1-Methyltryptophan treatment increases defense-related proteins in the apoplast of tomato plants

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~~KEYWORDS: apoplast, proteomics, induced resistance, *Pseudomonas syringae*, *Solanum lycopersicum*, 1-Methyltryptophan, resistance inducers.~~

ABSTRACT

The activation of induced resistance in plants may enhance the production of defensive proteins to avoid the invasion of pathogens. In this way, the composition of the apoplastic fluid could represent an important layer of defense that plants can modify to avoid the attack. In this study we performed a proteomic study of the apoplastic fluid from plants treated with the resistance inducer 1-methyll-tryiptophan (1-MT) as well as infected with *Pseudomonas syringae* pv. *tomato* (*Pst*). Our results showed that both the inoculation with *Pst* and the application of the inducer

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provokes changes in the proteomic composition in the apoplast enhancing the accumulation of proteins involved in plant defense. Finally, ~~several~~ one of the identified proteins that are overaccumulated upon the treatment have been expressed in *Escherichia coli* and purified in order to test their antimicrobial effect. The result showed that the tested proteins are able to reduce the growth of *Pst in vitro*. Taken together, in this work we described the proteomic changes in the apoplast induced by the treatment and by the inoculation, as well as demonstrated that the proteins identified has a role in the plant protection.

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INTRODUCTION

Plants are able to defend themselves against a vast number of pathogens. Among the different defensive mechanisms, the first layer of defense is composed of preformed barriers that provide the constitutive resistance against a broad spectrum of attacks. This layer includes non-specific defenses such as physical accumulation of wax, or chemical deterrents such as pyrethrins, phytoalexins, and phytoanticipins[1]. However, plants are also able to recognize the presence of certain pathogens and trigger a defensive response depending on the pathogen lifestyle. The recognition of the pathogen starts with the perception of microbial elicitors known as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs)[2]. A successful recognition activates the mechanism of defense known as PAMP-triggered immunity (PTI) inducing rapid changes in the phosphorylation of proteins, an increase in the calcium level of the cytosol, and the expression of defense-related genes[3].

However, pathogens have developed mechanisms to interfere with PTI by the release of effectors, resulting in the effector-triggered susceptibility (ETS). The effectors are intended to manipulate the host cell in a way that helps the pathogen to invade the plant[2]. Fortunately, plants also developed intracellular nucleotide-binding/leucine-rich-repeat (NLR) receptors that will detect and counteract the effectors, leading to the so-called effector-triggered immunity (ETI). Most of these mechanisms are controlled by phytohormones such as Salicylic acid (SA), Jasmonic acid (JA), and Ethylene (ET) which activate the induced defensive responses[4].

Despite the multilayered innate immune system of higher plants, some pathogens such as *Pseudomonas syringae* DC3000 (*Pst*) are capable of entering leaves through stomata or wounds in the epidermis. Once inside the plant, the nutrients present in the apoplast are usually sufficient to ensure a rapid colonization of the leaf. It has been reported that *Pst* is able to grow in leaf apoplast extract of *Arabidopsis* and tomato, suggesting that this bacterium is adapted to survive and use the C and N sources available in the plant apoplast and these nutrients are enough to allow the development of the bacteria[5,6].

When the population of the bacteria reaches certain levels on the leaf surface, the accumulation of N-acyl homoserine lactone (AHL) acts as quorum-sensing (QS) signal, resulting in a coordinated expression of virulence factors that mediates colonization of the host cells[7–9]. One of the most well-known virulence factor released by *Pst* is Coronatine (COR). COR is a phytotoxin with a structure that mimics J-jasmonic-isoleucine, which is the biologically functional conjugate of JA[10]. The binding of COR to Coronatine Insensitive 1 (COI1) leads to COR/COI1-dependent suppression of SA accumulation, reducing the resistance to *Pst* in *Arabidopsis* and tomato [11–13]. Moreover, COR also inhibits the stomatal closure of plants induced by the recognition of flagellin, facilitating the bacterial entry into the leaves[14].

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63 The mechanisms of infection of *Pst* have been usually studied in whole leaf tissue. However,
64 several of the previously described processes are located in the apoplast and cause changes in the
65 composition of the apoplastic fluid. Despite of that, only a few studies have been conducted in
66 order to ascertain the changes in the apoplast composition during infection and the role of the
67 apoplast in plant defense[15,16].

