al., 2019). Systemin was also shown to be able to induce resistance against necrotrophic fungi in taxonomically distant species such as Arabidopsis (Pastor-Fernández et al., 2020) and *Vitis vinifera* (Molisso et al., 2021).

However, the molecular mechanism underlying the observed enhanced resistance induced by Systemin against this variety of biotic stressors is still unclear. Using omics approaches, Corrado *et al*., (2007) found significant increases in the total amount of volatile emissions in PS+ plants and identified a group of monoterpenes, which made PS+ plants more attractive to parasitoids. Rocco *et al*., (2008) performed a proteome analysis of PS+ tobacco plants and observed that differentially accumulated proteins were involved in oxidative stress; pathogen and pest defence; energy production and carbon metabolism. During a study of the transcriptome of transgenic tomato plants overexpressing the PS gene, it was found that amino acid metabolism and response to stress pathways were strongly upregulated whereas carbon metabolism and transport had

a minor impact (Coppola et al, 2015). More recently, a metabolomic profiling of PS+ showed increased levels of SA and OPDA but not JA, and confirmed the impact on the amino acids metabolism as well as in the lignan biosynthesis (Pastor *et al*., 2018). Studies in tomato cell suspension cultures, deciphered changes in the phosphoproteome during the early signalling cascade triggered by Systemin (Ahmad *et al.*, 2019). Note that none of the previous mentioned studies focused on the metabolic response enhanced by Systemin upon a biotic challenge.

Systemin has demonstrated to be an interesting phytocytokine involved in defense against a broad spectrum of biotic challenges including herbivores and pathogens. However, very little is known about its effect as a defense elicitor when applied exogenously. Since we previously demonstrated that it is able to induce resistance against necrotrophic fungi in taxonomically distant species that do not produce systemin, we acknowledge its potential to be applied as a general agent of disease control. In this research, we aim to further characterise the molecular mechanisms underlying Systemin-Induced Resistance (Sys-IR) against necrotrophic fungi in tomato. We also attempt to find metabolic fingerprints that may be associated to its mode of action linked to enhanced resistance. We have performed comparative proteomic, metabolomic and enzymatic studies which are indicative of different levels of regulation of cell metabolism. Systemin has a different impact in the different cellular metabolic processes. It may be perceived as a signal of an imminent stress although the presence of the pathogen induces additional responses in treated plants.

Materials and methods

Plant materials and Systemin treatment

For all the experiments tomato plants of CastleMart cultivar (*Solanum lycopersicum cv CM*) were sown in 200 cm^3 pots in a vermiculite substrate and grown in a greenhouse. Plants were watered with Hoagland solution (Hoagland and Arnon, 1950) twice a week. Four weeks after germination plants were treated with Systemin by adding it to the substrate diluted in water reaching a final concentration of 20 nM in the pot. The same amount of water was added to the control plants.

Pathogen inoculation and disease quantification

Botrytis cinerea CECT2100 (Spanish collection of type cultures, Universidad de Valencia, Burjassot, Spain) was grown for 2 weeks in plates with PDA (potato dextrose agar) medium supplemented with tomato leaves (40 mg ml^{-1}). *Botrytis cinerea* spores were collected and pre-germinated in Gambor's B5 medium supplemented with 10 mM sucrose and 10 mM KH_2PO_4 for 2 h in the dark without shaking. Plant infection was performed on intact plants at 100% relative humidity as described by Vicedo *et al.* (2009). 24h after Systemin treatment plants' third and fourth leaves were inoculated by spraying them with a 10^6 ml^{-1} spore suspension.

72h after the pathogen inoculation leaves were collected to assess the phenotype analysis. Leaf necrosis was stained using lactophenol trypan blue as previously described (Flors et al., 2008). The infection levels were quantified using a damage rating, assigning numbers to different percentages of necrotic leaf surface (0 = healthy leaves; 1= leaves with less than 25% of necrotic surface; 2= leaves with a necrotic surface between 25-50%; 3= leaves with a necrotic surface between 50-75%; 4= leaves with more than 75% necrotic surface), according to previous reports (Luna et al., 2012; Agut et al., 2014; Wilkinson et al., 2018).

Infection quantification was also determined by measuring fungal biomass in the leaf tissue by measuring the expression of a fungus constitutive gene relative to a plant constitutive gene. Arabidopsis tissue of plants treated either with water or 20 nM Systemin was collected for DNA extraction 48h after pathogen infection. A simple and rapid protocol was followed for DNA extraction (Edwards et al 1991). Quantitative Real-Time PCR (qPCR) was performed using a Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific) in a StepOne instrument (Applied Biosystems). A ratio was calculated of the amount of *BcTUBULIN*, as a constitutive gene of *Botrytis cinerea*, relative to the amount of *SlEF1*, a constitutive gene of tomato, following the ΔCt method. Primer sequences used for qPCR were the following:

- SlEF1 forward primer: GATTGGTGGTATTGGAACTGTC
- SlEF1 reverse primer: AGCTCGTGGTGCATCTC
- BcTUB forward primer: CCGTCATGTCCGGTGTTACCAC
- BcTUB reverse primer: CGACCGTTACGGAAATCGGAA

Protein extraction and proteomic analysis

Plant third and fourth leaves were collected 24h after *Botrytis cinerea* infection in liquid nitrogen and then stored at -80ºC for subsequent proteomic analysis. Proteins were extracted from 100mg grinded fresh tissue by adding a protein extraction buffer in a 1:3 ratio (100 mg:300 µl). Buffer contained TBS, Triton, PMSF 1 mM, DTT 10 mM and 5ul/mlof a protease and phosphatase inhibitor cocktail (Sigma–Aldrich). Samples were mixed with vortex for 15" and incubated on ice for 10 min. Then, the supernatant was collected after centrifugation (10 min at maximum speed). Laemmli sample buffer was added to the protein extract, and the mix was boiled for 5 min. The protein concentration was determined via the Bradford method using BSA as a standard.

Protein identification was performed in the proteomic facility PROTEORED© of the University of Alicante (Spain) as detailed in Belchí-Navarro et al. (2019) with some modifications. In brief, protein extract (30 µg) was digested with trypsin, and later peptides were passed through a C18 column (Pierce® C18 Spin Columns, Thermo Scientific) and resuspended in formic acid (0.1%). Peptides were analysed with an Agilent 6550 iFunnel Q-TOF mass spectrometer (Agilent Technologies) coupled to an Agilent 1290 UHPLC chromatograph using an Agilent AdvanceBio Peptide mapping column (2.1 mm × 250 mm, 2.7 µm particle size, operated at 50 °C). Analytes were eluted with a linear gradient of 3 - 40% ACN in 0.1% formic acid and with a constant flow of 0.4 ml/min. The LC-MS output files were loaded in Progenesis QI for Proteomics (Nonlinear Dynamics) v4.0 label-free analysis software and a protein quantification based on the MS peak intensity was performed. For the gene ontology analysis of the identified proteins Blast2GO was used, selecting only the samples displaying a fold change over 2 in a pairwise comparison.

MetaboAnalyst (Chong et al., 2019) was used to create the coloured heatmap and the sparse partial least squares discriminant analysis (sPLS-DA) and Venn diagrams were plotted using eulerr and reshape2 packages in R (Wickham, 2007; Larsson & Gustafsson, 2018).

Measurement of enzymatic activities

Enzymatic activities were measured in the *robot-based* HitME platform at INRAE facilities (Bordeaux). 20 mg of fresh tissue was weighted and aliquoted in micronic tubes

and randomised in a 96-wells plate, leaving empty spaces for the blanks. Soluble enzymes were extracted by using a protein extraction buffer. Continuous (direct) and discontinuous (indirect) assays were performed to measure the enzymatic activities as described in previous reports (Gibon et al 2004; Bénard and Gibon 2016).

Prior to enzymatic activities measurement in our samples, protocols for specific enzymes measurement were optimized using a biological standard (*Solanum lycopersicum cv Money Maker leaves'* tissue) according to three different criteria: the optimal dilution of the extract, the evaluation of the recovery of the standard and the coefficient of variation between replicates (Bénard and Gibon 2016). Raw data was uploaded to the PLATO database (https://services.cbib.u-bordeaux.fr/plato/).

Non-targeted metabolomic analysis with LC-ESI full-scan mass spectrometry

200 mg of fresh grinded leaf material was extracted at 4 °C with 1 ml of MeOH:H_2O (30:90) containing 0.01% HCOOH. The supernatant was collected after centrifugation at full speed at 4 °C for 15 min and filtered through cellulose filters (Regenerated Cellulose Filter, 0.20 µm, 13 mm D. pk/100; Phenomenex). Metabolomic analysis was performed by injecting 20 µl into an Acquity UPLC system (Waters, Milford, MA, USA) interfaced with a hybrid quadrupole time-of-flight instrument (QTOF MS Premier).

Subsequently, a second fragmentation function was introduced into the TOF analyser to identify the signals detected. This function was programmed in a t-wave ranging from 5 to 45 eV to obtain a fragmentation spectrum of each analyte (Gamir *et al.*, 2014; Agut *et al.*, 2014). Positive and negative electrospray signals were analysed independently to obtain a global view of the data. To elute the analytes, a gradient of methanol and water containing 0.01% HCOOH was used. Six independent biological replicates per sample were randomly injected. LC separation was performed using a 2.1 × 50 mm Kinetex C18 analytical column, 1.7 µm particle size (Phenomenex). Chromatographic conditions and solvent gradients were set as described by Gamir *et al.*, (2014) and Agut *et al.*, (2014).

To accurately identify the metabolites, an internal library of plant metabolites was created with chemical standards. The specific retention time, exact mass, and spectrum fragmentation of the compounds in the library were characterized according to Schymanski *et al.* (2014). Eighty-four compounds were prepared at a final concentration of 100 ppb in the same solution (Rivero *et al.*, 2015). The standard solution was injected

through the UPLC in positive and negative ion mode for electrospray ionization (ESI) while applying the same conditions used with the plant samples. For those compounds that were not represented in the library, the signals obtained in the non-targeted metabolomic analysis were assured by comparing the fragmentation spectra in the Massbank or Metlin databases (www.massbank.jp; www.masspec.scripps.edu).

Full-scan data analysis and bioinformatic processing

Supervised principal component analysis (sPLSDA) and heat map analysis were performed using MetaboAnalyst software (https://www.metaboanalyst.ca/). Data acquired with Masslynx 4.1 software (Masslynx 4.1, Waters) in the raw format were transformed into .cdf files using the Databridge tool from Masslynx 4.1.. Chromatographic signals were statistically analysed in R environment (http://www.r-project.org/). Signals from positive and negative electrospray ionization (ESI+; ESI−) signals were analysed independently. The peak, grouping, and signal corrections were obtained using the XCMS library (www.bioconductor.org; Smith *et al.*, 2006). The amounts of the metabolites were calculated as the normalized peak area units relative to the dry weight. A nonparametric Kruskal–Wallis test (*$p<0.05$) was performed to test differences between treatments. Adduct and isotope correction, and clustering were performed with the Mar-Vis Suite 2.0 software packages MarVis Filter and MarVis Cluster (Kaever et al., 2012; 2014).

Callose staining and quantification

Callose deposition was measured at 24 and 48 hpi by staining the leaves with aniline blue as previously described (Ton and Mauch-Mani 2004). Stained leaves were photographed under an epifluorescence microscope with a UV filter (Nikon Eclipse 80i). Callose deposition was determined by the quantification of yellow pixels with respect to total pixels of the leaf using GIMP 2.10.8 image analysis free software (GIMP - GNU Image Manipulation Program).

Starch measurement

For the starch measurement the pellet obtained after an ethanolic extraction of fresh material was used. Firstly, the pelletwas dissolved by adding NaOH 0.1M and heating at 95ºC during 30min, then it was neutralised with HCl. Secondly starch was hydrolysed by adding a mix of amyloglucosidase and α-amylase and incubating O.N. at 37ºC. Then glucose levels were determined by adding hexokinase and measuring NADPH as the product of the reaction, at OD 340 nm, before and after the addition of the enzyme. Results were expressed as equivalent of glucose.

Physiological parameters measurement

Some plant growth and development-related parameters were evaluated in plants treated once with 20 nM Systemin. Plant height was evaluated 4, 10 and 15 days after treatment. Leaf area was evaluated 4 and 15 days after treatment, and fresh and dry weight were measured 15 days after the treatment. For the leaf area measurement, plant leaves were cut and placed over a white surface. Photos of the different conditions were taken from the same height and images were analysed using imageJ software (ImageJ (nih.gov).

Statistical analysis

Bioassays included 3 to 6 biological replicates, each containing a pool of three plants that were randomized in the greenhouse. Each experiment was repeated 3 times. Graphs show the average of all experiments with the standard error. Statgraphics-plus software for Windows V.5 (Statistical Graphics Corp., MD, USA) was used to determine the statistical analysis by one-way analysis of variance (ANOVA, Fisher's Least Significant Difference (LSD) test; $P < 0.05$ n=6). Different letters indicate statistically significant differences.

For the phenotype confirmation experiments using a disease rating, leaflets of the third and fourth leaves of 4 plants per treatment were analysed. Statgraphics-plus software was again used to perform the comparative analysis using a non-parametric Kruscal-Wallis test analyses (*$p<0.05$, n=4). Asterisk denotes statistically significant differences.

Results

Sys-IR is effective against botrytis in tomato plants inducing priming in callose deposition.

Exogenous Systemin treatments induce resistance against necrotrophic pathogens (Coppola et al, 2019; Pastor-Fernández et al., 2020; Molisso et al 2020). Thus, first of all, we confirmed the Sys-IR activity of exogenously applied Systemin in our experimental conditions. Tomato plants were treated by adding a solution of 20nM of Systemin in the substrate and 24h later plants were inoculated with *Botrytis cinerea*. Disease levels were assessed by trypan blue staining and quantification of the fungal biomass by the relative expression of a *Botrytis* constitutive gene in infected leaves (Figure 1A). The two methods showed that Systemin treated plants displayed a reduced disease severity confirming the Sys-IR phenotype against *Botrytis cinerea* in tomato plants.

Callose is known to function as one of the first defensive barriers against pathogens (Ellinger and Voigt, 2014). This 1,3-β-glucan is one of the main components of the papillae. During induced-resistance processes, the starch degradation provides sugar structures that are transported through a vesicular trafficking process (Maekawa et al., 2014; Gamir et al., 2018) and assembled firstly in the inner cell wall and later in the outer side of the cell wall (Ellinger and Voigt, 2014). Priming of callose accumulation was also shown of key relevance in BABA-Induced resistance in Arabidopsis, grape and other plant species (Flors et al., 2008; Gamir et al., 2018). To decipher whether callose deposition is also involved in the Sys-IR, we measured callose in both treated and non-treated plants 24 and 48 h after *B. cinerea* infection (Figure 1B). Systemin treated plants showed enhanced levels of callose accumulation compared to water treated control plants at both time points suggesting that it may also be an important mechanism behind the Sys-IR. To shed some light on this result, we studied the starch degradation as a possible source of sugars availability. Starch degradation rate was also higher in plants treated with Systemin (Figure 1C and Figure S1) reinforcing the hypothesis that the Systemin treatment could facilitate the availability of sugars for the accumulation of callose in order to block fungal penetration (Figure 1B).

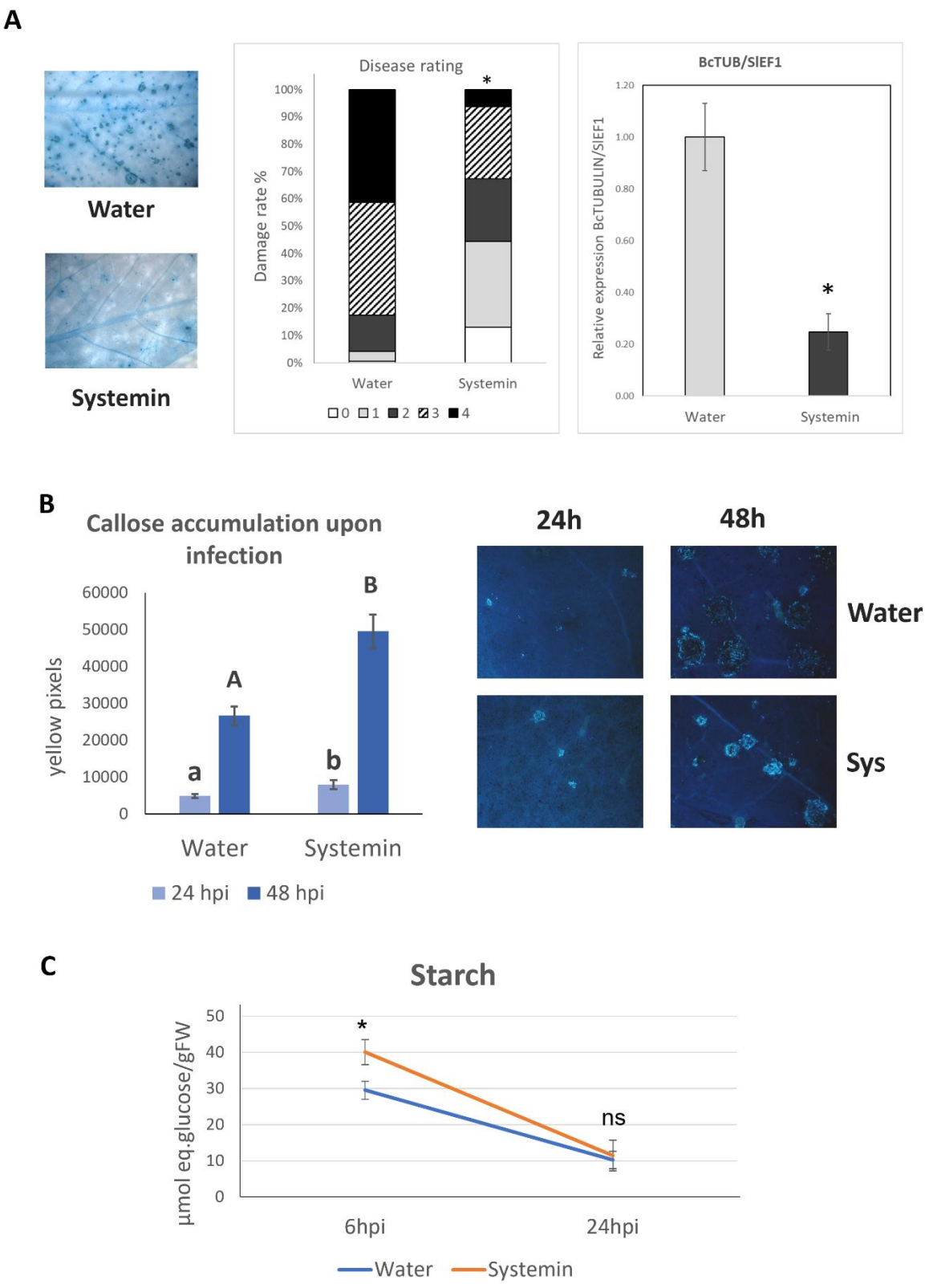


Figure 1. Systemin induces resistance against Botrytis through priming of callose. *(A) Phenotype of tomato plants inoculated with 10^6 spores/ml of Botrytis cinerea 24h after Systemin 20nM or water treatment. Infection was quantified either by necrotic area staining or by*

quantification of fungal biomass by qPCR. (B) Callose deposition in Water or Systemin-treated plants quantified at 24h and 48h after pathogen inoculation. (C) Starch levels in water or Systemin-treated plants quantified at 6h and 24h after pathogen inoculation. Asterisk indicates statistically significant differences by a Kruskal-Wallis test ($p<0.05$, $n=4$). Different letters indicate statistically significant differences (ANOVA, Fisher's least significant difference (LSD) test; $p < 0.05$ $n=6$).

Systemin treatment has a different impact in the plant proteomic and metabolomic profile

To shed light on the possible mechanisms behind Sys-IR in tomato plants, we performed non-targeted analysis of the proteome and metabolome in leaves of tomato water- and Systemin-treated plants in the absence or presence of *Botrytis cinerea* infection. A total of 902 proteins were detected in all treatments with a score higher than 6. Of these proteins, 513 showed a score above 15. A non-targeted metabolomic analysis of plants under the previously mentioned experimental conditions was performed by using an UPLC interfaced with a Q-TOF MS. Following a Kruskal Wallis statistical analysis, 3264 and 1160 features in ESI^+ and ESI^-, respectively, were found to significantly change ($p<0.05$).

To gain an overall insight of the Systemin impact in the plant's metabolism we performed a sPLSDA analysis of both proteins and metabolites separately. The proteomic analysis showed that prior to infection, water and Systemin-treated plants clustered separately whereas both overlapped after the challenge, hence the major changes in the proteome were triggered by Systemin treatment itself rather than by the infection (Figure 2A). Conversely, the metabolomic analysis showed the opposite behavior following a general priming profile. Although with a low percentage of features explaining the two main components (PC1 and PC2); the Systemin- and water-treated plants completely overlapped prior to the challenge, but once the infection was present, they clustered separately (Figure 2B). In order to get a comprehensible overview of the proteomic profile, Venn diagrams analysis of the detected proteins comparing Systemin- versus water-treated plants before and after *B. cinerea* infection were performed, plotting separately over-accumulated (threshold:1) and under-accumulated (threshold:-1) proteins (Figure 3A). In line with the sPLSDA results, the proteome changes after Systemin treatment were bigger than after the infection. Without infection, 224 proteins were over

accumulated in Systemin-treated plants vs water-treated plants, whereas only 10 proteins were over accumulated in the presence of infection (Figure 3A; Table 1). Regarding down accumulated proteins, we found 178 in Systemin treated plants relative to control plants were reduced, although only 4 proteins were under-accumulated following infection (Figure 3A; Table 1).

Since most changes occurred before the infection, we performed a pathway ontology classification of this group of proteins (Figure 3B) which showed that most proteins belonged to pathways of the primary metabolism, whereas only 6 proteins out of the total were related to the secondary metabolism (Figure 3B). Subsequent studies were focused on understanding those specific changes at the protein and metabolite levels to further clarify the mechanisms behind Systemin-IR.