68 The apoplast is defined as the extracellular matrix, including the cell wall and intercellular
69 spaces that contain the apoplastic fluid[17]. This space between cell membranes is involved in
70 several biological processes, such as water transport or plant defense[18]. The apoplastic fluid is
71 mostly composed of inorganic ions, metabolites, and proteins[15,19]. Several studies have analyzed
72 its characteristics and observed that its content is highly influenced by the processes occurring in
73 the surrounding cells as well as in the xylem and phloem, and by changes in plant physiology,
74 nutrition or response against biotic or abiotic stress[18,20]. Thus, its composition can change and
75 adapt to each particular situation.

76 Among the different pathogen control strategies in plants, the search for molecules which are
77 able to induce plant innate defense, is one of the most promising. This search is usually linked to
78 a general characterization of the response of both the plant and the pathogen[9,21–23]. Previous
79 studies have demonstrated the efficacy of the molecule 1-methyltryptophan (1-MT) in the control
80 of *Pst* in tomato[24]. Recently, Scalschi et al.[25] showed that the application of 1-MT induces
81 changes in the levels of ABA which lead to more closed stomata as well as to a blocking of the
82 JA pathway which can impair the effect of COR. Moreover, it was also observed that the growth
83 of the bacteria in the apoplastic fluid obtained from treated plants was lower when compared to
84 the growth of the bacteria in the apoplast extracted from untreated plants. Although possible
85 causes of this reduction of bacterial growth were studied, neither the sugar nor amino acid and

hormonal content explain fully this inhibition[25]. For this reason, we hypothesize that the application of 1-MT can induce the accumulation of defensive proteins changing the composition of the apoplast.

In this way, Parker et al.[26] compared the proteomic profile of tomato against *Pst* using the resistant cv. Rio Grande-PtoR (RG-PtoR) and the susceptible cv. Rio Grande-prf3 (RG-prf3). The comparison of both cultivars showed that the resistant RG-PtoR genotype had a higher content of stress response proteins, related to both biotic and abiotic stresses, as well as other proteins that could be playing an important role in the resistance against the bacteria. This higher level of proteins related to defense may be involved in the higher resistance to pathogen attack.

However, it is not known whether the plant is able to block essential nutrients away from the apoplast as a defense mechanism[27] or to accumulate different metabolites and proteins to avoid the colonization of the apoplast. Moreover, there are no studies about the influence of the application of resistance inducers in the plant defense at the apoplastic level and how the activation of the innate defenses mediated by the resistance inducer can modify the apoplastic content. For these reasons, the main objective of this study is to elucidate the changes in the proteomic composition of the apoplast induced by the treatment with 1-MT and its possible role in the control of the infection. To achieve this objective, we obtained apoplast washing fluid ~~(AWF)~~ of plants treated with 1-MT and inoculated with *Pst* using the infiltration–centrifugation technique[28]. The comparison of the proteome of treated and untreated plants, with or without infection, allowed us to study the different responses of the plant under these situations as well as to select the proteins that may play an active role in the control of the pathogen.

MATERIAL AND METHODS

Microbial strains, growth conditions and plant material

P. syringae pv. *tomato* strain used in the present study was DC3000. Rifampicin was added to King B medium (KB) at 50 µg–mL^{-1}. Tomato seeds (*Solanum lycopersicum* Mill. cv. Ailsa Craig) were germinated in vermiculite in a growth chamber under the following environmental conditions: light/dark cycle of 16/8h, temperature of 24/18 °C, light intensity of 200 µmol m^{-2} s^{-1}, and 60 % relative humidity. Seeds were irrigated with distilled water for a week and the next 3 weeks with Hoagland solution[29]. The pH of the nutrient solution was adjusted to 5.8 - 6.0 with KOH 1 mM.

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***Pseudomonas syringae* bioassays**

Four-week-old tomato plants were divided into four groups: Plants treated with 1-MT and inoculated (MTI), plants treated with 1-MT and non-inoculated (MT), plants non-treated and inoculated (CI), and plants non-treated and non-inoculated (C). Treatment was performed with a nutrient solution amended with 1-MT (5 mM) at pH=6, 72 hours before inoculation. Control plants were treated with a nutrient solution. *P. syringae* pv *tomato* DC3000 was grown in ~~KingB~~ (~~KB~~) medium at 28 ºC for 24 h. Bacterial suspensions were adjusted to 5 x 10^{5} colony-forming units (cfu)~~/~~mL^{-1} in sterile $MgSO_4$ (10 mM) with 0,01 % of Silwet L-77 surfactant (Osi Specialties, Danbury, CT, USA). Tomato plants were inoculated by dipping third and fourth leaves with *P. syringae* as previously described by Scalschi et al.[22]. The disease rate was scored by determining the percentage of leaves showing dark-brown spots and the number of colony forming units (cfu) at 72 hpi. Each experiment was independently conducted at least three times.