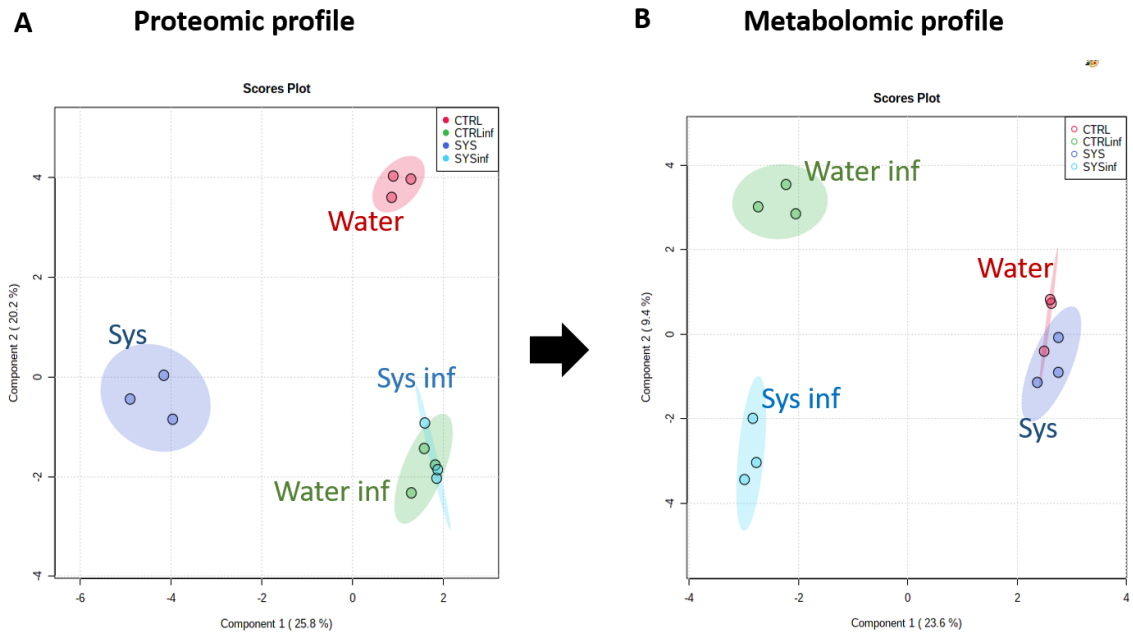


Figure 2. Proteomic and metabolomic fingerprint of Systemin treatment in tomato upon pathogen infection. *Sparse partial least squares discriminant analysis (sPLSDA) of the (A) proteomic analysis vs the (B) metabolomic analysis of water and Systemin-treated plants in the absence and presence of infection (24hpi).*

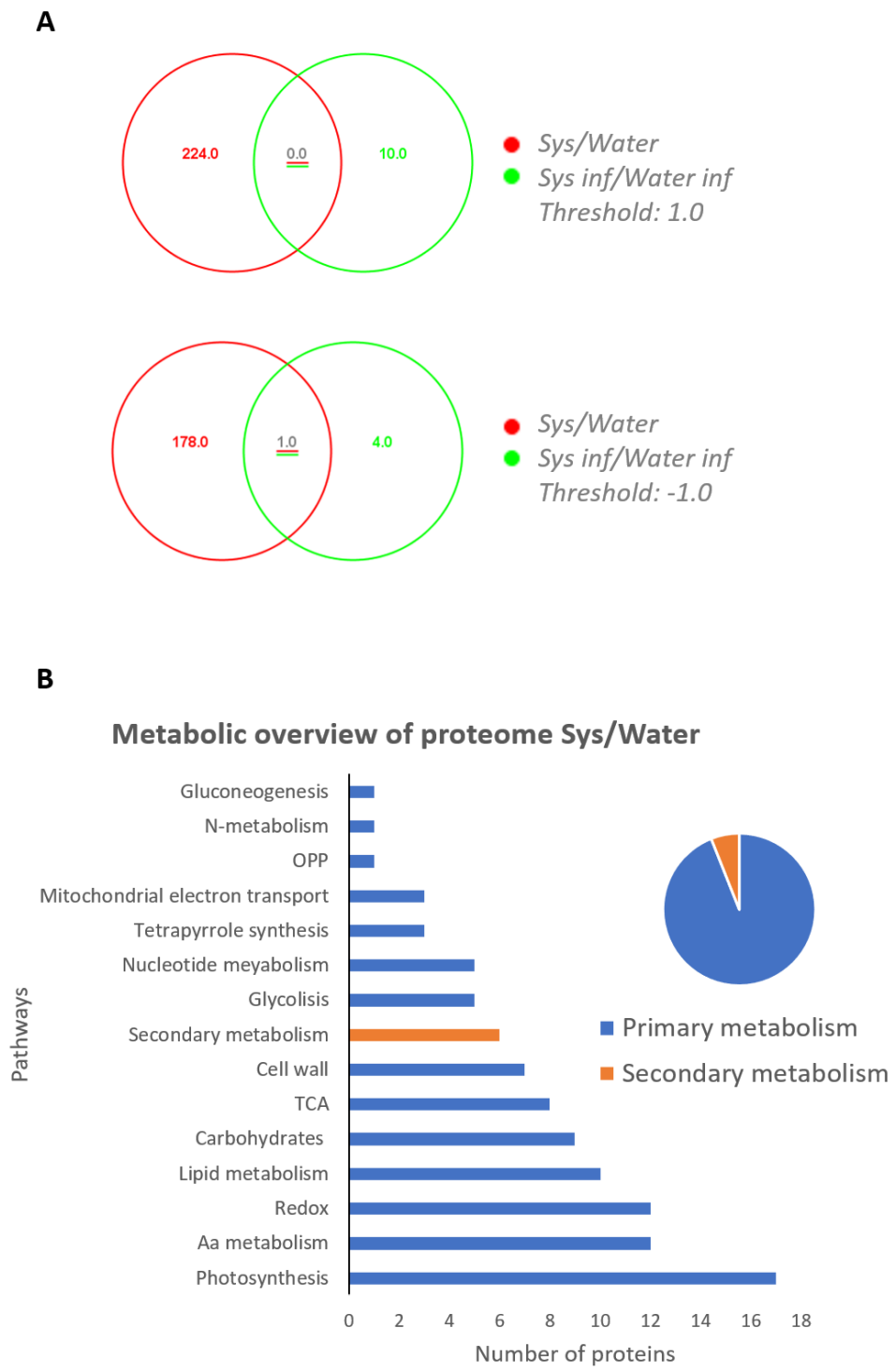


Figure 3. General proteomic changes induced by Systemin treatment in tomato. *(A) Venn diagrams of over-accumulated(upper) and under-accumulated (lower) showing common and specific proteins of Systemin treatment over water-treated plants in the abscence (Sys/water) and presence of the infection (Sys inf/water inf). (B) Metabolic pathways showing protein hits affected by the Systemin treatment in comparison with water-treated plants.*

Specific changes in the proteome during Systemin-IR

Reasonably, since Systemin has a strong impact on the proteome before the infection, we first focused in analyzing specific proteins affected by the Systemin treatment compared with water-treated plants. Primary metabolism processes with differential protein accumulation due to the peptide treatment included photosynthesis, carbohydrate and cell wall metabolism, glycolysis, TCA cycle, Amino acid metabolism, and redox reactions (Figure 3B and Figure 4).

Among the proteins with strongest changes found in the primary metabolism, photosynthesis showed the highest number of protein hits in Systemin-treated plants. Interestingly the RuBisCO large subunit was upregulated by the treatment together with a few chlorophyll binding proteins, two ATP synthases and a NAD(P)H-oxidoreductase subunit (Figure 4, Table S1). This suggests that Systemin treatment enhances photosynthetic activity, thus likely increasing the amount of energy and available photoassimilates for the plants.

Regarding the major CHO metabolism, sucrose phosphate synthase was under-accumulated in Systemin-treated plants, whereas hexokinase and starch phosphorylase, which are responsible for sucrose and starch degradation, were over-accumulated (Figure 4). As for minor CHO metabolism, 8 proteins were differentially accumulated by the Systemin treatment. It is worth noting that a glucan synthase like protein was over-accumulated, which is likely involved in the building of glucan polymers, such as callose for the cell wall reinforcement. In fact, many proteins involved in the cell wall synthesis and modification were affected by the treatment, some of them, such as the cellulose synthase and three expansin proteins were over-accumulated in Systemin-treated plants. On the other hand, except for a PGM-like protein, all proteins found related to the glycolysis were down-accumulated. Four Glucose-6-phosphate isomerase (GPI) and two 2,3-bisphosphoglycerate-dependent phosphoglycerate mutases (PGM) were down-accumulated in Systemin-treated plants compared to control plants. GPI catalyses the conversion of glucose-6 phosphate to fructose-6-phosphate. Although both sugars belong to the hexose-phosphate pool, fructose-6-phosphate is the one that continues to the glycolysis pathway towards the pyruvate formation. On the other hand, PGM catalyses the conversion of 3-PGA into 2-PGA and vice versa. Note that 3-PGA is one of the photosynthetic products. These results support the idea that the surplus of available sugars

induced by the treatment instead of being driven to the glycolysis are reallocated to other non-energetic purposes, such as cell wall modification and callose accumulation.

Regarding the TCA cycle, we found that three components to the pyruvate dehydrogenase complex and the malate dehydrogenase were over-accumulated by Systemin. Contrastingly, 2-oxoglutarate dehydrogenase and succinate dehydrogenase are down-accumulated, likely facilitating the accumulation of oxaloacetate, 2-oxoglutarate, and succinate (Figure 4). These results indicate that the TCA cycle is providing carbon skeletons to other metabolic pathways rather than energy to the cell. As for the aminoacid metabolism, out of the 11 proteins affected only 3 were down- accumulated; the mitochondrial alanine aminotransferase, the acetolactate synthase involved in the synthesis of valine from pyruvate, and the gamma-glutamyl phosphate reductase. This last down-accumulated protein is responsible for the glutamate degradation to proline. However, the ornithine carbamoyltransferase protein, which catalyzes the glutamate biosynthesis from ornithine, was over-accumulated. Regarding the biosynthesis of aspartate family amino acids, one phosphoserine aminotransferase and two s-adenosylmethionine synthases were over accumulated, which are involved in the threonine and methionine metabolism respectively. In addition, a cysteine synthase that leads to cysteine production was over accumulated by the peptide treatment.

Another cluster of proteins largely affected by Systemin treatment was redox-related metabolism. Most proteins related to redox reactions were over accumulated (Figure 4 and Table S1), being mainly thioredoxins and ascorbate/glutathione peroxidases. On the other hand, among the 6 proteins of the secondary metabolism we found 2 proteins that were strongly induced in response to Systemin which correspond to a 4-coumarate coA ligase, involved in phenylpropanoid biosynthesis and a chalcone-flavonone isomerase involved in the flavonoids biosynthesis, suggesting that phenolic compounds may be relevant in the Systemin triggered responses. The relevance of the phenolic and indolic compounds in the Sys-IR was already stated in Arabidopsis plants against necrotrophs (chapter 2) suggesting that there is a common fingerprint in the Sys-IR response which is conserved across the plant species.

Collectively, the results described above suggest that Systemin treatment is preparing the plant for a future challenge through the activation of the primary metabolism, accumulating available sugars and carbon structures for the synthesis of specific

proteinogenic amino acids and some defense components, such as compounds of redox and phenolic metabolism.

On the other hand, we were interested in the analysis of those proteins that were differentially accumulated in the presence of infection (Table 1) which might help explain the observed enhanced resistance. Since we found only 10 protein hits up-accumulated and 4 down-accumulated (Table 1), we hypothesized that Systemin triggers very targeted proteomic fingerprints upon infection. Indeed, when we studied the identity of these proteins, we found that most of them were defense-related, including pathogen related protein 1 (PR1b), pathogen-related protein 4 (PR4) and β-1,3-glucanase (PR2) (Table 1). Among these proteins were also a Guanine nucleotide-binding protein alpha-1 subunit (GPA), a G protein involved in defense signalling, and a Leucine aminopeptidase, which is involved in the last step of Systemin processing during defense responses (Belishitov et al., 2018). In addition, a Ribosomal RNA processing protein and a CoA ligase involved in fatty acid biosynthesis were also identified. As for down-accumulated proteins we found two non defense related proteins; one involved in lipid metabolism and one involved in DNA synthesis. Finally, three proteins that are yet uncharacterised were found, one of them assigned as a tentative protease inhibitor (PI) based on its high sequence similarity to a PI of potato (Table 1). These results suggest that the Systemin effect on the proteome upon infection is very specific, leading towards pathogen counteracting.

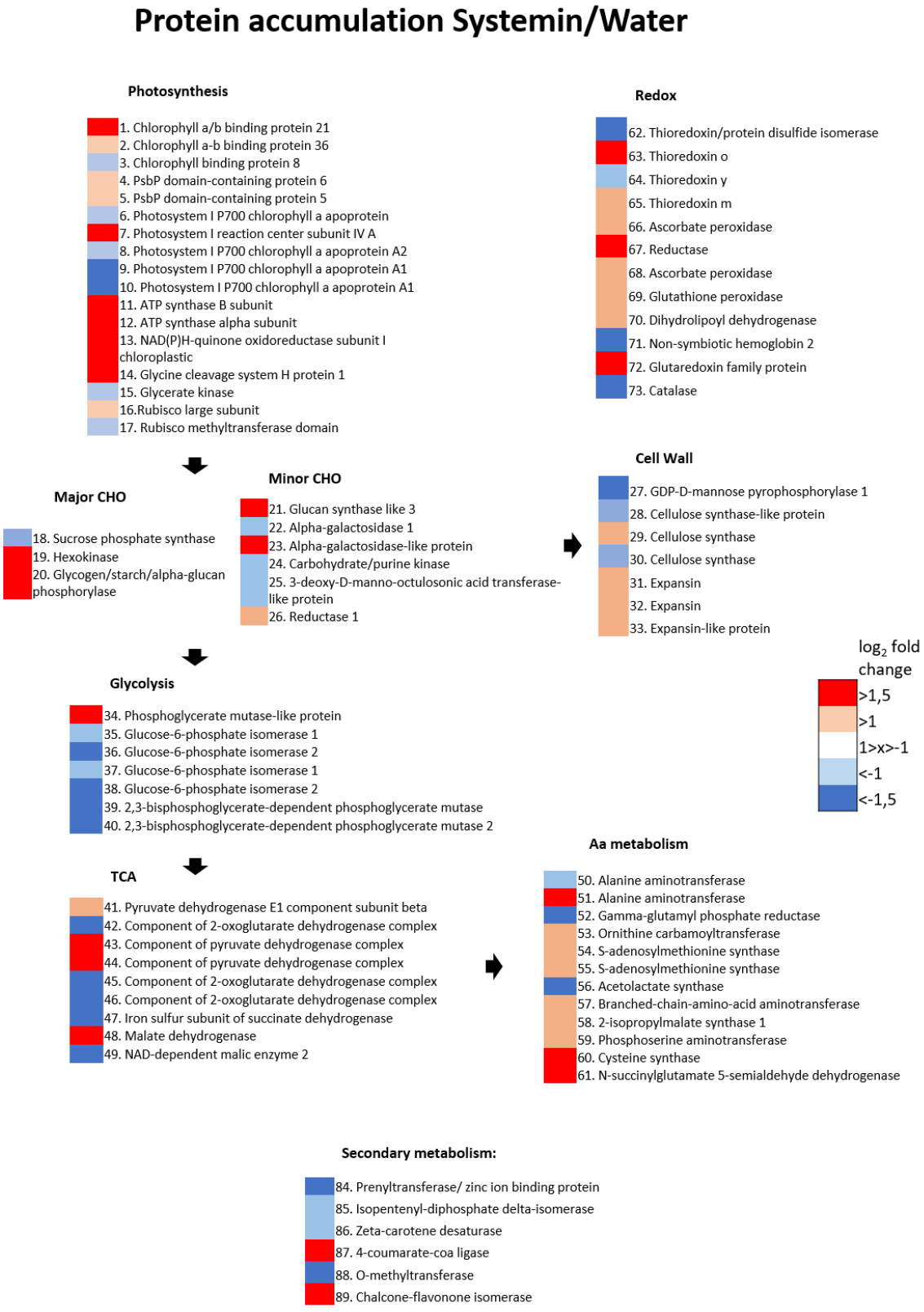


Figure 4. Protein accumulation levels upon Systemin treatment in tomato. *Log_2 fold change of specific proteins which's levels changed upon Systemin treatment at 24hpi. Colours legend is indicated in the figure.*

Table 1. Protein accumulation in Sys inf vs Water inf

Accession	Description	Log_2 Fold change Sys inf/Ctrl inf
	PR proteins	
Solyc00g174340.1.1	Pathogenesis-related leaf protein 6 (PR1B1)	1.27
Solyc09g007010.1.1	Pathogenesis-related leaf protein 4	1.21
	Beta glucanase	
Solyc01g008620.2.1	Beta-1 3-glucanase (PR2)	1.05
	Proteolysis	
Solyc12g010020.1.1	Leucine aminopeptidase 1, chloroplastic (LAPA)	1.71
	Signalling	
Solyc08g061220.2.1	Guanine nucleotide-binding protein alpha-1 subunit (GPA1)	1.09
	Regulation of transcription	
Solyc07g042380.2.1	Ribosomal RNA processing protein, NUC173 domain-containing protein	1.13
	Lipid metabolism	
Solyc08g075800.1.1	FA synthesis and FA elongation, acyl coA ligase	2.61
	Uncharacterised proteins	
Solyc01g056350.1.1	Unknown protein	1.25
Solyc09g089540.2.1	Tentative proteinase inhibitor	2.48
Solyc00g230080.1.1	Electron transport in photosystem II	1.28
Solyc09g009570.1.1	**Lipid metabolism**.Acyl CoA reductase	-1.09
Solyc01g073970.2.1	**DNA synthesis**. Histone	-1.54
Solyc03g044620.2.1	Unknown	-1.26
Solyc05g009850.2.1	Unknown	-1.24

Enzymatic activities of the primary metabolism are regulated by Systemin at early time-points.

In the proteomic analysis we observed that Systemin produced major changes at the primary metabolism. To have a better understanding of how these pathways are modified by the peptide treatment, we performed a time-course analysis of enzymatic activities in the central metabolism at 6, 24 and 48h after *Botrytis cinerea* inoculation in Systemin-treated plants.

In the absence of infection, the strongest impact of Systemin treatment was observed at 6hpi (which corresponds to 30 hours after treatment), affecting the activity of enzymes from the central metabolism and photosynthesis (Figure 5 and 7), whereas its impact was reduced to control levels at later time points (24h and 48h) in all enzyme activities affected by the Systemin treatment. Thus, in the absence of infection we will comment only the changes found in the enzymatic activities at 6hpi since at a later time points the changes were not statistically significant. At 6hpi, Systemin treatment induced an increase in the photosynthesis measured by the higher activity of the RuBisCo enzyme (Figure 5). The proteomic data suggested that the surplus of photoassimilates were being

used to synthesize other metabolites rather than energy reserves and this hypothesis was reinforced by the enzymatic data. Regarding the major carbohydrate metabolism, neutral invertase and fructokinase activities were higher in Systemin treated samples. Neutral invertase catalyzes the degradation of sucrose into glucose and fructose while fructokinase (FK) catalyzes the conversion of fructose in fructose-6-phosphate which enters to the hexose phosphate pool. Furthermore, the enzymes responsible for maintaining the hexose phosphate pool; phosphoglucose isomerase (PGI) and phosphoglucomutase (PGM) were also higher in Systemin-treated samples (Figure 5 and 7) indicating that there was higher availability of hexose phosphate in those samples.

On the other hand, since the photosynthesis increased in Systemin-treated samples, the content of triose phosphates, specifically 3-phosphoglycerate (3-PGA) in these samples is expected to be higher. 3-PGA can either follow the catabolic glycolysis pathway and be converted to 2-PGA by the PGA mutase or be diverted towards the triose phosphate pool by phosphorylation to 1,3 bisphosphoglycerate (1,3-BPGA). Here we found a higher phosphoglycerokinase (PGK) activity in Systemin-treated plants, this enzyme catalyzes the conversion of 1,3-BPGA to glyceraldehyde-3-phosphate. Since the PGA mutase was under-accumulated at the proteomic level, the higher activity of phosphoglycerokinase in Systemin-treated samples was rather expected. Following the central metabolism, in the absence of infection, Systemin treatment triggered the degradation of pyruvate by entering to the TCA cycle by the pyruvate kinase activity or transaminated to alanine by the alanine aminotransferase (AlaAT) enzyme. As for the TCA cycle, both citrate synthase and malate dehydrogenase activities were high upon Systemin treatment. These results reinforced the hypothesis that the TCA cycle in this case supplies carbon skeletons to other metabolic pathways rather than providing energy.

Most of the enzymatic activities that were found to be upregulated by Systemin without infection at an early time point (6h), supports the results obtained at the proteomic level. The induction of enzymatic activities produced by Systemin at the early time points were reduced along time. Activity levels of Systemin-treated plants at 48h were similar to those of control plants or even lower. This suggests that Systemin impact on the enzymatic regulation of the central metabolism is very fast and wears off within hours.

In contrast with the previous results, when the infection occurs, most enzymatic activities do not change or are downregulated in Systemin treated plants compared to control plants,

especially at the latest time point (48hpi) (Figure 6 and 7). Regarding the major carbohydrate metabolism, most of the enzymatic activities upregulated before the infection in Systemin treated plants did not show differences between control and Systemin treated samples after the challenge. The exceptions to this profile were both hexokinases belonging to the glycolysis metabolism, fructokinase (FK) and glucokinase (GK). They remain upregulated by Systemin treatment upon infection at 48 hpi. These results suggest again that treated plants are producing a surplus of available sugars that could be provided for other purposes, since they seem not to be provided for glycolysis. Indeed, the glycolysis enzymatic activities of phosphofructokinase, FBP aldolase, NAD-GAPDH, PK, and PEPC did not show differences between both samples at 6 and 24 hpi but were higher in control plants at 48hpi. Regarding the TCA cycle, the citrate synthase activity also showed the same activity level in both samples at 6 and 24 hpi and raised at 48 hpi in control plants compared to Systemin treated plants. Remarkably, the AlaAT and Aspartate transferase (AspAT) activities also followed the same pattern that the glycolysis activities after the challenge, that is, there were no significant differences between the two samples at 6 and 24 hpi but at 48 hpi control plants showed higher activity than Sys treated plants. (Fig 6, 7). Upon infection, enzymatic activities of both control and Systemin treated plants do not show much difference at early time points. On the contrary, at 48 hpi, control plants showed higher activities in primary metabolism than Systemin-treated plants, especially in glycolysis, TCA and amino acid metabolism. However, the infection rate in Systemin-treated plants indicated that they performed better than control plants upon infection. This indicates that the metabolism of Systemin-treated plants may have been conditioned before the challenge so that they responded to the infection earlier than non-treated plants at the enzymatic level.

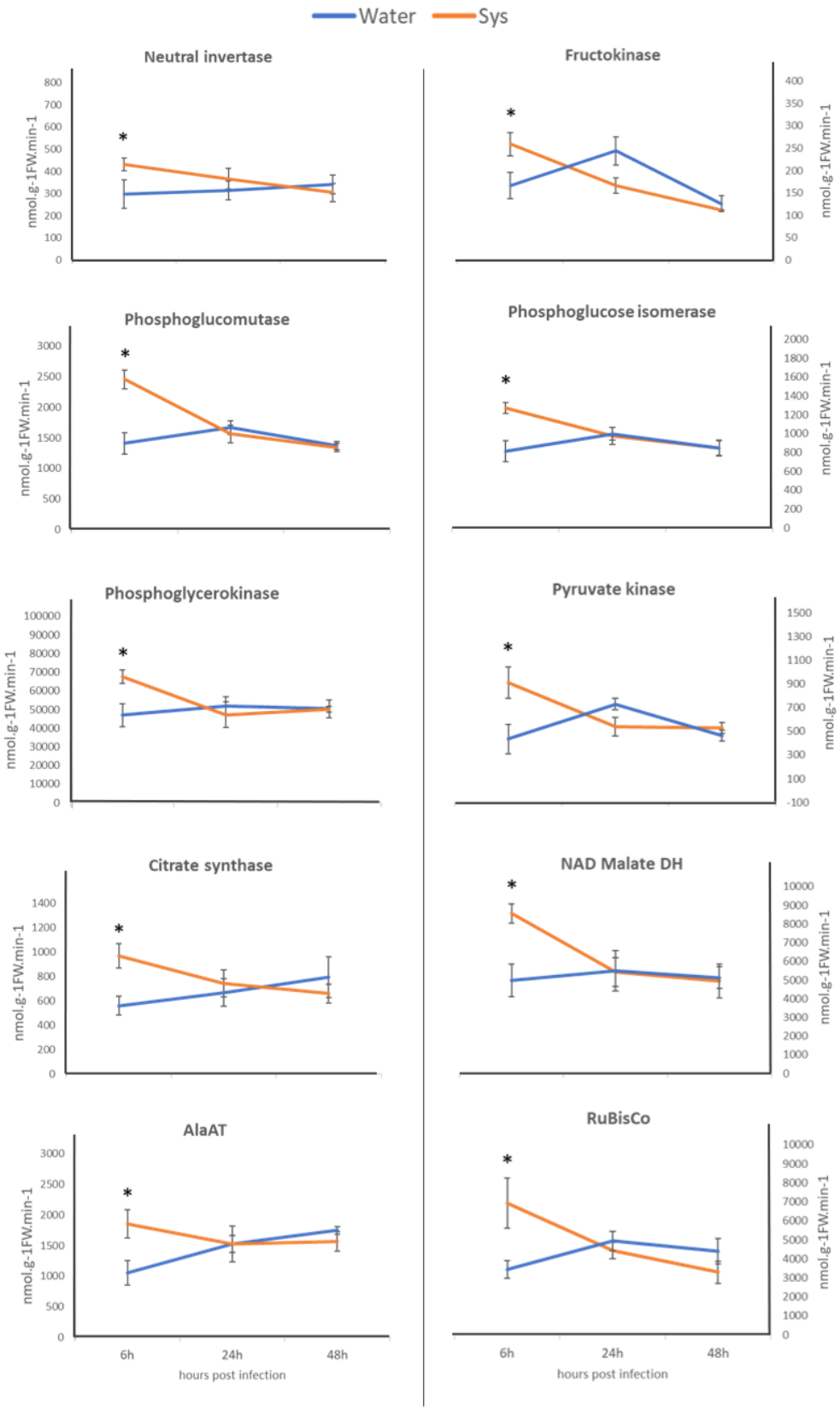


Figure 5. Systemin treatment impact on enzymatic activities of the primary metabolism. *Time-course of enzymatic activities in water and Systemin-treated plants at 6, 24 and 48hpi. Asterisk indicates statistically significant differences by a Student t test ($p<0.05$, $n=6$)*

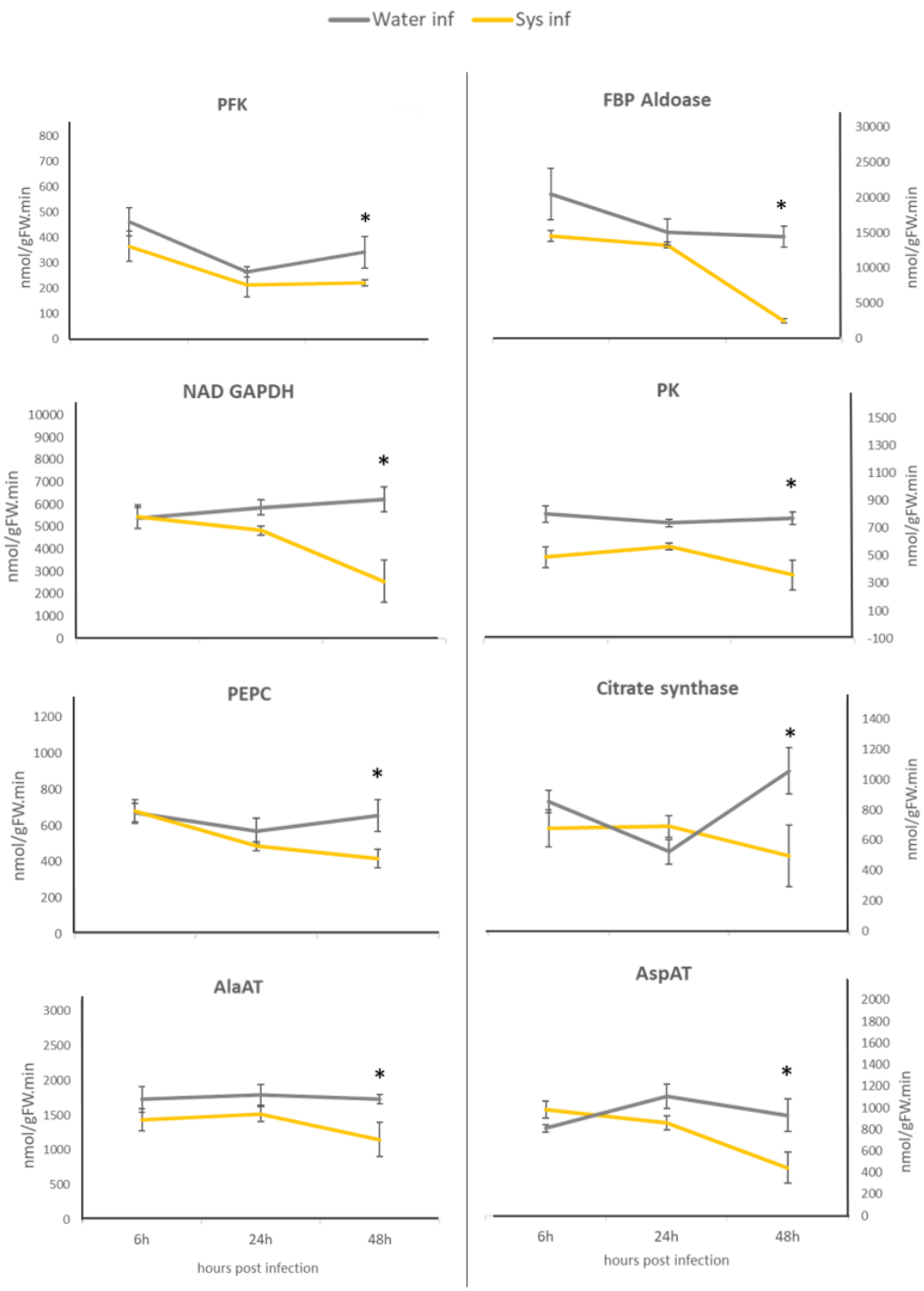
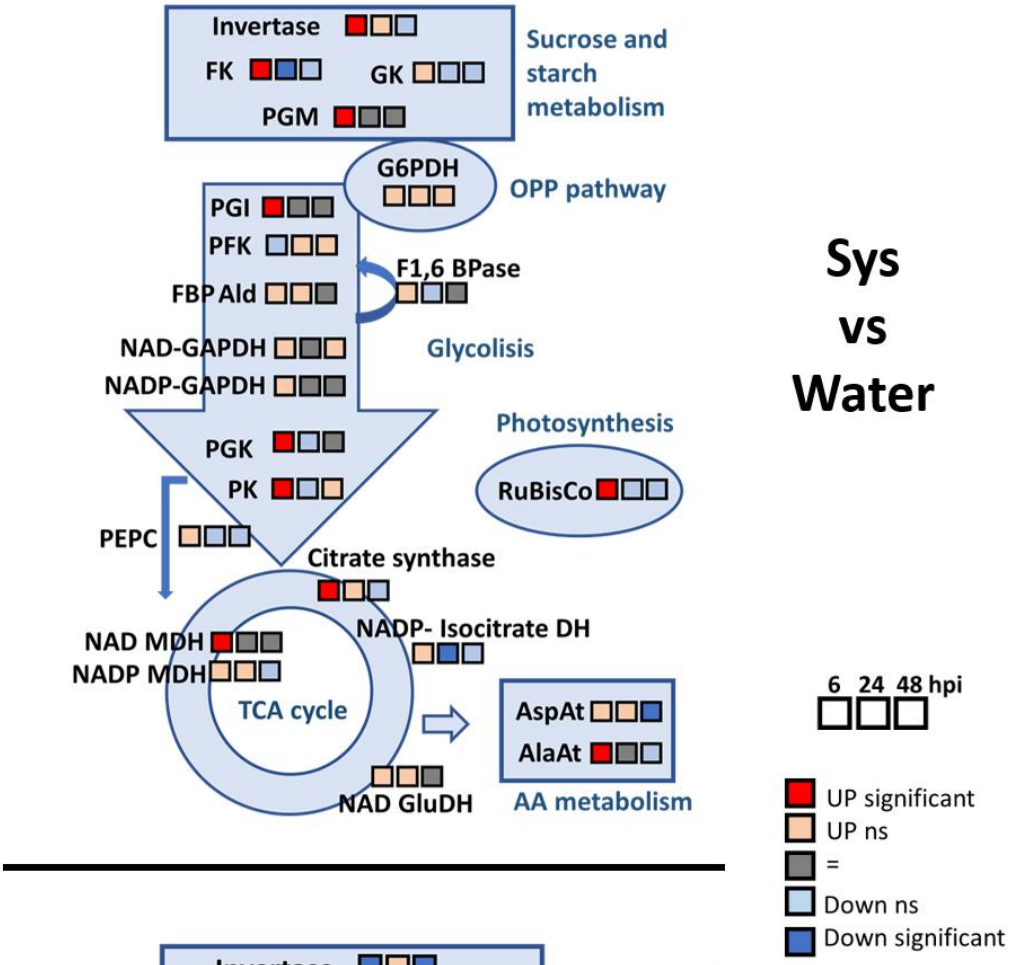


Figure 6. Enzymatic activities profile on Systemin-treated plants upon infection. *Time-course of enzymatic activities in water and Systemin-treated infected plants at 6, 24 and 48hpi. Asterisk indicates statistically significant differences by a Student t test ($p<0.05$, $n=6$)*


Invertase
FK
GK
PGM
Sucrose and starch metabolism
G6PDH
OPP pathway
PGI
PFK
FBP Ald
F1,6 BPase
NAD-GAPDH
NADP-GAPDH
Glycolisis
Photosynthesis
PGK
PK
RuBisCo
PEPC
Citrate synthase
NADP- Isocitrate DH
NAD MDH
NADP MDH
TCA cycle
AspAt
AlaAt
NAD GluDH
AA metabolism
Sys vs Water
6 24 48 hpi
UP significant
UP ns
=
Down ns
Down significant

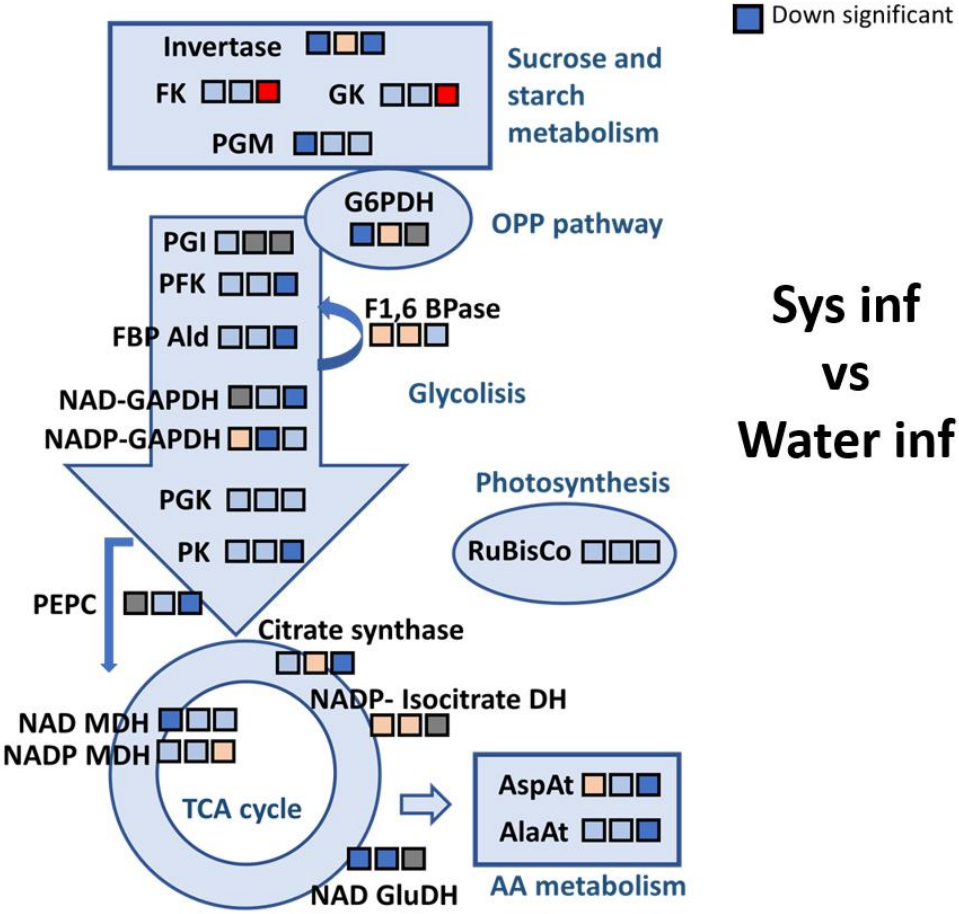

Invertase
FK
GK
PGM
Sucrose and starch metabolism
G6PDH
OPP pathway
PGI
PFK
FBP Ald
F1,6 BPase
NAD-GAPDH
NADP-GAPDH
Glycolisis
Photosynthesis
PGK
PK
RuBisCo
PEPC
Citrate synthase
NADP- Isocitrate DH
NAD MDH
NADP MDH
TCA cycle
AspAt
AlaAt
NAD GluDH
AA metabolism
Sys inf vs Water inf

Figure 7. Overall impact of Systemin treatment on enzymatic activities in the primary metabolism. *Time-course of enzymatic activities in the absence (left) or the presence (right) of infection. Protein accumulation is measures as a Log_2 fold change of Systemin over water-treated plants. Color legend is indicated in the figure. Statistical significance is analyzed by performing a t Student test ($p < 0.05$, n=6).*

Specific changes in the metabolome during Systemin-IR

Proteomic and enzymatic activity studies indicated a significant changes following Systemin treatments. To better understand the pattern observed in the sPLSDA we performed a heatmap analysis of the metabolomic data (Figure S2). Despite the sPLSDA analysis suggesting a priming profile (Fig 2), a heatmap analysis revealed a significant number of changes occurring due to the Systemin treatment in the absence of infection. Hence, Systemin clearly modifies the plant metabolism at protein and metabolite levels (Fig 2A and Fig S3). Using Metaboanalyst software, we performed an ANOVA to select those features with significant changes. A heatmap analysis of these features showed three major groups of metabolites. Firstly, those showing a priming profile, these are features over accumulated in Systemin-treated infected plants. Secondly, another group of compounds that antagonises or buffers the changes imposed by the infection, accumulated in water-treated plants following infection but unaltered or even less accumulated in Systemin-treated infected plants (Figure S2). And finally, a set of compounds that are directly induced by the Systemin irrespective of the infection. Either using internal libraries of standards or by contrasting exact mass and fragmentation spectrum using the Pubchem database, we identified two sulphur-containing amino acids, a hydroxycinnamic acid, a flavonoid and two alkaloids (Fig 8 and Table S2) that changed in abundance after Systemin treatment. Cysteine, methionine and hesperetin-7-O-glcoside were induced by Systemin treatments whereas the other three identified compounds followed a priming profile. Note that the alkaloids were identified by matching RT, parental exact mass and at least 3 fragments with a mass deviation lower than 0.05, since for alkaloids identified as thebaine and ajmalicine, so far there were no reports of their presence in tomato plants, they were assigned only as alkaloids.

In addition, we also found 12 compounds that either were induced by Systemin or followed a priming profile (Fig S3), however we were not able to identify them. Other relevant set of compounds included those that were induced by the infection in control

plants but remained unchanged or even reduced in Systemin-treated plants (Fig. S4). In this case, Systemin had a likely buffering effect on this set of metabolites indicating either these metabolites were not of utmost importance to mount an effective resistance, or the Systemin-treated plants performed better upon the challenge and were less stressed. Considering the latter, the plants did not need to invest more energy accumulating those metabolites. Unfortunately, we did not succeed in identifying such compounds.

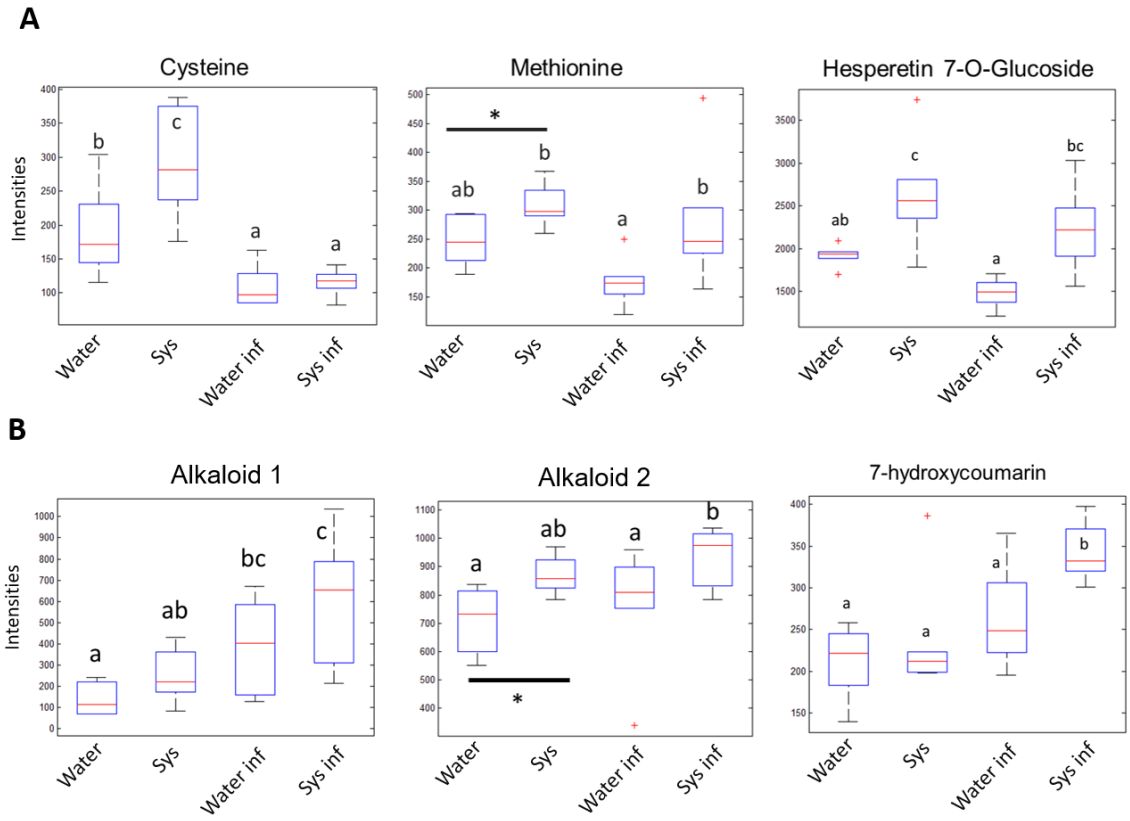


Figure 8. Identified metabolites induced and primed by Systemin. *Boxplots of identified metabolites using internal libraries or online databases in water or Systemin-treated plants in the absence and presence of infection with Botrytis cinerea. (A) Metabolites that were induced by the Systemin treatment. (B) Metabolites showing a priming profile. Different letters indicate statistically significant differences (ANOVA, Fisher's least significant difference (LSD) test; $p < 0.05$ n=6). Asterisk indicates statistically significant differences by a Student t test ($p<0.05$, n=6)*

Systemin Impact in the plant metabolism is not associated with fitness cost

The direct induction of many proteins and metabolites following Systemin treatment in the absence of infection lead us to hypothesise that plant fitness may be affected by the peptide treatment. For this reason, we measured some physiological parameters including plant height, leaf area, and leaf fresh and dry weight at different time points after Systemin treatment (Figure S5). Considering the fast growth of tomato plants, we determined growth parameters in the range between 4 and 15dpt. It is noticeable that plants almost doubled the height and leaf area along this period. All measured physiological parameters showed no differences between control and Systemin treated plants (Figure S5). In fact, there was a positive non-significant trend due to Systemin treatments in most measured parameters. These results suggest there is no fitness cost associated with the Systemin treatments in spite of the impact that it has on the plant primary metabolism.