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Apoplast extraction

Apoplast extraction was carried out 48 h after *Pseudomonas syringae* inoculation based on previous observations[22] since we observed that, in our inoculation conditions, the plant showed greater response at this time point. The extraction was performed using the infiltration-centrifugation method as described by O'Leary et al.[28].– Briefly, this technique is a two-step method that essentially involves the replacement of the apoplastic air space with sterile distilled water, which mixes with the native apoplastic fluid, followed by recovery of the infiltration/apoplastic mixture by gentle centrifugation of the leaves. The cytoplasmic contamination of apoplast was estimated as described by Rico and Preston[6]. Prior to subsequent analyzes, the apoplast extract was diluted twice in distilled water and filtered on a cellulose syringe filter (0.2 µm pore size), in order to avoid bacterial contamination. Four biological replicates of apoplast extracted from each group of plants were performed.

Protein preparation

The proteomic analysis was performed in the proteomics facility of SCSIE University of Valencia that belongs to ProteoRed, PRB2-ISCIII. For the analysis of the protein fraction by SWATH, 10 µg of each sample was loaded on a 1D_SDS_PAGE gel in order to remove contaminants, clean samples and proceed with the gel digestion of the proteins. Gel fraction was cut and the sample was digested with sequencing grade trypsin (Promega) as described previously by Shevchenko et al.[30]. 250 ng of trypsin in 150 µL of ABC solution was used. The digestion was stopped with trifluoroacetic acid (TFA) (1% final concentration), a double extraction with acetonitrile (ACN) was done and all the peptide and protein solutions were dried in a rotatory evaporator. The sample was resuspended with 10µL of 2% ACN; and 0.1% TFA.

LC-MSMS Analysis

1,5 µg of the pooled sample (proteins fraction samples) were loaded onto a trap column (NanoLC Column, 3µ C18-CL, 75umx15cm; Eksigent) and desalted with 0.1% TFA at 3µl /min^{-1} during 5 min. The peptides were loaded onto an analytical column (LC Column, 3 µ C18-CL, 75umx12cm, Nikkyo) equilibrated in 5% ACN 0.1% FA (formic acid). Proteins elution was carried out with a linear gradient of 5 to 35% ACN for 180 min at a flow rate of 300 nl /min^{-1}. Peptides were analyzed in a mass spectrometer nanoESI qQTOF (5600 TripleTOF, ABSCIEX). Eluted peptides were ionized applying 2.8 kV to the spray emitter. The tripleTOF was operated in swath mode, in which a 0.050s TOF MS scan from 350– 1250 m/z was performed, followed by 0.080s product ion scans from 350–1250 m/z on the 32 defined windows (3.05 sec/cycle). The rolling collision energies equations were set for all ions as for 2+ ions according to the following equations: |CE|=(slope)x(m/z)+(intercept). The raw data (.wiff) files obtained from SWATH experiment was analyzed by PeakView v.2.1 software (Sciex) under restricted criteria and settings: five peptides, five transitions, 95% peptide confidence threshold and 1% false discovery threshold. The quantitative data obtained by Peak View were analyzed with Marker View 1.3 (Sciex). First, areas were normalized by total areas summa, and then PCA analysis and t-test were done. In order to determine the differences between the different types of samples proposed in this study, we performed a logarithmic transformation of the data. Glmnet library of the R was used to apply a logistic regression with Lasso penalty and the Elastic net as selection methods for variables (proteins) that show significant differences between the different groups. Once the samples have been normalized, a Heatmap was performed before applying the two methodologies. CI vs C, MT vs C, MTI vs CI and MTI vs MT.

Protein annotation, gene ontology (GO) categories and analysis of localization

The differentially expressed proteins were annotated using Blast2GO software version 5.1.13 (https://www.blast2go.com). Protein sequences were compared against SwissProt database using public NCBI Blast service (QBlast). The blast program was set as blastp with blast expectation value (E-value) 1×10^{-5}. The meaningful matches from Blast2GO analysis were subjected to GO categories (cellular component, molecular function, and biological process). The classical secretion which derived by N-terminal signal peptides, and non-classical secreted protein were predicted using SignalP (version 4.1) (http://www.cbs.dtu.dk/services/SignalP/), and SecretomeP (version 2.0) (http://www.cbs.dtu.dk/services/SecretomeP/).