Discussion

Exogenous Systemin treatments induce an array of defense-related responses in tomato plants including the accumulation of proteinase inhibitors (PI) in response to wounding, an increase in ethylene biosynthesis, and the induction of oxidative burst (Pearce et al., 1991; Felix & Boller 1995; Ryan 2000). Recent studies suggested that Systemin can be perceived not only by tomato plants but also in phylogenetically distant species such as Arabidopsis depicting an enhanced resistance to necrotrophic pathogens (Pastor-Fernandez et al., 2020). Intending to decipher the molecular mechanisms behind the Sys-IR against the necrotrophic fungi in tomato plants, we performed proteomic, metabolomic and enzymatic analysis to have a holistic overview of the Systemin-induced responses.

As previously reported, exogenous Systemin treatment protected tomato plants against *B. cinerea*. Priming of callose accumulation is a quick defense response shown to be of utmost importance in several IR (Flors et al. 2008; Maekawa et al. 2014). Recently some papers have described the pivotal role of starch degradation in providing sugar availability in the priming of callose accumulation (Gamir et al., 2018, Sanmartin et al., 2020). Thus, we hypothesised that Systemin treatment might enhance the availability of sugars for callose accumulation through a faster starch degradation. Indeed the enhanced protection of Systemin-treated plants involved the priming of callose accumulation and a more rapid starch degradation rate as a likely source of carbohydrates.

Systemin treatment induced changes of the proteomic and metabolic profile. However, the two omics layers showed contrasting behaviour. Metabolomics revealed a general priming profile with some metabolites displaying an enhanced accumulation or buffering the effect of infection in Systemin-treated plants. Surprisingly, the main changes in the proteomic profile were due to the Systemin treatment itself rather than the infection. The protein changes after the treatment before the fungal infection were much bigger than after the challenge where only 14 proteins were affected. These results could explain the better preparation of Systemin-treated plants for a subsequent pathogen attack.

A common trait after a pathogen infection is the alteration in photosynthetic activity as well as a modulation of the host carbohydrate metabolism. Alterations in photosynthesis and sugar metabolism play a key role in governing the outcome of plant-pathogen interaction (Kanwar and Jha, 2019). Necrotrophic pathogens form chlorotic and necrotic areas that lead to a decrease in photosynthesis. Whether this photosynthetic reduction is merely the consequence of the necrosis areas or a plant defense strategy to limit the sugar availability to fungal growth is not clear. In the present study, the over-accumulation of photosynthetic proteins and the enhanced activity of Rubisco found in Systemin-treated samples could increase the energy and the number of available photoassimilates helping the plant to overcome the challenge once it is present. In this sense, some studies reported a severe inhibition of photosynthetic activity in the direct vicinity of the infection sites but also a stimulation of the photosynthesis surrounding the infection in tomato plants challenged with *B. cinerea* (Berger et al. 2007; Smith et al. 2014).

Regarding the Sys-IR, Rocco et al (2008) studied the proteome profile of ProSystemin overexpressing tobacco plants and found alterations in the major carbon/energy metabolism, specifically ATP synthase, starch synthase and some light reaction proteins. In our work, Systemin treatment produced an enhancement of photosynthesis and starch metabolism that likely leads to a surplus of available sugars. This fact is not reflected in an increase in the glycolysis metabolism, suggesting that these sugars could be used for other purposes. Indeed, a starch phosphorylase, which prepares it for degradation and a GL3, involved in callose synthesis were also upregulated by the treatment, suggesting that these free sugars could be ready to be allocated for callose accumulation as well as for other biosynthetic pathways to provide defense-related metabolites. This hypothesis is supported not only by the proteomic data but also by enzymatic activities found in

Systemin-treated plants. Invertase and fructokinase activities as well as those enzymes responsible for maintaining the hexose phosphate pool were upregulated by Systemin.

The link between carbohydrate metabolism and defense responses has been largely studied (reviewed by Kanwar and Jha, 2018). In Arabidopsis plants, the induction of PR1 and PR5 by glucose is dependent on the hexokinase activity through the glucose signalling-glycolysis-dependent pathway (Xiao et al. 2000). We show that Systemin treatment leads to an enhanced starch degradation and an over accumulation of hexokinase. Interestingly once the challenge is present, of the few proteins differentially affected in Systemin treated plants there were three over accumulated pathogenesis-related leaf proteins (PR1B1, PR4 and PR2). Going back to the carbohydrate metabolism, Systemin treatment resulted in a general down accumulation of the glycolysis-related proteins including GPI and PGM proteins. Considering the pivotal role of both fructose-6-phosphate and 3-PGA in plant metabolism (O'Leary and Plaxton 2020, Duminil et al., 2021) it is not surprising that both proteins catalyzing downstream glucose metabolism were highly regulated in our experiment. Likely, the entrance of both hexoses phosphate and trioses phosphate to the glycolysis towards the pyruvate formation is attenuated in Systemin treated plants compared to control plants.

Besides supporting higher energy demand during biotic challenges, TCA intermediates have been linked to defense priming in several plant-pathosystems. They act as defense signals inducing changes in defense gene expression or providing carbon skeletons for biosynthesis of defense metabolites (Wellen et al., 2009, Gauthier et al., 2010, Finkemeier et al., 2013). In the present study both, enzymatic activity of pyruvate kinase and protein accumulation of the pyruvate dehydrogenase complex were high after Systemin treatment suggesting that the entrance to the TCA cycle was facilitated in Systemin-treated plants. But again, the results indicate that the main output of TCA cycle in response to the treatment is the production of carbon backbones rather than generation of reducing energy. The enzymes catalysing further steps into the TCA cycle were down accumulated except for malate dehydrogenase and the citrate synthase activity suggesting that the plant is facilitating the accumulation of some TCA intermediates such as oxaloacetate, 2-oxoglutarate, and succinate. These intermediates could also diverge to the biosynthesis of other metabolites. Considering the proteomic data, one of these biosynthetic pathways is likely the biosynthesis of specific amino acids. Note that 2-oxoglutarate and oxaloacetate

are the precursors for the biosynthesis of amino acids of the glutamate and aspartate family, respectively (Sweetlove et al., 2010). Indeed, the biosynthesis of glutamate from ornithine is enhanced by Systemin whereas its degradation to proline is inhibited. Glutamic acid has been proposed as a signal molecule in defense responses (Goto et al., 2020).

In addition, phosphoserine aminotransferase which is part of the serine biosynthesis pathway (Wulfert et al. 2018) was over-accumulated by Systemin. Serine is the precursor of cysteine which in turn is the precursor of methionine. In the present study, two S-adenosylmethionine synthases were found over accumulated, indicating that methionine is being activated to S-adenosylmethionine (SAM), which is the most important donor of methyl group in many methylation reactions and also serves as a precursor in the formation of ethylene and polyamines (Loenen et al., 2018). Our metabolomic data also supports this hypothesis since both cysteine and methionine were increased in Systemin treated samples (Figure 8). Altogether, the conditioning of the primary metabolism observed after Systemin treatment, may enhance further metabolic defensive responses.

Once the challenge is present, all the differentially accumulated proteins between control and Systemin treated plants were defense-related proteins. Apart from the three PR proteins commented above, there was also a Guanine nucleotide-binding protein alpha-1 subunit (GPA), a G protein involved in defense signalling (Llorente *et al.*, 2005; Sanchez-Vallet *et al.*, 2010) and a leucine amino transferase (LapA). We already stated the major role of the G-proteins in Arabidopsis Sys-IR against necrotrophs (chapter 2) which reinforce the hypothesis that Systemin is activating PTI responses that are common in different plant species. On the other hand, according to previous reports, the Leucine aminopeptidase is very likely to be involved in the last step of Systemin processing, leading to the release of the active peptide (Belishistov et al., 2018). This suggests that Systemin could be inducing its own processing and release in order to keep amplifying the danger signalling. This mechanism of a positive feedback loop for the release of Systemin is a key feature of phytocytokines (reviewed in the introduction).

Systemin perception triggers several defense signalling cascades including ROS accumulation among others (Ryan 2000). Rocco et al. (2008) analysing the proteome of tobacco plants overexpressing ProSystemin gene and found over-accumulation of proteins related to oxidative stress; superoxide dismutases and a glutathione S-

transferase. In our study a large cluster of redox proteins was affected by Systemin treatment such as thioredoxin, ascorbate and glutathione peroxidases or glutaredoxin (Figure 4). Many studies have shown the association between defence responses in primary metabolism and redox metabolism (Xiao et al, 2000). Moreover, many of the carbohydrate metabolism proteins found to be affected by the Systemin treatment are known to be thioredoxin targets or redox regulated proteins (Montrichard, 2009; Balmant et al., 2015; O'Leary and Plaxton, 2020).

Since Systemin is a phytocytokine, in response to the Systemin exogenous treatment, plants are expected to activate specific metabolism affecting those pathways related to defense such as hormone signalling and biosynthesis and secondary metabolism. However, most of the changes we found were in proteins of the primary metabolism, such as photosynthesis, redox, lipids and carbohydrate metabolism, TCA cycle and cell wall. The changes of the protein abundance related to the primary metabolism observed in our study may be providing carbon structures to improve the defensive response once the challenge is present. These observations are indicative that when Systemin is perceived as a DAMP by the surroundings, cells not yet under attack, can better prepare for the upcoming infection. However, the synergistic effect of the DAMP and the pathogen, generates alternative responses directly related to defense metabolism. This hypothesis is also supported by the accumulation of enzymatic activities, which are in concordance with the proteomic data, and by the metabolomic data showing an accumulation of some amino acids and the priming profile of the identified phenolic compounds and alkaloids. Furthermore, among the few proteins that overaccumulated after Systemin treatment, related to the secondary metabolism, are two key proteins involved in the phytoalexin biosynthesis, the 4-coumarate-CoA ligase and chalcone-flavonone isomerase (Table 1). Copplola et al. (2015) in a transcriptomic analysis of the tomato transgenic lines overexpressing ProSystemin, also observed an upregulation of phenylpropanoids and flavonoids related genes. However, they reported that ProSystemin overexpression downregulates genes connected with carbon fixation and carbohydrate metabolism as well as photosynthesis. Some Systemin responses are conserved across plant families. The expression of ProSystemin gene in tobacco or Arabidopsis triggers similar responses to Systemin treatment in tomato plants. Similarly, when the Systemin receptor was cloned into Arabidopsis and tobacco plants Systemin-triggered responses were similar to those found in tomato (Wang et al., 2018).

There is a lack of agreement in the literature regarding the fitness cost of Sys-IR. Corrado et al (2011) using transgenic plants over-expressing the Systemin precursor prosystemin, found that Sys-IR in that context was costly to tomato plants whereas Luna-Martinez et al. (2021) using the same system reported even an improved plant production and yield in the overexpressing lines. Here, Systemin induced the over accumulation of many proteins and metabolites by itself in the absence of infection, however no differences in the physiological parameters measured were found between Systemin-treated and control plants.

In our study, we gathered evidence of the mechanisms behind Sys-IR by exogenously applied Systemin. As a secondary DAMP, it exhibits the typical responses of a danger signal perception, enhancing its own signal amplification and even mimicking an infection response either in the surrounding cells not yet attacked (Systemin's direct effects) or in cells directly infected (defense and priming reponses). This may explain why Systemin has such strong proteomic impact before the infection whereas after the infection, the major changes occured only at the metabolic level. According to our enzymatic, metabolomic and proteomic results, it is likely that Systemin treatment is perceived as a secondary DAMP and then conditioning the plant metabolism for a subsequent attack. This conditioning before the challenge takes place mainly on the primary metabolism providing more available sugars to fuel the defense responses by directly using them to strengthen the cell wall at the pathogen entrance site and/or providing carbon backbones to the biosynthesis of secondary metabolism.

Supplementary information

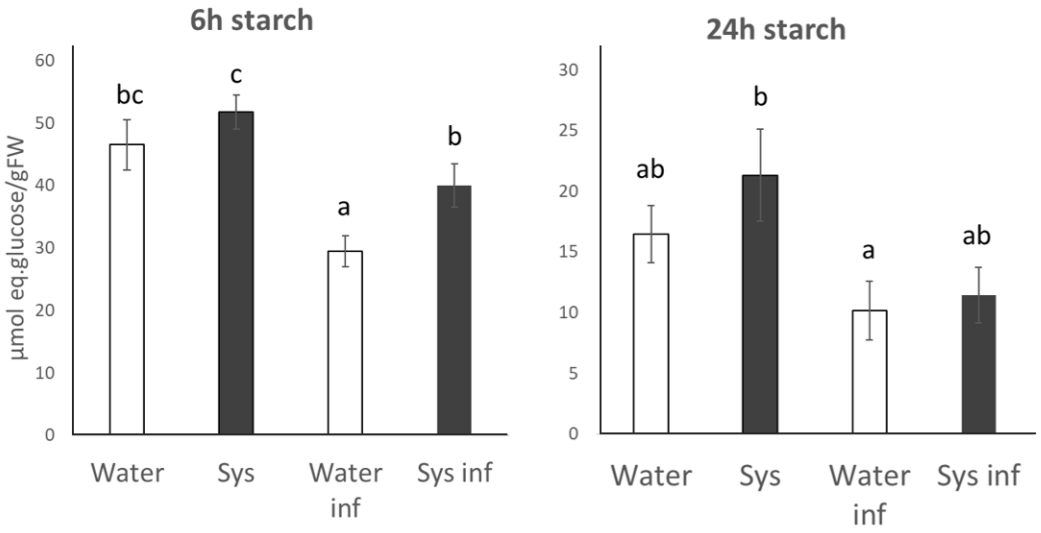


Figure S1. Starch amount. *Starch accumulation 6 and 24hpi in Water and Systemin-treated plants in the absence and presence of infection. Different letters indicate statistically significant differences (ANOVA, Fisher's least significant difference (LSD) test; $p < 0.05$ n=6).*

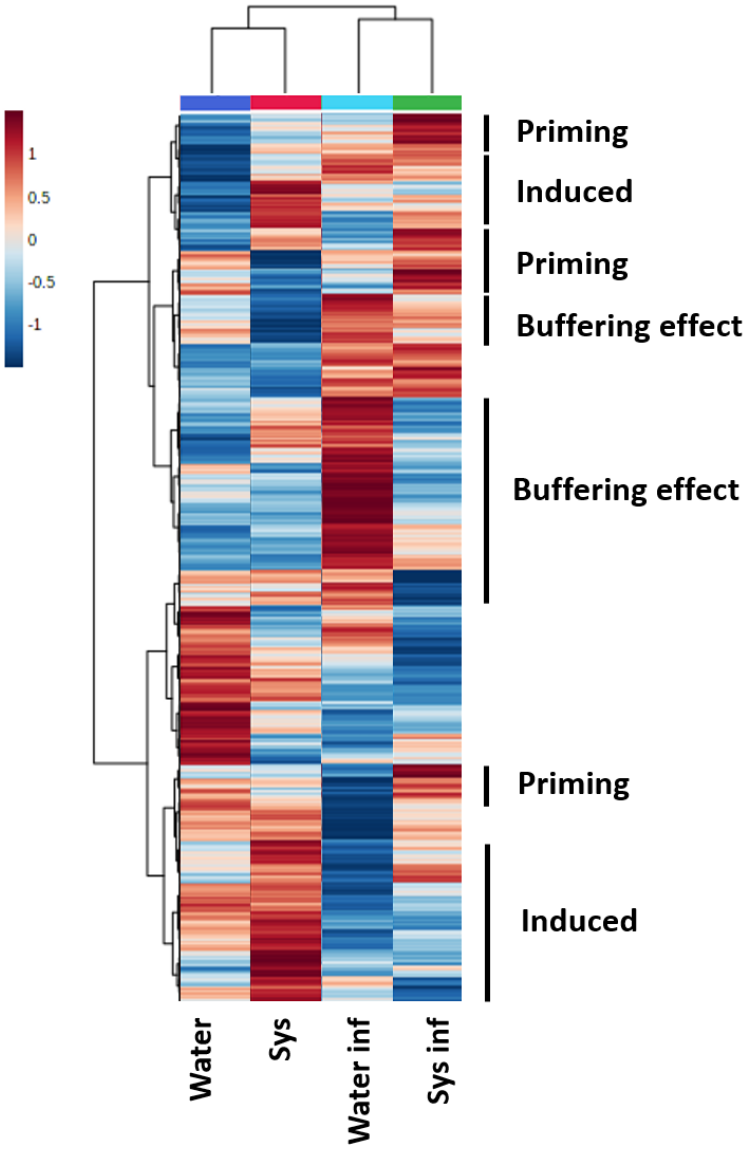


Figure S2. Heatmap analysis of the metabolomic data. *Heatmap analysis of the metabolomic data performed with Metaboanalyst online software using a Pearson clustering, of water and Systemin treated plants before and after the infection (24hpi). Red color shows overaccumulated metabolites whereas blue color indicates downaccumulated metabolites. Clusters of compounds showing a specific profile are indicated.*

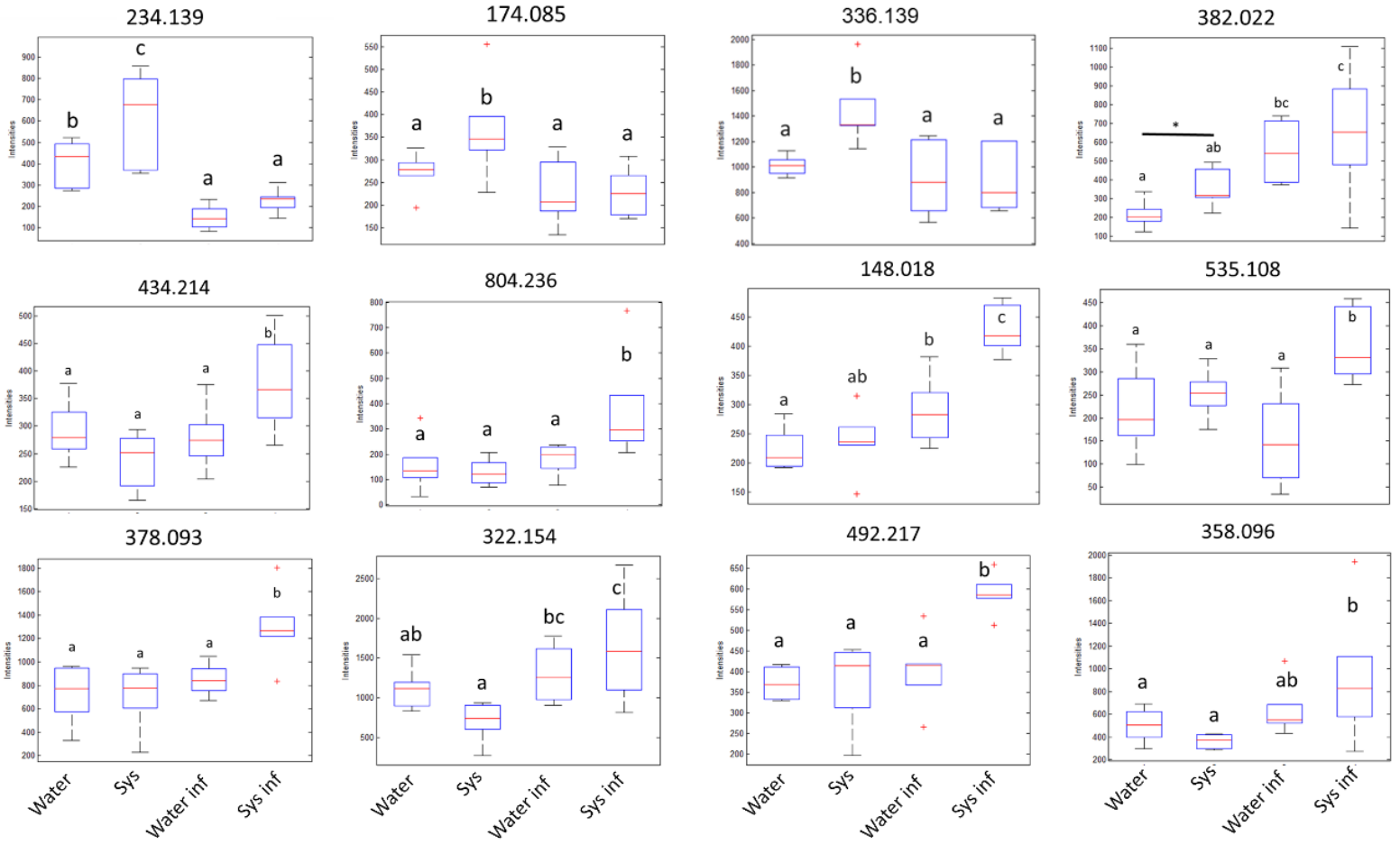


Figure S3. Systemin induced and primed compounds*. Boxplots of exact mases of compounds that were either induced or primed by the Sytemin treatment.* *Different letters indicate statistically significant differences (ANOVA, Fisher's least significant difference (LSD) test; $p < 0.05$ n=6).*

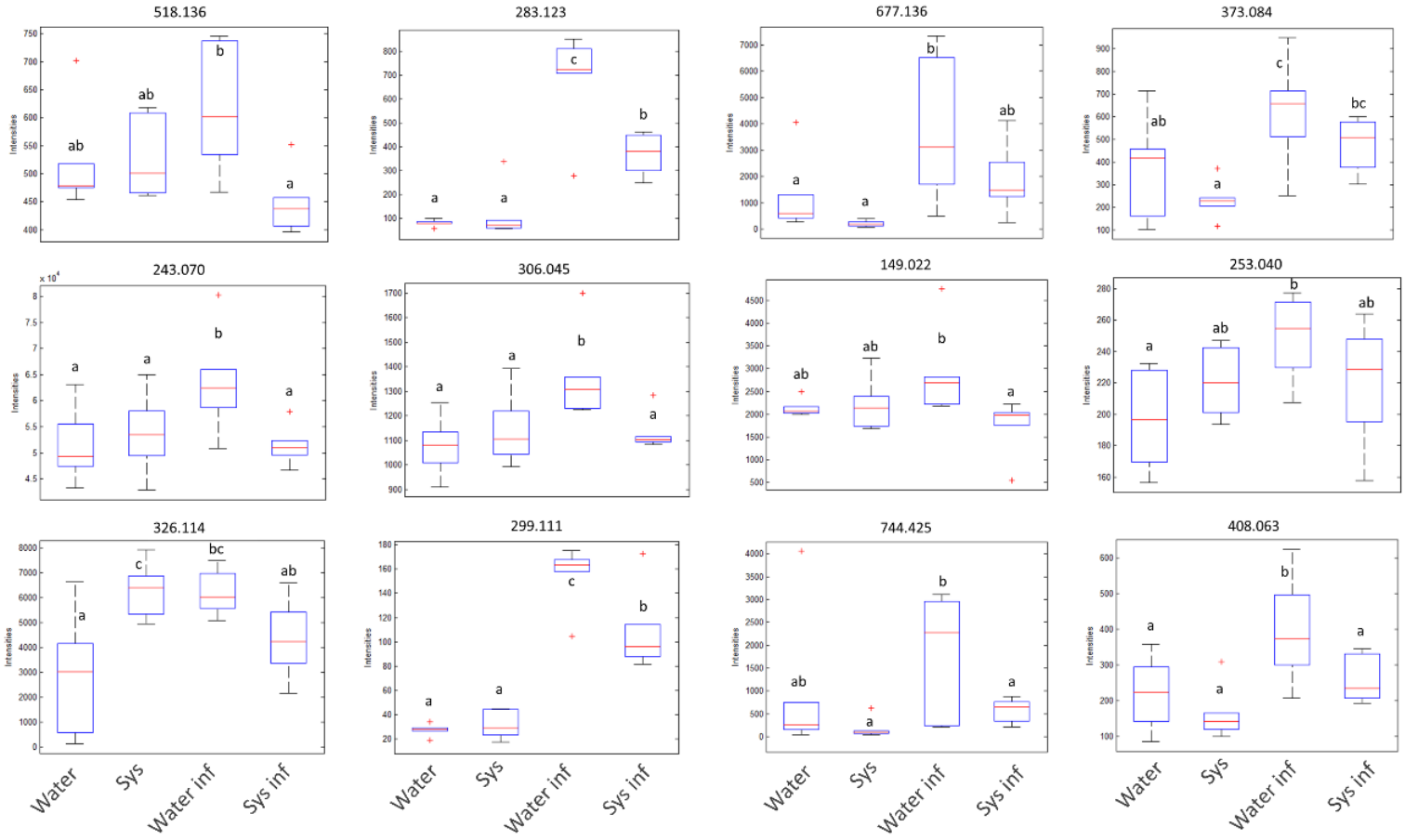


Figure S4. Compounds showing a buffering effect. *Boxplots of exact masses of compounds that were induced by the infection in water-treated plants but in systemin terated plants.* *Different letters indicate statistically significant differences (ANOVA, Fisher's least significant difference (LSD) test; $p < 0.05$ n=6).*

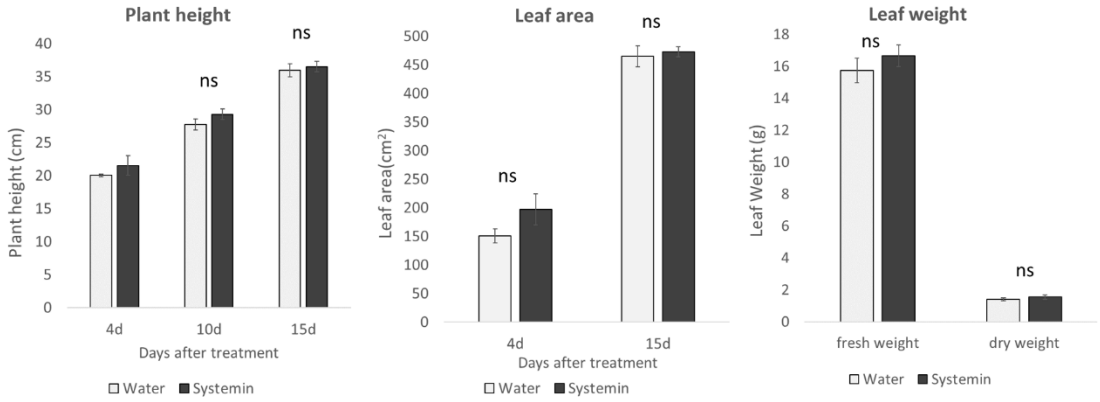


Figure S5. Measurement of physiological parameters in Systemin treated plants. *Measurements of plant height 4, 5 and 10 days after Systemin treatment; leaf area 4 and 25 days post treatment; and leaf fresh and dry weight 10 days after treatment. "ns" indicates no statistically significant differences (Student t test; p>0.05).*

Table S1. Proteins accumulation in Systemin-treated plants vs water-treated plants

ID	PATHWAY	ACCESION	DESCRIPTION	LOG2 FOLD CHANGE SYS/WATER
	Photosynthesis			
1	Lightreaction: photosystem II	solyc02g071000.1.1	Chlorophyll a/b binding protein 21	**1.799251**
2	Lightreaction: photosystem II	solyc07g047850.2.1	Chlorophyll a-b binding protein 36	**1.1472156**
3	Lightreaction: photosystem II	solyc10g007690.2.1	Chlorophyll binding protein 8	-1.0860567
4	Lightreaction: photosystem II	solyc06g065490.2.1	PsbP domain-containing protein 6	**1.2631848**
5	Lightreaction: photosystem I	solyc08g067840.2.1	PsbP domain-containing protein 5	**1.0622873**
6	Lightreaction: photosystem I	solyc10g017900.1.1	Photosystem I P700 chlorophyll a apoprotein	-1.2890962
7	Lightreaction: photosystem I	solyc06g083680.2.1	Photosystem I reaction center subunit IV A	**1.652199**
8	Lightreaction: photosystem I	solyc12g062610.1.1	Photosystem I P700 chlorophyll a apoprotein A2	-1.1194069
9	Lightreaction: photosystem I	solyc01g017320.1.1	Photosystem I P700 chlorophyll a apoprotein A1	-2.3040235
10	Lightreaction: photosystem I	solyc06g009940.1.1	Photosystem I P700 chlorophyll a apoprotein A1	-1.6079141
11	Lightreaction: ATP synthase	solyc06g065990.1.1	ATP synthase B subunit	**1.8127897**
12	Lightreaction: ATP synthase	solyc10g044540.1.1	ATP synthase alpha subunit	**1.6527498**
13	Lightreaction: Electron transport	solyc02g011780.1.1	NAD(P)H-quinone oxidoreductase subunit I chloroplastic	**1.672294**
14	Photorespiration	solyc06g061070.2.1	Glycine cleavage system H protein 1	**1.8795954**
15	Photorespiration	solyc03g120430.2.1	Glycerate kinase	-1.148419
16	Calvin cycle	solyc02g077860.1.1	Rubisco large subunit	1.2846563

17	Calvin cycle	solyc05g013160.2.1	Rubisco methyltransferase domain	-1.2507219
	Major CHO metabolism			
18	Sucrose synthesis	solyc09g092130.2.1	Sucrose phosphate synthase	-1.2238266
19	Sucrose degradation	solyc03g121070.2.1	Hexokinase	2.2445405
20	Starch degradation	solyc05g012510.2.1	Glycogen/starch/alpha-glucan phosphorylase	1.8076328
	Minor CHO metabolism			
21	Callose	solyc07g061920.2.1	Glucan synthase like 3	**2.331288**
22	Galactose	solyc04g008730.2.1	Alpha-galactosidase 1	-1.0451916
23	Galactose	solyc06g050130.2.1	Alpha-galactosidase-like protein	**2.3519113**
24	Others	solyc09g064240.2.1	Carbohydrate/purine kinase	-1.4602267
25	Others	solyc10g083710.1.1	3-deoxy-D-manno-octulosonic acid transferase-like protein	-1.4618655
26	Others	solyc09g015070.2.1	Reductase 1	**1.1913769**
	Cell wall			
27	Precursor synthesis	solyc03g096730.2.1	GDP-D-mannose pyrophosphorylase 1	-6.511614
28	Cellulose synthesis	solyc04g077470.2.1	Cellulose synthase-like protein	-1.0881197
29	Cellulose synthesis	solyc11g005560.1.1	Cellulose synthase	**1.106453**
30	Cellulose synthesis	solyc07g051820.2.1	Cellulose synthase	-1.784867
31	Modification	solyc02g081210.2.1	Expansin	**1.3893999**
32	Modification	solyc01g010890.2.1	Expansin	**1.034454**
33	Modification	solyc01g112000.2.1	Expansin-like protein	**1.2709222**
	Glycolisis			
34	Cytosolic branch	solyc04g076830.2.1	Phosphoglycerate mutase-like protein	**2.1763897**
35	Plastid branch	solyc12g014380.1.1	Glucose-6-phosphate isomerase 1	-1.4652609
36	Plastid branch	solyc04g076090.2.1	Glucose-6-phosphate isomerase 2	-7.0318556
37	Unclear/dually targeted	solyc12g014380.1.1	Glucose-6-phosphate isomerase 1	-1.4652609
38	Unclear/dually targeted	solyc04g076090.2.1	Glucose-6-phosphate isomerase 2	-7.0318556
39	Unclear/dually targeted	solyc02g078240.2.1	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	-2.0949664
40	Unclear/dually targeted	solyc06g074510.2.1	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2	-2.1560962
	TCA			
41	Organic acids transformation	solyc06g072580.2.1	Pyruvate dehydrogenase E1 component subunit beta	**1.0153985**
42	Organic acids transformation	solyc12g005080.1.1	Component of 2-oxoglutarate dehydrogenase complex	-2.8222432
43	Organic acids transformation	solyc07g006790.2.1	Ccomponent of pyruvate dehydrogenase complex	**2.586331**
44	Organic acids transformation	solyc05g009530.2.1	Ccomponent of pyruvate dehydrogenase complex	**1.9615886**

45	Organic acids transformation	solyc12g005080.1.1	Component of 2-oxoglutarate dehydrogenase complex	-2.8222432
46	Organic acids transformation	solyc05g054640.2.1	Component of 2-oxoglutarate dehydrogenase complex	-1.5444036
47	Organic acids transformation	solyc02g092730.2.1	Iron sulfur subunit of succinate dehydrogenase	-2.3247886
48	Organic acids transformation	solyc03g115990.1.1	Malate dehydrogenase	**2.001904**
49	Organic acids transformation	solyc08g013860.2.1	NAD-dependent malic enzyme 2	-1.9301001
	Amino acids metabolism			
50	Synthesis: central amino acid metabolism	solyc03g123610.2.1	Alanine aminotransferase	-1.305662
51	Synthesis: central amino acid metabolism	solyc03g123600.2.1	Alanine aminotransferase	**1.7524977**
52	Synthesis: glutamate family	solyc06g019170.2.1	Gamma-glutamyl phosphate reductase	-2.3973768
53	Synthesis: glutamate family	solyc12g089210.1.1	Ornithine carbamoyltransferase	**1.4314361**
54	Synthesis: aspartate family	solyc01g101060.2.1	S-adenosylmethionine synthase	**1.3134227**
55	Synthesis: aspartate family	solyc09g008280.1.1	S-adenosylmethionine synthase	**1.1019828**
56	Synthesis: branched chain group	solyc03g044330.1.1	Acetolactate synthase	-3.2334764
57	Synthesis: branched chain group	solyc02g091970.2.1	Branched-chain-amino-acid aminotransferase	**1.2210523**
58	Synthesis: branched chain group	solyc08g014130.2.1	2-isopropylmalate synthase 1	**1.4348718**
59	Synthesis: serine-glycine-cysteine group	solyc02g082830.1.1	Phosphoserine aminotransferase	**1.3936011**
60	Synthesis: serine-glycine-cysteine group	solyc01g097920.2.1	Cysteine synthase	**1.6985775**
61	Degradation: glutamate family	solyc06g071000.2.1	N-succinylglutamate 5-semialdehyde dehydrogenase	**1.9331796**
	Redox			
62	Thioredoxin	solyc01g100320.2.1	Thioredoxin/protein disulfide isomerase	-2.059238
63	Thioredoxin	solyc02g068500.2.1	Thioredoxin o	**1.6857018**
64	Thioredoxin	solyc04g071560.2.1	Thioredoxin y	-1.2537582
65	Thioredoxin	solyc01g108020.2.1	Thioredoxin m	**1.40866**
66	Ascorbate	solyc06g005160.2.1	Ascorbate peroxidase	**1.3288596**
67	Ascorbate	solyc08g081530.2.1	Reductase	**2.152831**
68	Ascorbate	solyc06g005150.2.1	Ascorbate peroxidase	**1.0791879**
69	Glutathione	solyc08g006720.2.1	Glutathione peroxidase	**1.1790127**
70	Glutathione	solyc01g100360.2.1	Dihydrolipoyl dehydrogenase	**1.7428**
71	Heme	solyc03g071690.2.1	Non-symbiotic hemoglobin 2	-1.6505129
72	Glutaredoxins	solyc06g082170.2.1	Glutaredoxin family protein	**2.792332**
73	Dismutases and catalases	solyc01g100630.1.1	Catalase	-1.9458207
	Lipids metabolism			
74	FA synthesis and elongation	solyc06g068010.2.1	Biotin carboxyl carrier protein of acetyl-CoA carboxylase	**1.0652397**

75	FA synthesis and elongation	solyc02g070790.2.1	Ketoacyl ACP synthase	-1.151206
76	FA synthesis and elongation	solyc08g016170.2.1	Ketoacyl ACP synthase	**1.0398574**
77	FA synthesis and elongation	solyc08g075800.1.1	Acyl coa ligase	-5.329569
78	FA synthesis and elongation	solyc01g088570.2.1	ACP protein	**1.7863034**
79	FA synthesis and elongation	solyc01g100360.2.1	Pyruvate DH	**1.7428**
80	Phospholipid synthesis	solyc10g008020.2.1	Methyltransferase	-1.6654131
81	Phospholipid synthesis	solyc08g076470.2.1	Glycerol-3-phosphate acyltransferase	-1.0022142
82	Multifunctional	solyc04g078920.2.1	Peroxisomal multifunctional enzyme type 2	**1.2717104**
83	Lipid degradation	solyc02g077110.2.1	Lipase-like protein	-1.7270311
	Secondary metabolism			
84	Isoprenoids	solyc04g049290.1.1	Prenyltransferase/ zinc ion binding protein	-1.9536098
85	Isoprenoids	solyc04g056390.2.1	Isopentenyl-diphosphate delta-isomerase	-1.3734972
86	Isoprenoids	solyc01g097810.2.1	Zeta-carotene desaturase	-1.1538819
87	Phenylpropanoids	solyc12g094520.1.1	4-coumarate-coa ligase	**4.0142264**
88	Phenylpropanoids	solyc06g064500.2.1	O-methyltransferase	-2.1209066
89	Flavonoids	solyc07g062030.2.1	Chalcone-flavonone isomerase	**2.084783**

Table S2. Chemical features used for compounds identification

Compound name	Molecular formula	RT	Ionization mode	Parental mass (Da)	Fragments (Da)
Cysteine	$C_3H_7NO_2S$	0.44	ESI+	122.028	102.096>74.057>72.095
Methionine	$C_5H_{11}NO_2S$	0.46	ESI+	150.059	-
Hesperetin-7-O-glucoside	$C_{22}H_{24}O_{11}$	7.47	ESI-	463.163	305.092
Thebaine (Alkaloid 1)	$C_{29}H_{21}NO_3$	4.21	ESI+	312.129	251.085>236.086>205.064 >190.075>178.035
Ajmalicine (Alkaloid 2)	$C_{21}H_{24}N_2O_3$	6.31	ESI+	353.232	355.163>354.182>353.192
7-hydroxycoumarin	$C_9H_6O_3$	4.75	ESI-	161.023	162.025>161.038>133.022

Table S3. Log2 fold change of enzymatic activities in the absence (Sys/water) or the presence of infection (Sys inf/Water inf). *Values in bold indicate statistically significant differences (Student t test, $p<0.05$, $n=6$)*

	Sys/Water			**Sys inf/Water inf**		
	6h	**24h**	**48h**	**6h**	**24h**	**48h**
Invertase	**0.54**	0.23	-0.16	-0.77	0.30	-0.52
GK	0.87	-0.51	-0.62	-0.42	-0.51	**1.11**
FK	**0.63**	**-0.55**	-0.18	-0.37	-0.10	**0.54**
PGM	**0.80**	-0.09	-0.03	**-0.52**	-0.29	-1.51
PGI	**0.65**	-0.03	0.00	**-0.35**	-0.06	-0.02
G6PDH	1.32	1.20	0.44	**-1.98**	0.50	-0.08
PFK	-0.72	0.88	1.03	-0.34	-0.30	**-0.63**
F1,6BPase	0.22	-0.90	0.03	**0.67**	**0.12**	-0.40
FBPAdoase	0.28	0.26	-0.08	**-0.50**	-0.19	**-2.57**
NAD-GADPDH	0.30	0.08	0.30	0.01	-0.28	**-1.28**
NADP-GADPDH	0.55	0.08	0.07	0.20	**-0.83**	**-0.76**
PGK	**0.53**	-0.14	-0.01	-0.36	-0.12	-0.20
PK	**1.12**	**-0.28**	0.34	**-0.53**	**-0.24**	**-0.84**
PEPC	0.20	-0.11	-0.37	0.01	-0.23	**-0.65**
Citrate syntase	**0.79**	0.16	-0.27	-0.24	0.50	**-1.00**
NAD-MDH	**0.78**	-0.02	-0.05	**-0.59**	-0.12	**-0.13**
NADP-MDH	0.23	0.61	-0.53	-0.37	-0.20	0.32
NADP-IsocitrateDH	0.15	**-1.87**	-0.24	0.17	0.26	-0.06
NAD-GluDH	0.12	0.29	0.02	**-3.06**	**-0.96**	**-0.03**
AspAT	0.25	0.13	**-1.22**	**0.28**	-0.36	**-1.07**
AlaAT	**0.82**	0.00	-0.15	-0.27	-0.24	**-0.59**
RubisCo	**1.01**	-0.16	-0.42	**-0.41**	-0.47	-0.59

CHAPTER 4

MPKs are essential in the mediation of Systemin-Induced resistance against *Botrytis cinerea*

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Abstract

Previously, we showed that Systemin induces resistance against *Botrytis cinerea* through a metabolic rearrangement at the proteomic and metabolomic levels. However, the early signalling elements that ensure and enable downstream responses upon Systemin treatment remain elusive. Here we investigate the role of MPK in the mediation of Systemin-triggered responses and Systemin-induced resistance (Sys-IR) against *Botrytis cinerea*. For that purpose, we used the Virus-Induced Gene Silencing (VIGS) technique. First, we confirmed that exogenous Systemin treatments induce MPK1, 2 and 3 gene expression and protein accumulation and conversely, Systemin-induced MPK 1/2 and 3 gene expression is abolished in silenced plants. In addition, we observed that expression of Systemin responsive genes was reduced when MPK was impaired. Then, to decipher the importance of MPKs in the Systemin signalling upon infection we tested the levels of phosphorylated MPKs upon fungal elicitor challenge. Noteworthy, we observed that activation of MPKs upon challenge was primed by the Systemin treatment. Moreover, MPK-silenced were not protected by Systemin against *B. cinerea* infection. Finally, we observed that Systemin induction of defence gene expression upon infection was impaired in the silenced plants. These results demonstrate that MPKs play a key role as early signalling elements in Sys-IR.

Keywords: MPKs, Systemin, Induced-Resistance, VIGS

Introduction

Signalling of many stresses in plants relies on Mitogen-activated Protein Kinases (MAPKs or MPKs) cascades, from plant growth and development to plant disease resistance (Meng and Zhang 2013; Xu et al., 2015). Regarding biotic stresses, plants' perception of pathogens via Pattern recognition receptors (PRRs), triggers Pattern triggered immunity (PTI) which activates MAPK, as one of the earliest events activated during PTI (Yu et al., 2017). Together with Pathogen- associated molecular patterns (PAMPs), endogenous danger molecules, termed damage-associated molecular patterns (DAMPS) also trigger MAPK cascades activation to amplify the plant immune response (Hou et al., 2019). Additionally, they are also important components of ETI (Pedley and Martin, 2005). MAPK cascades consist of three interdependent kinases, the MAPK

kinase kinases (MAPKKK) which activate MAPK kinases (MAPKK) by phosphorylation which, in turn, activates the MAPK (Ichimura et al., 2002; Jonak et al., 2002). They act as converging points between pattern recognition and downstream defense responses, such as defense gene activation, cell wall strengthening, phytoalexin biosynthesis and induced resistance (Meng and Zhang, 2013).

In fact, in tobacco, SA-induced protein kinase (SIPK) and wounding -induced protein kinase (WIPK), two MAPKs implicated in tobacco resistance to biotic stresses, phosphorylate a WRKY transcription factor to activate defense responses (Jin et al., 2003; Ishihama et al., 2011). In Arabidopsis MPK3, MPK6, MPK4 and MPK11 are known to participate in signalling of plant defense responses, which are activated upon PAMP challenge (Meng et al., 2013). AtMAPKs regulate defense gene expression and phytoalexin biosynthesis which mediates plant resistance to *Botrytis cinerea* infection (Ren et al., 2008; Tsuda et al., 2013). On the contrary, some MPKs negatively regulate defence against *Pseudomonas syringae* (Frei dit Frey et al., 2014) suggesting alternative roles of MPKs in plants interacting with pathogens. On the other hand, MPK3 and MPK6 were shown to regulate defense priming in Arabidopsis (Beckers et al., 2009). In tomato**,** *MAPK3*, the ortholog of *AtMPK3*, was also shown to be essential for resistance against the necrotrophic fungi *Botrytis cinerea* through regulating defense enzymes and SA/JA-related genes (Zhang et al., 2018).

MAPKs are also part of the signalling triggered events by Systemin in the tomato response to wounding and herbivory (Ryan et al., 2000). Systemin is a tomato phytocytokine that regulates herbivore response and defense to pathogens (Pearce and Ryan 1991; Coppola et al., 2015). Moreover, it functions as a potent resistance elicitor against a variety of attackers in several plant species (Coppola et al., 2019; Pastor-Fernández et al., 2020; Molisso et al., 2020). Systemin binding to its receptor triggers a cascade of signalling events that eventually leads to defense gene activation and anti-herbivore defense. These early signalling events include an increase in ion fluxes and intracellular calcium (Felix and Boller, 1995; Moyen et al., 1998), production of reactive oxygen species (ROS; Orozco-cadenas, 1999; Wang et al., 2018) and activation of MAPKs (Stratmann and Ryan, 1997; Holley et al., 2003). Downstream, MAPKs activation, the phospholipase A and the allene oxide cyclase are activated (Narvaez-Vazquez et al., 1999; Stenzel et al., 2003), leading to the release of linolenic acid that is

converted through several oxylipin intermediates to Jasmonic Acid (JA). Ultimately, wound response genes are activated.