Recombinant protein expression and antimicrobial assays

Apoplastic cysteine proteinase (CP3), induced by 1-MT in the absence and presence of infection, was cloned into pET-14b (Novagen) for recombinant protein expression. To construct the *E. coli* protein expression vector, the sequence encoding mature protein without the signal peptide was cloned into pET-14b to generate the expression vector pET-14b-cysteine proteinase-6×His. The *E. coli* strain BL21 (DE3) (New England BioLabs) was used to express the recombinant protein. Purification of the recombinant protein was performed using HisPur™ Ni-NTA Spin Purification Kit (Thermo Scientific) according to the supplier's instructions. The purified protein was further used to test its antimicrobial activity *in vitro*.

Experiments were performed in M9 minimal medium supplemented with purified recombinant protein at a concentration of 70 ng/ mL^{-1} or with PBS (Phosphate buffered saline) (Thermo Scientific). The pH of medium was adjusted to 5.8 to mimic the pH of the apoplast, before adding the bacteria. The strain was precultivated on KB plates containing the appropriate antibiotics for 2 days to obtain the inoculum. The bacterium was harvested in sterilized $MgSO_4$

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(10 mM). The growth assay was carried out in a Multiskan™ FC Microplate Photometer (Thermo Scientific) in a total volume of 200 µl in microtiter wells using an initial bacterial density of 10^6 cfu/ mlmL^{-1}. Bacterial growth was incubated at 28ºC with continuous agitation and monitored by measuring optical density every 10 min with periodic shaking for 72h. The results were printed out as growth curves.

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RESULTS AND DISCUSSION

Plants have a variety of inducible defenses that adapt the defensive mechanisms to the upcoming stress, making the plant a more unpredictable environment for the attacking pathogen. These inducible defenses can be stimulated by the application of resistance inducers such as 1-MT[24,25]. Previous studies showed that plants treated with 1-MT were less susceptible to *Pst*. Moreover, it has also been observed that *Pst* growth in the apoplast extracted from plants treated with 1-MT, with or without infection/in the presence or absence of infection, was reduced, suggesting that this molecule might induce changes in the apoplastic content.

When a pathogen attempts to infect a plant, the apoplastic space is the first area of contact between the host and the microbe. The apoplastic fluid is one of the first layers of inducible defenses, since, upon pathogen recognition, its composition may change by accumulating antimicrobial compounds in order to hinder pathogen survival[31]. Therefore, the ability of the invader to adapt and survive in the apoplastic fluid will determine the success of the invasion. In this way, it has been demonstrated that some pathogens such as *Pst* are able to adapt and proliferate in the apoplastic fluid using the nutrients present in it[6]. Furthermore, it has been suggested that the physiological environment that *Pst* encounters inside the plant leaf can induce the expression of *hrp* genes, that encode for the type III protein secretion system (TTSS),

~~responsible for delivering effectors into the plant cell. The expression of these genes can be induced in environments with low pH, high content of sugars, whereas it can be suppressed in environments rich in amino acids and tricarboxylic acid cycle (TCA) intermediates[33]. Therefore, in healthy plants, the apoplastic fluid is an acidic environment, low in nitrogen, and rich in plant-derived sugars such as fructose, providing a suitable environment for inducing the invasion machinery.~~

~~Previous studies on tomato-Pseudomonas interaction[26] performed an analysis of the proteome and the bacterial growth in leaves, comparing a cultivar resistant to *Pst* with a cultivar susceptible to *Pst*, showing a high complexity of the protein content in the leaves. This complexity hinders the study of plant-pathogen interaction. In this study, Parker et al.[26] observed that the proteomics response of the plant against *Pst* is different in susceptible or resistant cultivars, suggesting that the ability to activate the correct proteomic responses may be a key in the resistance. However, despite the importance of the apoplastic proteome during plant-pathogen interaction and infection establishment/during pathogen establishment and in the degree of infection, it is less characterized than the intracellular proteome.~~

~~In this way, as mentioned above, we have demonstrated in a recent study that the application of resistance inducers such as 1-MT is able to modify the apoplastic fluid, impairing the growth and survivor of *Pst* [25]. Since it was previously described that the proteomic composition of the apoplast can change under different stress situations[34,35], in the present work we performed an analysis of the changes of proteomic content of the apoplast extracted from plants treated with a resistance inducer, the 1-MT, inoculated or no with *Pst*.~~

Previous studies showed that plants treated with 1-MT are less susceptible to *Pst.* Moreover, in the apoplast extracted from plants treated with 1-MT, the *Pst* growth was reduced, suggesting that this molecule might induce changes in the apoplastic content[25]. To date, the studies on tomato-*Pseudomonas* interaction[26] showed a high complexity of the protein content in the leaves. However, despite the importance of the apoplastic proteome during plant-pathogen interaction and pathogen establishment, the proteome of apoplast plants treated with a resistance inducer has not been studied yet. For this reason, the aim of this work is to analyze the possible changes provoked by a resistance inducer, the 1-MT.