Several studies report the importance of MAPKs in the Systemin-triggered signalling cascade. Systemin supply to cut stems elicited the activity of a myelin basic protein kinase (MBPK) in *Solanum lycopersicum* plants, a member of the mitogen-activated protein kinases (MAPKs) in a similar extent to wounding, polygalacturonic acid and chitosan elicitors and herbivore feeding (Stratmann and Ryan, 1997). Similarly, Systemin induces MAPK activity when supplied to *Lycopersicum peruvianum* suspension cells. Two MPKs with high sequence homology, MPK1 and MPK2, but not MPK3, were activated by Systemin (Holley et al., 2003). Later it was demonstrated that MPK1 and MPK2 are essential for ProSystemin-mediated defenses against the herbivorous insect *Manduca sexta* (Kadoth et al., 2007). More insights on Systemin signalins were found in tobacco cells and calli, that also respond to Systemin supply by MAPK activation (Malinowski et al., 2009). Very recently, we also demonstrated that Systemin induced a higher phosphorylation of MAPK in Systemin non-producing plant species such as Arabidopsis in response to a PAMP challenge (see chapter 2).

As mentioned before, defense gene signalling in response to wounding and Systemin is mediated by the octadecanoid pathway (Constabel et al., 1995; Sun et al 2011). Systemin-induced synthesis of JA leads to the expression of defense genes, including protease inhibitors (PIs; Pearce et al 1991; Pearce, 2011). PIs interfere with insect gut digestive proteases, so they play a key role in the plant resistance against feeding hervibores (Orozco-Cardenas et al., 1993). These proteins also have antimicrobial properties (Giudici et al., 2000). Among other effects, Systemin mediates the production of JA, which upregulates the expression of the Systemin precursor gene, ProSystemin, leading to a second wave of JA production, resulting in a positive feedback loop (Lee and Howe, 2003; Sun et al., 2011).

On the other hand, ProSystemin overexpression (PS+) upregulates genes and metabolites of multiple signalling pathways including stress-related pathways as well as genes involved in the primary metabolism such as in amino acid and carbohydrate metabolism, and it confers resistance to different biotic stresses (Coppola et al., 2015; Pastor et al., 2018). Recently, Coppola et al., 2019 showed that exogenously applied Systemin triggers the upregulation of *WOUND-INDUCED PROTEINASE INHIBITOR I* and *II* (*PIN-I* and

PIN-II), *ALLENE OXIDE SYNTHASE* (*AOS*) and *PROSYSTEMIN* (*PROSYS*) genes from 3h after Systemin supply both locally and systemically. reasonably, upregulatiuon of these genes may contribute to the observed Systemin-triggered enhanced resistance against biotic challenges. However, there are no studies reporting the Systemin-induced gene expression upon a pathogen infection, which would mediate the observed Systemin-induced resistance (Sys-IR).

MPKs play a crucial role in plant disease resistance and defence signalling. However, the implication of MAPK in the underlying mechanisms of induced resistance is not fully understood. Although the Systemin-triggered MAPKs and defense gene activation has been studied, the role of MPKs in the Systemin-mediated signalling upon a pathogenic challenge has not yet been reported. Previously we demonstrated that Systemin induces resistance against *Botrytis cinerea* in tomato plants through the induction of a rearrangement of the plant metabolism at different metabolic levels. On the other hand, the Systemin perception has been recently elucidated by several authors (Wang et al., 2018; Xu et al., 2018). However, how the Systemin signalling is enabled to ensure downstream defense responses is less understood. Here we aim to decipher the role of MPKs as early signalling events mediated Systemin-induced defense responses and Systemin-IR against *Botrtis cinerea.*

Materials and methods

Plant materials and growth conditions.

For all the experiments tomato plants of MoneyMaker cultivar (*Solanum lycopersicum cv MM*) were sown in 200cm^3 pots in a vermiculite substrate and grown in a growth chamber with a 16h/8h of day/night photoperiod at 24ºC during the day and 18ºC during the night, and about 60% of relative humidity. Plants were watered with Hoagland solution (Hoagland and Arnon, 1950) twice a week. Four weeks after germination plants were treated with Systemin by adding it to the substrate diluted in water reaching a final concentration of 20nM in the pot. The same amount of water was added to the control plants.

Plasmids construction and plant infiltration

pTRV1 and pTRV2_ev (empty vector) and PTRV2_PDS vectors were obtained from Dr Eduardo Rodríguez Bejarano (Universidad de Málaga, Spain). pTRV2-SlPDS was

digested using EcoRI and XhoI restriction enzymes, and the SlPDS fragment was replaced by a 290bp and 400bp PCR-amplified fragment of SlMPK1 or SlMPK3 respectively, both containing EcoRI and XhoI sites (see primer sequences in Table S1). Best target sequences were selected based on the VIGS tool in Solgenomics (http://vigs.solgenomics.net/; see Table S2 for selected target sequences). MPK1 target sequence was used for the cosilencing of both SlMPK1 and SlMPK2 (pTRV2_MPK1/2). pTRV1, pTRV2_ev, pTRV2-SlPDS, pTRV2-MPK1/2 and pTRV2_MPK3 were introduced into Agrobacterium tumefaciens strain GV3101 pmp90 by heatshock transformation of competent cells.

Agrobacterium tumefaciens strains were grown on Luria–Bertani (LB) liquid culture supplemented with 10mM MES and 20µM acetosyringone for 24 h at 28°C, with appropriate antibiotics. The bacterial cells were harvested by centrifugation and resuspended in an infiltration buffer (10 mM MgCl2, 10 mM MES, pH 5.7, 200µM acetosyringone and mQ sterile water) reaching a final optical density at 600 nm (OD600) of 0.5 each and kept for 2-3 h at room temperature in darkness. Strains containing pTRV1 were mixed in a 1 : 1 ratio with strains containing the pTRV2 derivatives before infiltration. Tomato plants having two true leaves were infiltrated in the cotyledons by applying pressure with a 1ml syringe and through the stems at the axillary level using a 1ml syringe with a needle.

RNA extraction and RT-qPCR analysis

Plant leaves were collected 24h postinoculation (24 hpi), ground in liquid nitrogen and stored at -80 °C. For RNA extraction, 1 ml of TRIzol was added to 90 mg of powdered leaves. The supernatant was collected and transferred to a new tube after centrifugation, and 0.22 ml of chloroform (CHCl3) was added. The samples were centrifuged again, and the supernatant was transferred into a new tube; 0.35 ml of isopropanol and 0.35 ml of 0.8 M citrate/1.2 mM NaCl were added and gently mixed. The supernatant was removed after centrifugation, and the pellet was washed twice with 70% EtOH. Finally, the pellets were dried and dissolved in nuclease-free water.

The RNA was cleaned using a DNAse kit (DNAse I, RNAse-free, Fisher Scientific). Then, cDNA was synthesized using a retrotranscription kit (PrimeScript RT reagent, Takara). Quantitative real-time PCR (qPCR) was performed using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific) and a StepOne instrument

(Applied Biosystems). The ΔCt method was used to analyse the gene expression data. The housekeeping gene ELONGATION FACTOR 1 (EF1) was used to normalise the expression values. The primer sequences of measured genes are shown in Supplementary Table 1.

Protein extraction and Western blot analysis

Plant third and fourth leaves were collected 24h after *Botrytis cinerea* infection in liquid nitrogen and then stored at -80ºC for subsequent proteomic analysis. Proteins were extracted from 100mg grinded fresh tissue by adding a protein extraction buffer in a 1:3 ratio (100 mg:300 µl). Buffer contained TBS, Triton, PMSF 1 mM, DTT 10 mM and 5ul/mlof a protease and phosphatase inhibitor cocktail (Sigma–Aldrich). Samples were mixed with vortex for 15" and incubated on ice for 10 min. Then, the supernatant was collected after centrifugation (10 min at maximum speed). Laemmli sample buffer was added to the protein extract, and the mix was boiled for 5 min. The protein concentration was determined via the Bradford method using BSA as a standard.

A protein gel was prepared containing 4.5 ml of 12% resolving gel and 1 ml of 5% stacking gel. The gel was run for 1 h 30' at 100-150 V with an adjusted amount of 20 µg of protein in each well. One gel was stained with colloidal Coomassie stain (Bio-Rad) following the manufacturer's instructions to check the quality of the protein extract. Then, another gel prepared under the same conditions was used for protein transfer to a nitrocellulose membrane and immunoblot analysis. Phospho-p42/p44 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Werfen) was used for immunodetection. Peroxidase-conjugated goat anti-rabbit IG (Sigma–Aldrich) secondary antibody was used for blotting, and the membranes were revealed using ECL Prime Western blotting Detection Reagents (Cytiva).

Fungus inoculation and infection quantification

Botrytis cinerea CECT2100 (Spanish collection of type cultures, Universidad de Valencia, Burjassot, Spain) was grown for 2 weeks in plates with PDA (potato dextrose agar) medium supplemented with tomato leaves (40 mg ml^{-1}). *Botrytis cinerea* spores were collected and pre-germinated in Gambor's B5 medium supplemented with 10 mM sucrose and 10 mM KH_2PO_4 for 2 h in the dark without shaking. Plant infection was performed on intact plants at 100% relative humidity as described by Vicedo *et al.* (2009).

24h after Systemin treatment plants' third and fourth leaves were inoculated by spraying them with a 10^6 ml^{-1} spore suspension.

72h after the pathogen inoculation leaves were collected to assess the phenotype analysis. Leaf necrosis was stained using lactophenol trypan blue as previously described (Flors et al., 2008). The infection levels were quantified using a damage rating, assigning numbers to different percentages of necrotic leaf surface (0 = healthy leaves; 1= leaves with less than 25% of necrotic surface; 2= leaves with a necrotic surface between 25-50%; 3= leaves with a necrotic surface between 50-75%; 4= leaves with more than 75% necrotic surface), according to previous reports (Luna et al., 2012; Agut et al., 2014; Wilkinson et al., 2018).

Statistical analysis

Bioassays included 6 biological replicates and each experiment was repeated 3 times. Graphs show the average of all experiments results represented with the standard error. Statgraphics-plus software for Windows V.5 (Statistical Graphics Corp., MD, USA) was used to determine the statistical analysis by one-way analysis of variance (ANOVA, Fisher's Least Significant Difference (LSD) test; $P < 0.05$ n=6). Different letters indicate statistically significant differences.

For the phenotype confirmation experiments using a disease rating, leaflets of the third and fourth leaves of 4 plants per treatment were analysed. Statgraphics-plus software was again used to perform the comparative analysis using a non-parametric Kruscal-Wallis test analyses (*$p<0.05$, n=4). Asterisk means statistically significant differences.

Results

Systemin-induced *MPK1, 2* and *3* gene expression in the absence of challenge is abolished in silenced tomato plants.

In the present study, we aim to decipher the role of MPKs in the mediation of Systemin signalling and Systemin-Induced Resistance in tomato plants against fungal pathogens. In Arabidopsis, *MPK3* and *6* are two kinases involved in defense responses and defense priming in Arabidopsis (Beckers et al., 2009). The *AtMPK3* ortholog in tomato is *SlMPK3*, whereas *AtMPK6* shares high sequence identity with both *SlMPK1* and *SlMPK2* in tomato (Kong et al., 2012). The three mentioned tomato MAPK share also high homology with two major stress-responsive MAPK in tobacco, SA-induced protein kinase (*SIPK*) and wounding -induced protein kinase (*WIPK*) (Zhang and klesing 2000; Holley et al., 2003). Thus, *MPK1, 2,* and *3* were selected as targets for silencing for subsequent analysis. As expected, exogenous treatments with Systemin significantly induced *MPK2* and *3* and supregulated *MPK1* in the absence of challenge (Fig 1).

To determine the relevance of MPKs signalling in Systemic-induced resistance, Virus Induced Gene Silencing (VIGS) technique optimised for tomato plants was implemented (Buendia et al., 2016). Two *Agrobacterium* expression vectors carrying the bipartite genome of tobacco rattle virus (pTRV1 and pTRV2) were used for the undergoing experiments (Liu et al., 2002). Giving the high sequence homology (95%) of *SlMPK1* and *SlMPK2* a construct containing a single target sequence was designed for the cosilensing of both genes (Table S1). Separately, a construct containing a target sequence for silencing *SlMPK3* was selected (Table S1).

Control plants were infiltrated with a pTRV2 empty vector (pTRV2_ev) in order to exclude the possibility that some of the observed effects are due to general defense responses triggered by TRV or *Agrobacterium.* Additionally, a group of plants was infiltrated with *Agrobacterium* containing a pTRV2 construct with a fragment of *PHYTOENE DESATURASE* gene (pTRV2_PDS), involved in carotenoid biosynthesis. These plants display a photobleaching phenotype in leaves and serve as controls to confirm VIGS efficiency and to track plant silenced tissue (Figure S1; Buendia et al., 2016). In our experiments, maximum levels of silencing were observed 21d after infiltration and the 6th and 7th leaves showed the highest photobleaching areas, thus the same leaves in MPKs silence plants were collected for subsequent molecular analysis.

Expression of MPKs genes was measured by RTqPCR in plants that were infiltrated with the MPK1/2 and MPK3 containing vectors respectively and compared with the empty vector infiltrated plants to confirm silencing levels. Empty vector infiltrated plants will be from now on referred to as control plants. With this technique a significant reduction of MPKs expression of around 60% was achieved for the three targeted genes (Figure S2). However, no phenotypic difference between silenced plants in comparison with control plants was observed by naked eye (Figure S2).

The *MPK1, 2* and *3* induction following Systemin treatments was lost in the silenced plants (Figure 1). In fact, expression levels of MPKs upon Systemin treatment were similar to those of water-treated control plants (Figure 1). These results confirm MPKs as exogenous Systemin-responsive signalling elements in tomato.

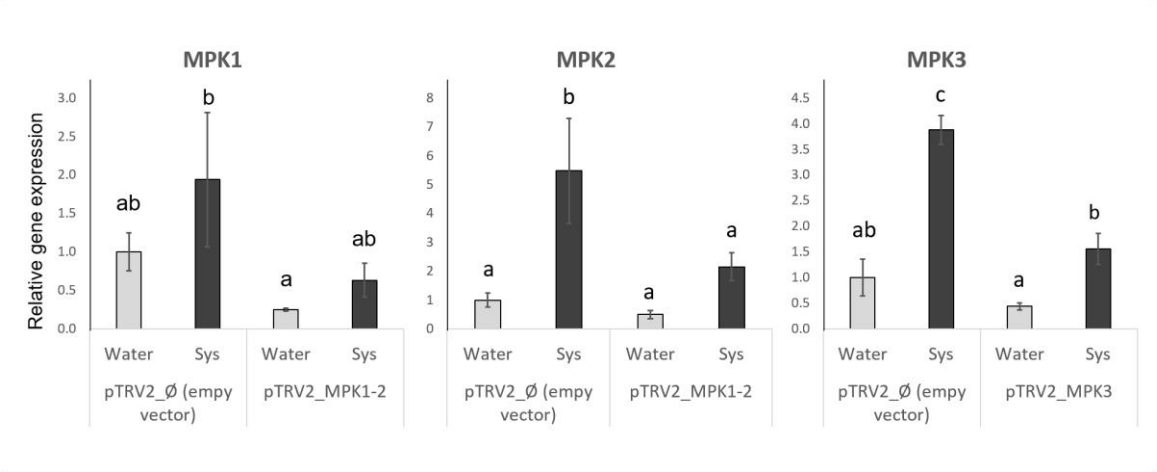

Figure 1. MPKs gene expression upon Systemin treatment in silenced plants*. RT-qPCR analysis of MPK1, MPK2 and MPK3 gene expression in tomato silenced leaves 24h after normally watering (Water) or Systemin treatment (Sys). Graphs represent averages of three experiments with standard errors each including 6 plants per treatment. Different letters indicate statistically significant differences (ANOVA, Fisher's least significant difference (LSD) test; P < 0.05 n=6).*

MPKs act upstream gene induction by the exogenous treatment of Systemin

To study the implication of MPKs in the Systemin downstream signalling we measured the expression of several defense genes of the octadecanoid pathway based on previous Systemin literature. Tomato plants with 4 fully developed leaves were treated with Systemin 20nM in the substrate and 24 after the treatment leaf tissue was collected for gene expression analysis. Three groups of genes were selected for analysis, two protease inhibitors, PINI and PIN II which are markers of JA signalling; 2 genes involved in the

biosynthesis of JA, LOXD and AOS; and two genes involved in the Systemin production and processing, its precursor protein ProSystemin and a Leucine Aminopeptidase likely involved in ProSystemin processing**.**

After 24h, Systemin treatment significantly induced PINI, LAPA and AOS in control plants, whereas the PINII, LOXD and PROSYS showed non-significant but increasing trend (Figure 2). In silenced plants this Systemin induction was no longer observed for most of the tested genes. Only LAPA remained significantly induced upon Sys treatment in MPK3-silenced plants although its expression was remarkably lower compared with Systemin-treated control plants (Figure 2). In addition, most gene expression levels were much lower in the silenced plants compared with control plants. Note that PINII, PROSYS and LOXD basal levels are lower in silenced plants irrespective of the Sys treatment. As in previous chapters we have observed that exogenous treatment of Systemin has an effect on the oxylipin plant metabolism. In addition, these results confirm that Systemin triggered induction of defense genes is MPK-dependent.

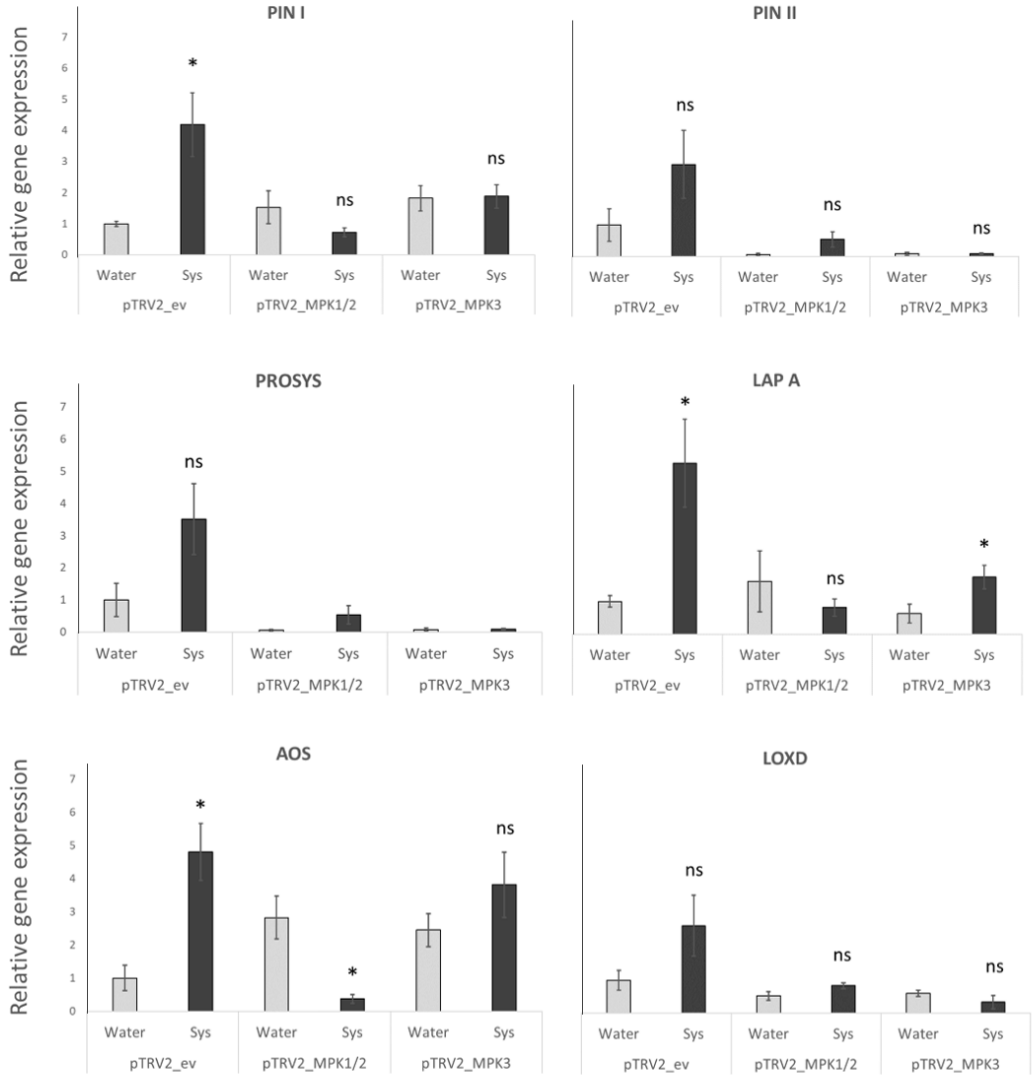

Figure 2. Gene expression analysis after Systemin treatment in silenced plants. *RT-qPCR analysis of PIN I, PIN II, PROSYS, LAP A, LOXD and AOS gene expression in tomato silenced leaves 24h after normally watering (Water) or Systemin treatment (Sys). Graphs represent averages of three experiments with standard errors each including 6 plants per treatment. Asterisks indicate statistically significant differences (Student t test; *p < 0.05 n=6); "ns" indicates no statistically significant difference.*

Systemin primes MPKs phosphorylation upon a biotic challenge

Although Systemin induction of MPK activity has been previously observed, there are no studies reporting the effect of Systemin treatment in MPK activation upon a challenge. Given the role of MPK in the induction of Systemin-responsive genes we speculate that MPK activation by phosphorylation might be important for Systemin-triggered responses upon biotic challenge. Therefore, we set up an experiment in which tomato plants were treated with a low and a high concentration of Systemin 24h before applying a PAMP challenge. Chitosan, a component of fungal cell wall, was used as the PAMP to mimic a fungal infection by spraying the leaves. Leaf tissue was collected in a time-course of 0, 15 and 60min after PAMP exposure. A western blot analysis using an antibody that binds to phosphorylated MPKs was performed.