Global proteome analysis of *Solanum lycopersicum* apoplast

Proteomic analysis was performed in the apoplast extracted from tomato plants ~~that have received the following treatments/ subjected to the following conditions: Control (C), infected (I), treated with 1-MT (MT), and treated with 1-MT and infected (MTI)~~, in order to examine proteome changes that occur ~~in~~ against *Pst* infection and the treatment with a resistance inducer such as 1-MT. Overall, 512 proteins and 40 peptides in the pooled samples of apoplast from *S. lycopersicum* were detected, with a false discovery rate (FDR) of 1% (Supplementary Table S1), which represented all the proteins found in the apoplast in all the samples. Similar results were obtained by Kim et al.,[31] and Yang et al.[32] when studying the proteomics of plants inoculated with pathogens. This list was compared with a database to detect possible contamination~~nt~~ in the samples[33], ~~resulting~~ which resulted in the detection of 20 possible contaminations reducing the list to 492 proteins. The high sensitivity of gel-free techniques compared with gel-based ones makes possible to identify a greater number of proteins in the same type of sample[34]. This higher

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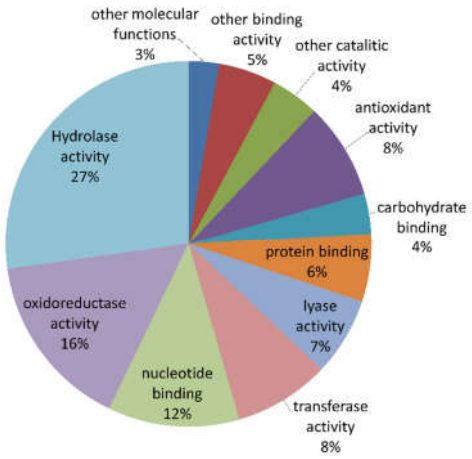
sensitivity would explain ~~T~~the high number of proteins obtained in this study.~~is probably due to the sensitivity of gel-free techniques compared with gel-based ones, which makes possible to identify a greater number of proteins in the same type of sample[38]~~. In this way, similar results have been found using gel-based proteomics yielded 150-300 proteins[35,36]

The 492 resultant proteins were classified by Gene Ontology (GO) enrichment analyses[37]. Categories were based on GO classification using AgBase[38], a unified resource for functional analysis in agriculture. Proteins were grouped according to plant GO-slim categories obtained for molecular functions. Some of the GO classes were merged in order to simplify the classification (Fig. 1). The molecular function classification showed a high proportion of proteins with hydrolase activity (27%). A similar percentage of proteins with this activity was observed in other plant species such as rice or grapevine[39,40]. It is well known that hydrolytic enzymes such as glycoside hydrolases are involved in the protection of plants against pathogens[41]. These proteins are mainly glycoside hydrolases with β-glucanase and chitinase activities and are classified as different families of pathogenesis-related proteins (PR-2, PR-3, PR-4, PR-8, PR-11)[41]. Although these proteins mainly act in the defense against fungi, it has also been described that plant chitinases are able to hydrolyze the peptidoglycan of the bacterial cell walls, inducing bacterial lysis[42]. In the same way, the overexpression of chitinases of fungal origin in tobacco plants enhances resistance against bacterial pathogens[43].



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279 **Figure 1.** Distribution of functional categories of the identified apoplastic proteins. A total of
280 492 proteins were identified in all four treatments Control, Infected, Treated with 1-MT and
281 treated with 1-MT and infected. The percentages of proteins of the functional categories are
282 shown.

283

284 The combination of oxidoreductase and antioxidant activities reaches 24% of the identified
285 proteins. These proteins may be related to the plant response to pathogen attack regulating the
286 transient burst of reactive oxygen species (ROS). The accumulation of ROS after a pathogen
287 attack can be toxic to plant cells and must be maintained at an appropriate level by
288 antioxidants[44]. On the other hand, the oxidative burst also acts as signaling for the activation of
289 plant defenses[45,46]. Taken together, our results showed that the two major functional ontologies

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observed can be related to plant protection, suggesting that the proteins present in the apoplast could play a major role in the defense against pathogens.

The presence of a signal peptide sequence in the 492 proteins was predicted using the TargetP software[47]. This analysis showed that 44.7% possess a classical signal peptide which is similar than previous observed in the apoplast of *Arabidopsis* (47%) and rice (37%) [48–50]. On the other hand, 34% of the proteins showed no signal peptide while 22% of the proteins showed a signal peptide for chloroplastic or mitochondrial localization. Previous reports showed that the presence of cytosolic, mitochondrial or vacuolar proteins is a common event in apoplast preparations in different plant species[40], which hints to the occurrence of non-classical secretory pathways for yet unknown apoplastic proteins[51]. On the other hand, Kaffarnik et al.[52] observed that the type III secretion system of *Pst* causes the accumulation in the apoplast of host proteins without classical signal peptides for secretion via the endomembrane system.

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~~**Confirmation of apoplastic localization of the proteins**~~

~~In order to confirm the apoplastic localization of the proteins, the presence of a signal peptide sequence was predicted using the TargetP software[52]. This analysis showed that 44.7% poses a signal peptide. A similar percentage of proteins with a predicted signal peptide sequence were found in the apoplast of Arabidopsis (47%) and rice (37%) whereas higher amounts were observed in the apoplast of soybean (65%) and grapevine (66%)[53–55]. On the other hand, 34% of the proteins showed no signal peptide while 22% of the proteins showed a signal peptide for chloroplastic or mitochondrial localization. The presence in the AWF of proteins from the cellular components has been observed in other experiments describing the proteomic content of~~

~~the apoplast (i.e 12% in grapevine)[45]. This might be a consequence of the bacterial attack that may provoke damage to the plant cell leading to the release of plant cytoplasmic proteins to the apoplast. On the other hand, Kaffarnik et al.[56] observed that the type III secretion system of *Pst* causes the accumulation in the apoplast of host proteins without classical signal peptides for secretion via the endomembrane system. Finally, a low number of bacterial proteins related to bacterium structures have also been detected. Among these proteins, flagellin, ABC transporter substrate-binding protein and Hcp family type VI secretion system effector, have been found. These proteins can play diverse roles in the development of the infection, such as adhesion to the host cells, secretion and translocation of virulence-related proteins, as well as the competition for nutrients during the stationary phase[57,58].~~

Treatment with 1-MT and infection provokes changes in the apoplastic proteins.

To understand how apoplastic proteins were regulated during 1-MT induced resistance in the presence and absence of the pathogen, quantitative proteomics was performed with label-free SWATH-MS to determine differentially expressed proteins (DEPs). Partial least Square discriminant analysis (PLS-DA) showed that the major changes in the proteomic content were induced by the infection with *Pst* (Fig. 2. X-var). In the same way, the treatment also provoked strong changes in the proteins of the apoplast that were clearly distinguishable from the ones found in the apoplast of the untreated plants.

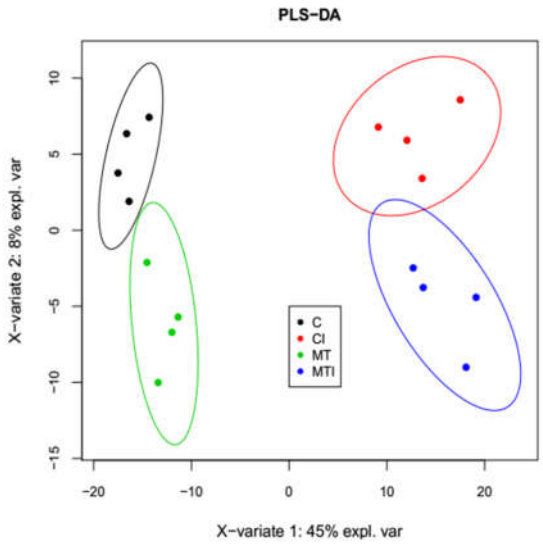

Figure 2. PCA plot based on the normalized proteome data. PC1 is plotted on the x-axis, PC2 is plotted on the y-axis. The PCA analysis was performed with all four treatments Control (C), Infected (CI), Treated with 1-MT (MT) and treated with 1-MT and infected (MTI).