Note, that Systemin either at 20 or 100 nM at 24h post treatments (0 h post chitosan challenge) did not induce direct phosphorylation of the proteins (Fig 3). The analysis showed that MPK phosphorylation was higher in Systemin-treated plants at 15min for both 20nm and 100nM Systemin concentrations in comparison with water-treated plants (Figure 3). For the 20nM treatment at 60min phosphorylation returned to basal levels. However, Systemin treatment at 100nM produced sustained induction in time, showing a higher phosphorylation level than water-treated plants even at 60min after PAMP challenge (Figure 3). Note that 100nM is a very high Systemin concentration that may result in some physiological alterations in the plant. Hence, Systemin treatment primes MPK phosphorylation upon a biotic challenge, indicating that these proteins and their phosphorylation are likely relevant in Systemin-IR.

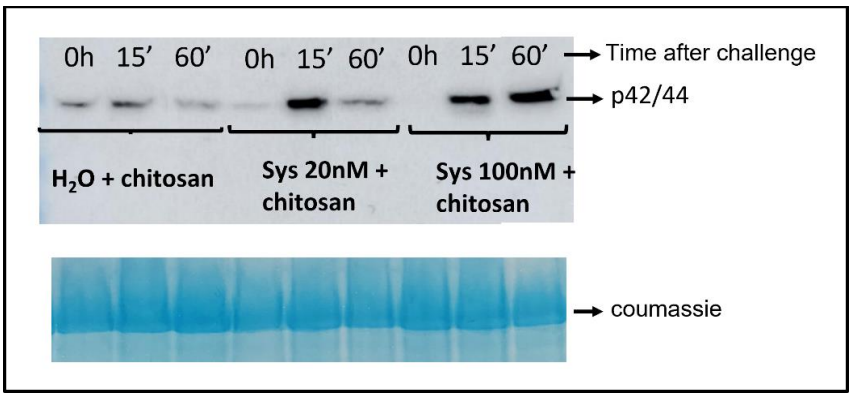

Figure 3. Measurement of MPK phosphorylation levels. *WB analysis of phosphorylated MPKs using the p42/44 antibody in tomato plants at 0 h, 15' and 60' after challenge with 0.05% chitosan*

solution 24h after water or treatment with either 20nM or 100 nM Systemin. Coumassie gel staining was used as a control of protein amount.

Silencing of MPK1/2 and 3 impaires a functional Systemin-Induced Resistance

In previous work, we have observed that Systemin is able to induce resistance against necrotrophic fungi in tomato plants. In addition, here we have shown that Systemin primes MPKs phosphorylation upon a chitosan challenge. Therefore we investigated whether the silencing of MPKs could have an impact on the Sys-IR phenotype observed in tomato plants. To follow this purpose tomato plants infiltrated with either the empty vector, the MPK1/2 or the MPK3 silencing constructs were treated with Systemin and 24h later inoculated with the necrotrophic fungus *Botrytis cinerea*. Disease quantification was performed 72h post-inoculation by measuring the necrotic areas in the leaves.

As expected, control plants, inoculated with the empty vector, showed enhanced protection against the fungus when previously treated with Systemin (Figure 4). However, the Sys-IR phenotype was lost in plants in which the MPKs were silenced (Figure 4). Silenced plants showed no symptomatology difference between water and Systemin treatments, suggesting that MPK1,2 and 3 are essential to express functional Sys-IR. Note that MPK3-silenced plants were more susceptible than control plants (Figure 4) irrespective of the treatment, demonstrating that MPK3 is essential for basal levels of resistance against *Botrytis cinerea* infection. This is in consonance with previously published work, where knock out MPK3 tomato plants showed enhanced susceptibility to the same fungus (Zhang et al., 2018).

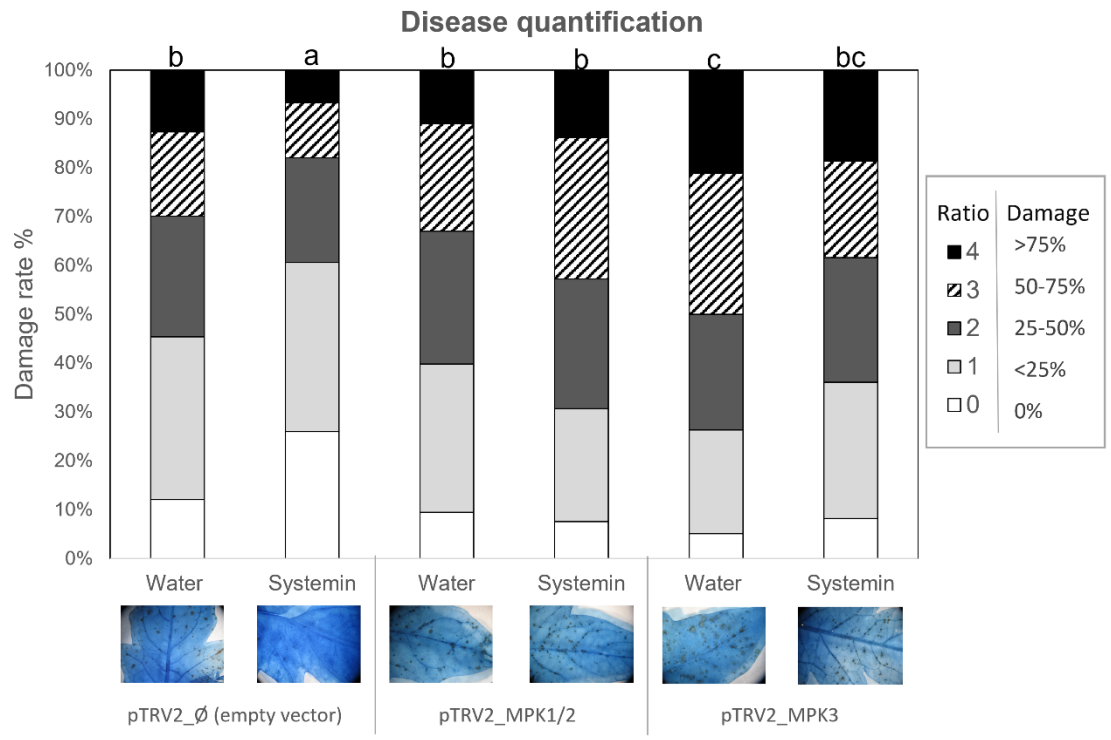

Figure 4. Induced Resistance bioassays in MPK silenced plants. *Infection levels of normally watered (Water) and Systemin-treated (Systemin) silenced plants quantified 3 days after infection (10^6 spores/ml) using a disease rating and trypan blue staining. Infection levels were measured using a scale based on the percentage of infected leaf surface (0 = 0%; 1= less than 25%; 2= 25-50%; 3= 50-75%, 4= more than 75%). Graphs represent averages of three experiments each including 4 plants per treatment and 2 infected leaves per plant. Asterisks indicate statistically significant differences performing Kruskal-wallis test (*P<0.05, n=4).*

MPKs mediate Systemin-induced gene expression of JA-related defenses upon infection

Earlier we showed how MPKs were required for the Systemin induction of some JA and defense-related genes. Later we have demonstrated that they are essential for the Sys-IR against *B. cinerea*. To better understand the possible function of MPKs in Systemin-induced resistance, we silenced MPK1/2 and 3 and challenged the plants with *B. cinerea* infection. Gene expression analysis of the main genes related to JA-dependent defences were analysed under infection conditions. The transcripts were assessed at 24 after pathogen inoculation.

Systemin treatment boosted the expression of all measured genes except for *AOS* (Figure 5) compared with control infected plants. Unlike the Systemin treatment alone, upon infection we observed a stronger Systemin-induction of genes, more remarkable for the protease inhibitors and Systemin production-related genes. These observations confirm the importance of JA-related defenses in the mechanisms underlying Sys-IR. Note that PROSYS and LAPA showed the highest induction levels showing the highest significance level (**$p<0.01$; Figure 5). As previously mentioned, these genes are involved in the Systemin production and release, indicative of a positive feedback loop for the release of Systemin to amplify the immune signalling response, a typical trait for DAMPs. On the other hand, silencing of both MPK1/2 and 3 resulted in a clear loss of the enhanced expression of genes triggered by the Systemin treatment (Figure 5). Therefore, here we demonstrate the key role of MPK in the Systemin-induced defense signalling cascade that leads to enhanced resistance.

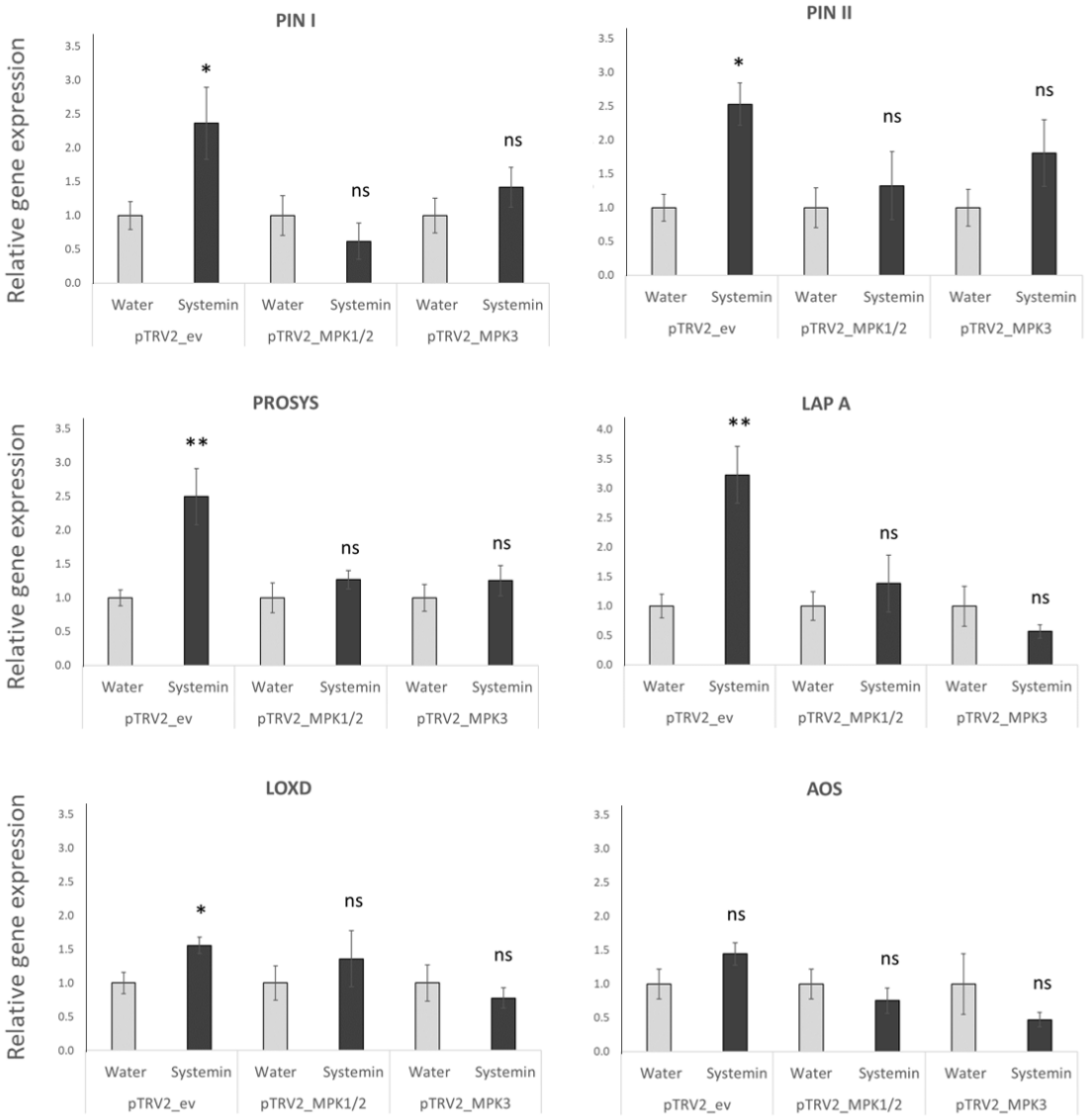

Figure 5. Gene expression levels upon infection. *RT-qPCR analysis of PIN I, PIN II, PROSYS, LAP A, LOXD and AOS gene expression in tomato silenced leaves 24h after B. cinerea infection with 10^6 spores/ml (inf) either in normally watered (Water) or Systemin-treated (Sys) plants. Graphs represent averages of three experiments with standard errors each including 6 plants per treatment and 2 infected leaves per plant. Asterisks indicate statistically significant differences (Student t test; *p < 0.05, **p<0.01, n=6); "ns" indicates no statistically significant difference.*

Discussion

MAPKs cascades are key elements in defense signalling which enable activation of defense responses downstream of challenge perception (Meng and Zhang, 2013). In tomato they mediate the Systemin signalling elements likely participatin in the response to wounding and herbivory (Ryan et al 200, Kadoth et al., 2007). However, further research is needed to decipher the implication of MPK in Systemin-induced resistance. In this study we aimed to investigate whether MAPKs are associated with signalling of defensive genes triggered by Systemin and their participation in the Systemin-Induced Resistance (Sys-IR). We confirmed that MPK1, 2 and 3 regulate the Systemin induction of JA-related genes and that indeed these MPKs are essential elements of Systemin-Induced Resistance against fungi. On the one hand, Systemin treatment primed the phosphorylation of MPKs upon a fungal PAMP challenge. On the other hand, a silencing of MPK1/2 and 3 resulted in an impairment of the Sys-IR against *Botrytis cinerea* and a loss of the Systemin-induced enhancement of defensive genes.

To study the implication of MPKs in the observed Sys-IR against Botrytis cinerea, we selected *SlMPK1, 2* and *3* based on previous literature, that suggests they participate in plant defense and in the Systemin-triggered responses**.** *SlMPK1* and 2 share a very high sequence identity (approximately 95%) and are homologous to *AtMPK6* whereas *SlMPK3* shares homology with *AtMPK3* (Kong et al., 2012), being these two Arabidopsis MPKs key regulators of denfende priming (Beckers et al., 2009). Similarly, *SlMPK3 is highly* homologous to the tobacco *WIPK*, whereas *SlMPK1* and *SlMPK2* show very high sequence homology to *SIPK* and other tobacco *MAPK*, like *Ntf4*, all of them associated the regulation of tobacco resistance against biotic attackers (Zhang ad Klessing 2000, Jin et al 2003). *MPK1* and *2* were also reported to mediate the observed enhanced resistance triggered by ProSystemin overexpression against herbivores (Kadoth et al 2007). Additionally, *SlMPK3* was shown to be essential for tomato defense against *Botrytis cinerea*. Thus, we hypothesised that *MPK1, 2 and 3* may be of key relevance in signalling Sys-IR against *B. cinerea*.

In our study, we observed that Systemin primed MAPK activation upon a challenge. Similarly Holley et al. (2003) observed that pretreatment with Systemin affected the MAPK response to subsequent challenges since *S. peruvianum* cells pretread with Systemin and subsequently exposed to UV-B light showed stronger MAPK activity

compared to non-treated plants at prolonged time points (90min after exposure su second challenge). These results suggest that similar to what occurs in Arabidopsis, tomato MPKs could be regulating priming responses to different challenges in tomato.

Our results show that Systemin primes MPKs phosphorylation and this is partially related to the concentration of the phytocytokine, since none of the concentrations used induced phosphorylation in the absence of challenge. However, both 20nM and 100 nM primed phosphorylation at 24h post challenge, note that the highest concentration led to a longer lasting MAPK activation. Contrastingly, Coppola et al., (2019) demonstrated that lower doses of Systemin had a stronger effect on defense genes induction than the higher doses. Similarly, induction of a myelin-based protein linakse (MBPK) activity and PI-I accumulation by Systemin was shown to be dose-dependent since increasing concentration of Systemin leads to increasing activity or accumulation (Stratmann and Ryan, 1997). Note that these analyses were performed in the absence of a challenge. Thus, Systemin directly triggered-responses seems to be differently regulated in the presence or in the absence of challenge.

We have confirmed that during Systemin-Induced resistance, the transcription and likely the activation by phosphorylation of MPKs functions upstream the induction of defense related genes both in the absence and the presence of infection. However, the stronger impact was shown after the infection, since Systemin boosts expression of JA-related genes, which is abolished when MPK were silenced. These observations demonstrate the implication of MPKs in the mechanisms following exogenous treatments with Systemin in tomato. Similar to our observations on gene expression, MBPK activity elicitation also occurred upstream to the octadecanoid pathway since a mutant of this pathway, ths was concluded by using *def1*, which was not impaired in Systemin-induced MBPK activity (Stratmann et al., 1997). Our observations are in consonance to previous results obtained by Kadoth et al. (2007). They observed that the expression of marker genes *PI-I* and *PI-II* was reduced in *MPK1/2*-silenced PS+ plants. These suggest that overexpression of ProSystemin shares common mechanisms with exogenously applied Systemin regarding the enhancement of marker gene expression However, the MPKs silencing in the PS+ background did not affected early genes *LOXD*, *AOS* and AOC gene expression, whereas we observed an impairment of JA biosynthesis genes in silenced plants either in the

absence or in the presence of infection, suggesting that the silencing of MAPKs also shows a different impact in PS+ or in Systemin-treated plants.

Additionally, Kaddoth et al., 2007 observed that co-silencing of *MPK1 and 2* reduced the enhanced resistance displayed in PS+ plants against *Manduca sexta*. As shown in this report, we also observed that Sys-IR against *Botrytis cinerea* was lost when both *MPK1 and 2* were silenced. Silencing of MPK3 also impaired Sys-IR against the fungus, even to a higher extent than co-silencing of MPK1/2. Note that basal levels of resistance in silenced MPK3 plants were already reduced irrespective of the peptide treatment in consonance with previous observations by Zhang et al., (2018). This suggests that *MPK1 and 2* may be conserved signalling mechanisms against pests and pathogens, whereas MPK3 might be more specific for pathogen defense. Concluding, despite the mechanisms regulating PS+ responses and Systemin treatmets share similarities, there are relevant differences. Note that recent studies demonstrate a direct function of the unprocessed protein, in fact, endogenous levels of ProSystemin are also important for defense against biotic challenges in tomato (Orozco-Cadenas 1993; Coppola et al., 2015) and other species (Rocco et al., 2008; Zhang et al., 2017). These observations support the idea of differential regulation of defenses in PS+ and Systemin-treated plants.

Collectively, our results along with previous observations indicate that MAPKs might be important regulators of Induced-Resistance and priming reponses in tomato plants. On the other hand, Systemin induction of transcription of MAP kinases and activity seems to be very important for the Systemin mediation of defense responses and for Sys-IR, although we cannot exclude the participation of additional protein kinases. A study of the Systemin triggered phosphoproteome upon a biotic challenge would elucidate the importance of kinase activity in the underlying mechanisms of Sys-IR.

Supplementary information

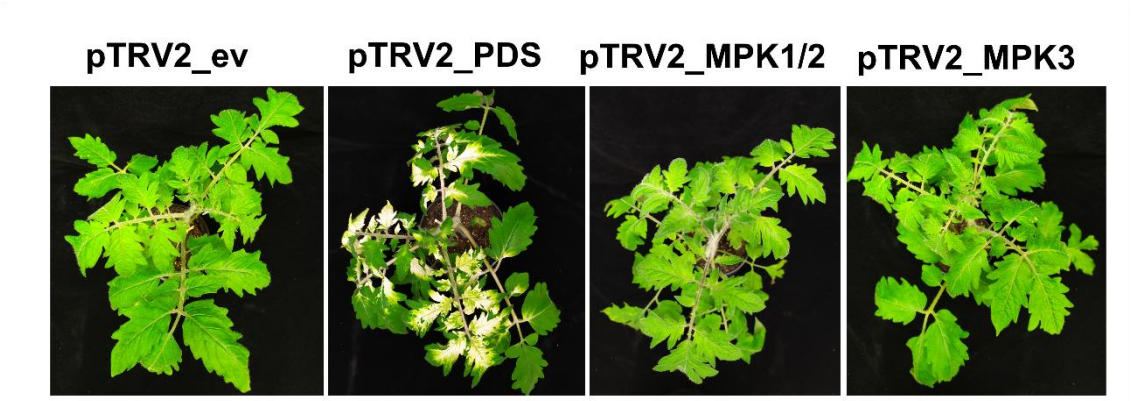

Figure S1. Phenotype of silenced plants. *Photos of tomato plants (Solanum lycpersicum cv MoneyMAker) taken 21 days after infiltration with a pTRV2 empty vector (pTRV2_ev) and the vector containing a target sequence for either PDS pTRV2_PDS), MPK1 and 2 (pTRV2_MPK1/2) or MPK3 (pTRV2_MPK3).*

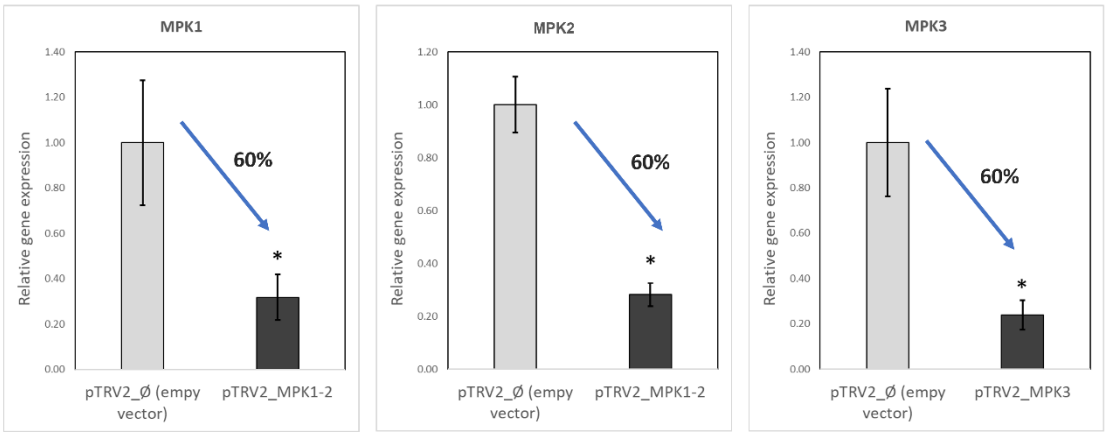

Figure S2. MPK1, 2 and 3 gene silencing levels. *RT-qPCR analysis of MPK1, MPK2 and MPK3 gene expression in tomato silenced with either pTRV2 empty vector or the vector containing the corresponding target sequence. Graphs represent averages of three experiments with standard errors each including 6 plants per treatment. % indicate percentage of gene expression reduction of the silenced plants in comparison with the control plants. Asterisks indicate statistically significant differences (Student t test; *p < 0.05, n=6).*