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Identification of defensive proteins induced by the inoculation or treatment with the resistant inducer 1-MT

The comparison between CI vs C showed from the original proteins, 150 are differentially expressed, whereas the comparison between MTI vs MT showed 163 proteins. The comparison of these two sets of proteins shows that there are 96 common proteins, regardless of the

treatment (Fig. 3). Interestingly, ~~40~~ 39 of these proteins are upregulated upon infection (34 plant proteins and ~~56~~ 56 bacterial proteins). Moreover, this common core of infection-induced proteins is composed of 80% of proteins related to plant defense, which include proteins classified according GO as response to wounding, defense response and response to biotic stimuli. Between these proteins, we can highlight the higher abundance in infected plants of the papain-like C1A –which belongs to the cysteine proteases and are important regulators involved in numerous plant biological processes, including leaf senescence and Programmed Cell Death, PCD [53]~~(Liu et al., 2018)~~. Interestingly, most of the common observed proteins are down-regulated upon infection, indicating that the presence of *Pst* provokes a strong downregulation of proteins in the apoplast (57 proteins, Fig. 3). The downregulated proteins in the common proteins, as well as in the unique proteins found are related with primary metabolism and cellular development. From this, near the 50% of proteins are related with carbohydrate or amino acid metabolism or proteins involved in chemical reactions with small molecules which include monosaccharides but exclude disaccharides and polysaccharides. From all the downregulated proteins, less than a 4% are related to defense against stress. These results agree with our previous work, in which the apoplast from tomato plants inoculated with *Pst* showed a reduction in the sugar content[25]. ~~These results are according to a previous work which suggested~~Other authors suggested two different possibilities to explain the decrease of proteins mediated by *Pst* infection[52]. In the first scenario, the plant after the recognition of MAMPs would activate a set of proteins with protease activity against bacterial effectors. In this way, the plant will destroy the proteins that could be beneficial for the pathogen. In the second scenario, it could well be an effect of pathogen-derived proteases which can act in destroying any protein with beneficial activity for the plant host.

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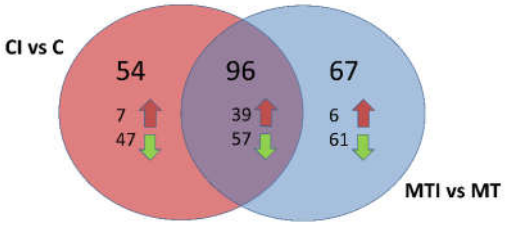

Figure 3. Venn diagram of differentially expressed proteins. Venn diagram of differentially expressed proteins representing all four treatments: Control (C), Infected (CI), Treated with 1-MT (MT) and treated with 1-MT and infected (MTI). Red arrows represent upregulated proteins and green arrows represent downregulated proteins in the comparison between CI vs C and MTI vs MT.

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When we focus on the unique proteins, results showed 54 unique responsive proteins when comparing CI vs C plants and 67 unique responsive proteins when comparing MTI vs MT plants (Fig. 4). This large number of unique proteins for each group suggests that the treatment induces a different mechanism of response in plants treated compared to untreated under challenging inoculation. Moreover, from the unique proteins observed, 7 ~~proteins~~ are upregulated in C plants and 6 proteins are upregulated in MTI. In both cases, the proteins are ~~proteins~~ involved in plant defense such as subtilisin-like protease, miraculin or miraculin-like proteins and wound-induced proteinase inhibitors or 12-oxophytodienoate reductase 3. Previous results in plants treated with the resistance inducer hexanoic acid showed that the accumulation of miraculin-like proteins could contribute to the hexanoic acid priming effect in this Solanaceae species[54].

When we focus on the effect of the treatment with 1-MT on the proteomic content of the apoplast, the comparison between MT vs C showed 30 differentially expressed proteins whereas the comparison between MTI vs CI showed 12 proteins (Fig. 4). In this case, results showed only 3 common proteins, from which two of them are strongly induced by the treatment in the absence of inoculation. These two proteins have been described as heme-binding protein 2-like and a cysteine proteinase 3-like, related to plant defense against pathogens[55]. Interestingly, the downregulated proteins found, belong to the same groups observed above (carbohydrate or amino acid metabolism, and small molecules process).