Table S1. Primer sequences

Primer name	Primers sequences
pTRV_MPK1/2	Fw - CGCGCGAATTCATTCAATACAATATTTTTGG
	Rv - GCGCGCTCGAGTGTATTTCAACCCAC
pTRV_MPK3	Fw - CGCGCGAATTCATGGTTGATGCTAATATGGG
	Rv - GCGCGCTCGAGTAATTATTTGGTGAAGATCA
EF1	Fw - GATTGGTGGTATTGGAACTGTC
	Rv - AGCTCGTGGTGCATCTC
MPK1	Fw - ATTGGCACTCCTTCAGAGGC
	Rv - TGGGTTTACATGCGGGAACT
MPK2	Fw - CATGCCCTTACCGAGGAACA
	Rv - AATCCAACAGCAAACGAGCG
MPK3	Fw - AACATCCACGCCAGCAGTTA
	Rv - TTTGCGAGGTAGGGATGAGC
PIN-I	Fw - GCAACTTCCTTTGAAACTCTC
	Rv - GTACACCAATAAGTTCTGGCC
PIN-II	Fw - CGTTCACAAGGAAAATCGTTAAT
	Rv - CTTGGGTTCATCACTCTCTCC
PROSYS	Fw - AATTTGTCTCCCGTTAGA
	Rv - AGCCAAAAGAAAGGAAGCAAT
LAP A	Fw - ATCTCAGGTTTCCTGGTGGAAGGA
	Rv - AGTTGCTATGGCAGAGGCAGAG
LOXD	Fw - GACTGGTCCAAGTTCACGATCC
	Rv - ATGTGCTGCCAATATAAATGGTTCC

AOS	Fw – CACCTGTTAAACAAGCGAAAC
	Rv – GACCTGGTGGCATGTTCG

Table S2. Target gene sequences for VIGS

Targeted gene	**Targeted sequence used for VIGS**
MPK1 and MPK2	GGAGACAAATGAATCTGTAGCAATTAAGAAAATTGCTAATGCTTTTGATA ACAAGATTGATGCTAAGAGGACTTTGAGAGAGATCAAGCTTCTTCGACAT ATGGATCATGAAAATATTGTTGCGATCAGAGATATAATTCCACCACCACA GAGAGAAGCCTTTAACGATGTTTACATTGCGTATGAGCTTATGGATACTG ATCTCCATCAAATTATTCGCTCGAATCAGGGTTTATCTGAGGAGCACTGC CAGTATTTCTTGTATCAGATCCTCCGTGGGTTGAAATACA
MPK3	ATGGTTGATGCTAATATGGGTGCTGCTCAATTTCCTGATTTTCCTAAAATT GTCACTCATGCTGGACAATATGTTCAGTATGACATTTTTGGTAATCTTTTT GAGATTACTAACAAGTATCAACCTCCTATCATGCCTATTGGCCGTGGCGC TTATGGAATCGTCTGCTCTGTGTTTAATGCGGAGCTGAATGAGATGGTTG CAGTTAAGAAAATCGCCAATGCTTTTGATAATTACATGGATGCTAAGAGG ACGCTCCGTGAAATTAAGCTTCTTCGCCATTTAGACCATGAAAACGTAAT TGGTTTAAGAGATGTGATTCCTCCGCCCTTACGAAGGGAGTTTTCTGATGT TTACATTGCTACTGAACTCATGGATACTGATCTTCACCAAATAATTA

DISCUSSION

DISCUSSION

The discovery of new plant peptides regulating plant metabolic processes is now rapidly increasing (Albert, 2013; Marmiroli et maestri, 2014; Tavormina, 2015; Gust et al., 2017). Although there are many studies describing the role of certain peptides in plant defense, the study of their use as defense elicitors is rather scarce. In this work, we reported how the supply of heterologous peptides from different plant species to Arabidopsis roots confers systemic defense in leaves against a necrotrophic fungus. By developing an analytical method to measure peptides we demonstrated their presence in the leaves confirming that indeed they are uptaken by the roots and transported to the leaves. Moreover, we established that the observed induced resistance is through the amplification of the plant immune system since none of them displayed direct antimicrobial activity. Finally, like with other IR stimuli, we observed that the concentration range of the peptides used for the treatment is of utmost importance to obtain beneficial effects. These results provided evidence of the potential of peptides to be used as general biocontrol agents.

Then we selected Systemin for further studies based on its ability to induce resistance in Arabidopsis from very low concentrations (0.1nM to 1nM). Interestingly Sys-IR shows an optimal threshold of action losing the protection at concentrations above 1nM. This behavior resembles that of phytohormones and other resistance inducers such as BABA or BTH (Katz et al., 1998; Müssig et al., 2003; Floeryszak-Wieczorek et al., 2015). In fact, many authors refer to plant peptides as peptidic hormones (Matsubayashi and Sakagami, 2006; Roy et al., 2018). Following our goal, we characterized for the first time the mechanisms underlying induced resistance by a peptide in a taxonomically distant species. We observed some commonalities between Systemin-mediation of defense responses in Arabidopsis and tomato since we found the hormone JA, but not SA, participated in the regulation of Sys-IR in Arabidopsis. On the other hand, we observed that Systemin was functioning as PTI enhancer, similar to the Arabiodpis peptide Pep1 (Flury et al., 2013). Systemin treatment did not induce any ROS production *per se*, whereas ROS production was boosted by Systemin when a challenge was present, meeting the defense priming requierement (Mauch-Mani et al., 2017). The same pattern was followed by the gene expression of components of the signal transduction *BAK1*,

BIK1, G heterotrimeric proteins biosynthetic genes and *MPKs*, as well as MPK phosphorylation, together with downstream metabolites of the tryptophan-derived pathway.

Remarkably Sys-IR in Arabidopsis functioned through the induction of conserved PTI components, whereas at the same time boosted innate defenses against *Plectsopaherella cucumerina*. BAK1 and BIK1 form a complex with multiple PRRs activated upon danger perception leading to the initiation of the first layer of defense responses such as ROS production and defense signaling through MPKs (Lu et al., 2010; Liu et al., 2013). This complex and the MPKs, MPK3 and MPK6, were necessary to express functional Sys-IR. On the other hand, β and γ subunits of the G heterotrimeric proteins, as well as tryptophan derivatives, were described in the literature to be components of the basal immunity of Arabidopsis against *Plectosphaerella cucumerina* infection (Llorente *et al.*, 2005; Sanchez-Vallet *et al.*, 2010; Delgado-Cerezo *et al.*, 2012; Gamir *et al.*, 2018; Kosaka *et al.*, 2021). Both proteins and compounds were primed by Systemin and essential for Sys-IR.

Most studies in phytocytokines focus only on the early defense responses, common for PTI. However, in our work, we wanted to go further and study downstream defense responses triggered by Systemin by performing a non-targeted metabolic analysis. This study revealed two main groups of compounds affected by the Systemin treatment. Phenolic compounds, including some phenylpropanoids and flavonoids, were induced upon the Systemin treatment, whereas indolic compounds derived from tryptophan showed a priming profile. Both groups of compounds resulted to be essential for Sys-IR, since mutants impaired in their production failed to express functional Sys-IR. As previously mentioned, tryptophan-derived metabolites, including indole-glucosinolates (IGs), indole carboxylic acids (ICAs) and the phytoalexin camalexin, are among the most important secondary metabolites required for Arabidopsis resistance against *P. cucumerina* (Sanchez-Vallet *et al.*, 2010; Gamir *et al.*, 2018; Kosaka *et al.*, 2021). On the other hand, phenolic compounds including flavonoids and phenylpropanoids have been previously linked to defence (Zaynab *et al.*, 2018) although they were never reported in response to *P. cucumerina* or in Peptide-IR.

In tomato, the perception of Systemin has been recently elucidated (Wang et al., 2018), as well as new elements involved in the signal transduction (Xu et al., 2018). However,

very few studies report on the impact of Systemin treatment in the whole plant metabolism which may lead to the enhanced resistance observed against different attackers. In our work we found that Systemin-IR against *Botrytis cinerea* was likely mediated by a priming in callose deposition. Then, we proceeded to analyse the molecular changes occurring after Systemin treatment and upon *Botrytis cinerea* infection in tomato plants that may explain the observed enhanced resistance and priming of callose. To reach this goal, we followed a holistic approach by integrating non-targeted omic analysis at different metabolic levels including proteomics and metabolomics complemented with the analysis of enzymatic activities. The effect of Systemin in tomato plants have been generally associated with defensive responses against wounding and herbivory which are mediated by the JA pathway (Sun et al., 2011). Surprisingly, in our study we found that the major impact of the Systemin treatment was observed in proteins of the primary metabolism whereas minor changes were observed after infection. Proteomics, together with analysis of enzymatic activities revealed that Systemin modulates the primary metabolism towards the production of available sugars and carbon skeletons accumulation. Therefore, we hypothesize that this surplus of available sugars may be allocated to the strengthening of the cell wall, which leads to the observed priming of callose deposition. In fact, this was corroborated by the upregulation of a starch degradation protein as well as callose synthase together with a more rapid degradation of starch in Systemin treated plants upon infection. On the other hand, the increase in carbon structures might serve as a reservoir to mount a faster response against a future challenge via rapid production of downstream compounds. In fact, in our omics analysis we found very few changes observed at the proteomic level upon infection, whereas at the level of metabolites major changes were observed after infection, suggesting that the accumulated carbohydrates are being allocated to the rapid production of defensive metabolites. In this regard, we were able to identify a few defense-related metabolites that were either induced or primed by Systemin, including phenolic compounds and alkaloids.

Contrary to the high impact of Systemin treatment in the plant proteins of the primary metabolism very few changes were observed in the presence of infection. However, although scarce, proteins accumulated upon infection in Systemin-treated plants were very specific for pathogen defense, including pathogen-related proteins, a G protein and a tentative protease inhibitor. These results suggest that Systemin-treated plants rearrange their proteomic metabolism upon a pathogen challenge in order to interfere with pathogen

infection. Remarkably, although we observed that Systemin treatment has a great impact on the plant metabolism, it did not have a cost on plant growth and development. We hypothesize that the increase in photosynthesis may be behind the lack of fitness cost observed in Systemin-treated plants in spite of the enhanced sugar availability and the general primary metabolism higher rate found in these plants.

Finally, we shed light on the signaling elements that enable downstream responses after Systemin perception in tomato and eventually lead to Systemin-Induced Resistance. We demonstrated that MAPK, and specifically MPK1, MPK2 and MPK3, are key elements of the early signaling triggered by Systemin upon a challenge and essential to express functional Sys-IR. A reduction of these MPKs lead to a loss in the Systemin priming of defensive genes and impairs the observed enhanced resistance state triggered by the peptide. These observations demonstrated the importance of intact signaling and phosphorylation protein cascades to ensure that proper downstream defense responses occur. However, further research is needed to elucidate the importance of kinase cascades behind Sys-IR.

It is worthy noting that along this thesis we have found several defense-releated elements behind Sys-IR which are shared by both Arabidopsis and tomato. These elements include JA-related defenses, MPKs, G proteins and phenolic compounds, suggesting that they are conserved molecular fingerprints underlying Sys-IR in both species. Thus, it would be very interesting to study if these common features are conserved across other different plant species as well.

In this thesis we show the use of omics techniques in order to have a global picture of how plant metabolism is affected at different metabolic levels. Combination of omic techniques is a useful tool in order to identify common or differentiating metabolic fingerprints in response to different stimuli. It confers a holistic approach that could be applied for the study of plant responses to different environmental changes. Thus, we encourage its use in future research.

This work gathers conclusive evidence to claim that peptides act as resistance inducers. More specifically, Systemin is able to induce resistance against necrotrophs in both tomato and Arabidopsis. However, whether it can be considered a priming stimulus needs further discussion. Defense priming mechanisms may differ depending on the nature of

the stimulus, however, it shares common key characteristic features that help to recognise the presence of priming in plants (Martínez-medina et al., 2016). Firstly, primed plants have memory, which refers to the ability of the plant to store information of the priming stimulus until the exposure to a subsequent challenge, when the activation of defensive traits occurs. In our experimental setup we can claim that there is indeed a plant memory between Systemin application and fungal infection of at least 24h, the time span between the two events. Although not tested, we cannot exclude a longer memory upon Sys treatment . Priming can also be inheritable to the primed-plant descendants, displaying what is called "transgenerational priming", however, whether subsequent generations of Systemin-treated plants also display induced resistance against pathogens was not tested in our work and remains elusive.

Secondly, priming is associated with an absent or very low fitness cost, which results in a positive cost-benefit equilibrium upon stress (Martínez-medina et al., 2016). On the contrary, direct induction of defenses is often associated with a growth and developmental alterations due to energy allocation to the production of defensive elements. In our work we have observed that although Systemin treatment itself produces major changes in the plant metabolism and they are mostly associated with the primary metabolism, no fitness penalty could be measured in the physiological parameters of Systemin-treated plants.

Finally, primed plants exhibit a more robust defense response due to a faster and stronger activation of plant defenses that leads to a plant better performance upon a biotic challenge (Martínez-Medina et al., 2016; Mauch-Mani et al., 2017). In this regard, Systemin boosted many defense-related signalling components in Arabidopsis upon the challenge which were not induced in the absence of the challenge. Gene expression of *BAK1*, *BIK1*, *AGB1* and *AGG2*, *RBOHD* as well as *MPK3* and *MPK6* were primed by Systemin. In addition, *MPK3* and *MPK6* phosphorylation was also higher in Sys-treated plants upon PAMP challenge 15 minutes after the PAMP presence. Similarly, downstream defense-related metabolites were also primed by Systemin in Arabidopsis upon *P.cucumerina* infection which include tryptophan derivatives such as *IAN* and *I3CA*, as well as the genes involved in their synthesis.

Like in Arabidopsis, Systemin induced resistance against a necrotroph in tomato, which in this case was linked to a priming of callose deposition. As previously mentioned, Systemin treatment induced a direct effect in the plant proteomic profile which correlated

with changes in components of the plant primary metabolism, this was further corroborated by enzymatic activities measurement. Interestingly, these changes occurred towards the production of more available sugars which may be a preparation for the plant for a future attack. Sugars are a useful source to be allocated for callose formation whereas carbon structures could be allocated to defense compounds biosynthesis. The accumulation of inactive potential defense cues is a key feature of priming to ensure a more rapid activation of a defense response upon a subsequent stress (Conrath et al., 2015; Mauch-mani et al., 2017). In fact, at metabolomic level major changes due to Systemin occurred after infection, showing a typical priming profile. On the other hand, Systemin also primed MPK phosphorylation upon a PAMP challenge in tomato plants. This response was longer-lasting in plants treated with a high concentration of Systemin, which, unlike in the water-treated plants, was maintained 1h after challenge, being also a characteristic feature of priming.

Consequently, both Arabidopsis and tomato plants treated with Systemin showed a better performance against necrotrophic fungi displaying a lower susceptibility phenotype compared to non-treated plants. Further priming features include broad spectrum activity. Although in our work we only tested the Systemin effect against necrotrophic fungi, other authors have tested its effectiveness against different biotic and even abiotic stresses, which include herbivores, aphids, virus and salinity (Orsini et al., 2010; Bubici et a., 2017; Coppola et al., 2019). But, besides the broad spectrum activity, very interestingly we have seen that it is effective in heterologous species that do not produce it. In consonance, it was recently reported that Systemin induces resistance against *Botrytis cinerea* in *Solanum melongea* and *Vitis vinifera* plants (Molisso et al., 2020). These evidences poses the potential of Systemin to be a general resistance inducer in plants.

For all the above-mentioned hallmarks of Sys-IR, although Systemin was shown to produce a direct effect, we consider Systemin as a priming inducer candidate, in fact, the IR definition was revisited recently and considered to be a sum of both direct and primed defence activation (De Kessel et al., 2021). Thus, here we propose a model for the mechanisms of Sytemin-IR against infection (Figure 1). Systemin, as a secondary DAMP or phytocytokine (Gust el al., 2017) is perceived by the plant as a danger signal and induces direct responses, although the challenge is absent. Many of these triggered responses are not final but serve as preparation for the plant for future attack (priming

phase). Then when the infection occurs the plant responds in a more effective and rapid manner displaying defense priming. At the same time infection induces again the release of endogenous Systemin (in tomato) in a positive feedback loop that keeps amplifying the defense response in order to effectively cope with the pathogen attack.

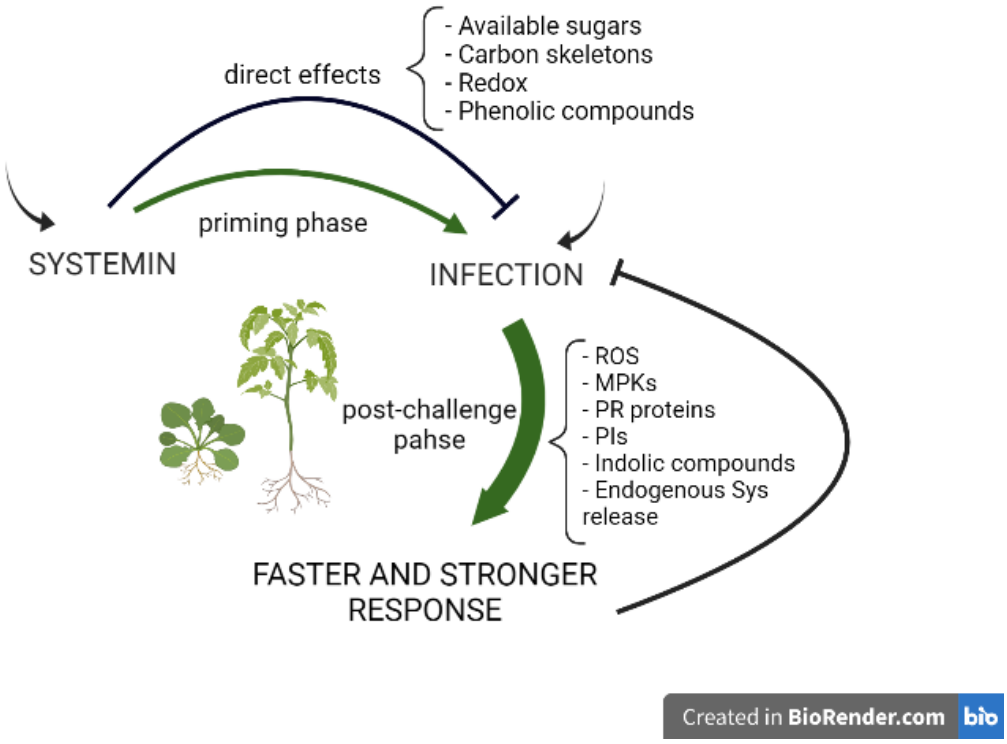

Figure 1. Mode of action of Systemin as a priming elicitor. *Exogenously applied Systemin induces direct metabolic changes in the plant, but when the infection is present a faster and stronger induction of defense mechanisms occurs leading to more robust response that counteracts the infection. Parallelly, the release of endogenous peptides (Systemin in tomato) is produced, which keeps amplifying the immune responses. Black arrows indicate exogenous stimuli. Green arrows indicate sequential events occurring in the plant starting from the exogenous peptide treatment. Inhibitor arrows indicate pathogen counteracting.*

CONCLUSIONS

CONCLUSIONS

Based on the proposed objectives in this thesis, the following conclusions can be drawn:

1. Arabidopsis plants are able to sense peptides from different plant species which confer protection against the necrotrophic fungi *Plectosphaerella cucumerina* through the promotion of specific immune responses.

2. Systemin primes conserved defense signaling elements in Arabidopsis upon a pathogen challenge including *BAK1 and BIK1* gene expression and *MPKs* as well as elements of the specific signaling elements against *Plectosphaerella cucumerina*, as are the β and γ subunits of G heterotrimeric proteins. Moreover, Systemin primed *RBOHD* gene expression as well as ROS production upon a PAMP challenge. All these elements are essential to display Sys-IR.

3. Systemin induces a metabolomic rearrangement in Arabidopsis plants that includes the accumulation of phenolic compounds whereas it primes the production of specific tryptophan derivatives in Arabidopsis. Flavonoids ampelopsin, cateching and naringin together with phenylpropanoids chlorogenic acid and sinapic acid were induced by the Systemin treatment. Indolic compounds IAM, IAN and I3CA as well as the indolic compounds biosythetic gene*s CYP71A13, CYP71B6 and AAO1* were primed by Systemin. Both groups of compounds were shown to be essential for Sys-IR against *Plectosphaerella cucumerina* since mutants on their biosynthesis displayed impaired Sys-IR.

4. Systemin protects tomato against *Botrytis cinerea* through a priming in callose deposition which is linked with a more rapid degradation of starch upon infection, an increased availability of sugars and an upregulation a starch phosphorylase, a glucan synthase-like and a cellulose synthase-like proteins.

5. Systemin treatment has a great impact in the tomato proteomic profile, upregulating proteins of the primary metabolism in the absence of infection, leading towards the production of carbon structures and available sugars, which is not associated with a fitness cost. Conversely, upon the pathogen challenge, very few

pathogen defense related proteins are upregulated in Sys-treated plants, including PR1, PR2, PR4 and a tentative protease inhibitor.

6. Unlike in the proteomic analysis, the metabolomic data of tomato plants showed a clear priming profile.The two amino acids cysteine and methionine, two phenolic compounds, 7-hydroxycoumarin and herperetin-7-O-glucoside and two alkaloids were identified among the Systemin induced and primed metabolites. On the other hand, many compounds having a buffering effect over infection were found but specific metabolites could not be identified.

7. MPK1, MPK2 and MPK3 are key signaling elements to ensure proper Systemin-triggered defense gene expression. Systemin primes MPKs phosphorylation upon PAMP challenge. In addition, silencing of *MPK1/MPK2 and MPK3* impaires Sys-IR against *Botrytis cinerea* infection in tomato plants.

8. Systemin is a potent IR elicitor, functional in taxonomically distant species, that fits most features of a priming agent. Thus, it could be a useful tool to control plant biotic stressors in plants and crops.

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