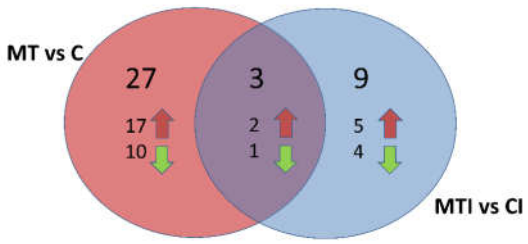

Figure 4. Venn diagram of differentially expressed proteins. Venn diagram of differentially expressed proteins representing all four treatments: Control (C), Infected (CI), Treated with 1-MT (MT) and treated with 1-MT and infected (MTI). Red arrows represent upregulated proteins and green arrows represent downregulated proteins in the comparison proteins between MT vs C and MTI vs CI

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In this comparison, the unique proteins showed that the application of 1-MT in absence of inoculation induced changes in 27 proteins, 17 of which were upregulated by the treatment and 12 of them are involved in defensive process. Between the upregulated proteins we found some proteins involved in carbohydrate metabolic process. Moreover, some of the proteins are described as involved in plant defense such as miraculin-like proteins or wound-induced proteinase inhibitor 1 (Fig. 4). On the other hand, when we compare the inoculated plants, the treatment only induced the accumulation of 5 proteins, such as a multi-cystatin that could be related with plant defense[56].

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General proteome changes against the pathogen infection

When we analyze the differences between the four conditions tested (C, CI, MT and MTI) using the Elastic net penalty, from the 492 proteins detected, results showed 68 proteins with significant differences that are either induced or repressed in, at least, one of the tested condition (Fig. 5, Supplementary Table S2). The CI plants showed 20 over-accumulated proteins, from which, 16 are also observed in MTI ~~plants~~ but at lower levels than in CI. In the 16 common proteins in CI and MTI proteinase inhibitor proteins, miraculin like proteins or defensive proteins such as PR2, have been found. Only two proteins of this group, kynurenine formamidase-like, and auxin responsive proteins, were observed also in non-infected plants treated with MT plants. The kynurenine formamidase-like is part of the L-tryptophan degradation pathway, transforming the N-formylkynurenine in kynurenine. In the same way, although the auxin-responsive proteins

were implicated in developmental process, several works show an implication of these in plant stress[57,58].

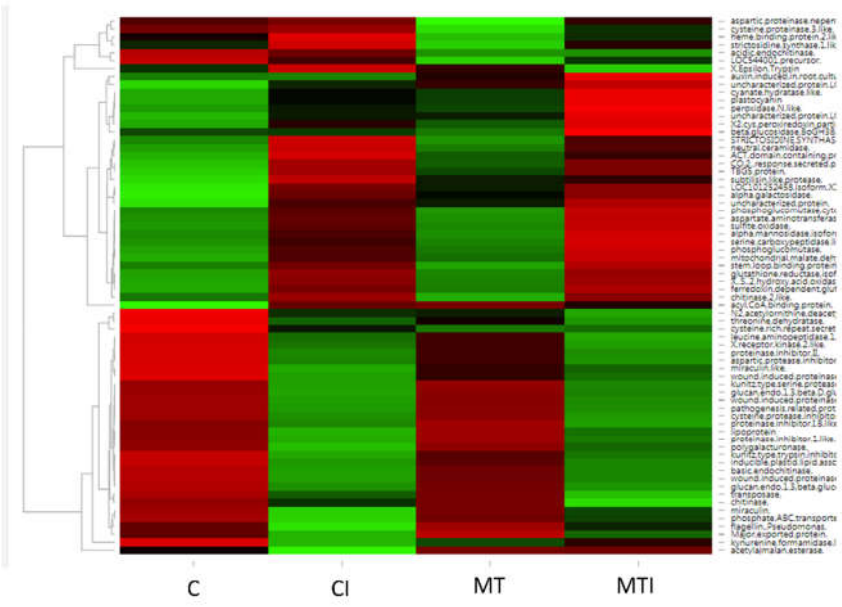

Figure 5. Heat map presenting differentially expressed proteins after Elastic net penalty analysis. Proteomic profiling was carried out for all four treatments Control (C), Infected (CI), Treated with 1-MT (MT) and treated with 1-MT and infected (MTI).

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A total of 20 proteins were over-accumulated upon the treatment with 1-MT in both infected and uninfected plants. 6 of these proteins are described as having peptidase activity, 1 with

chitinase activity, 2 with hydrolase activity. It is well known that peptidase and hydrolase activity have an important role in the plant immune system, and proteins with this activity found in the apoplast have been related to priming, signaling, and defense[55]. The fact that the application of 1-MT promotes the accumulation of such proteins in the apoplast suggests the implication of the changes in the apoplastic content during induced resistance. This enhancement of protein accumulation after the treatment could indicate an activation of a priming mechanism, which is preparing the plant for a more active defense when the pathogen attack occurs.

Upon infection, 1-MT treatment highly induced the accumulation of 8 proteins related with plant defense such as threonine deaminase, N2-acetylornithine deacetylase, cysteine proteinase Inhibitor 8-like, chitinase, aspartic protease inhibitor 1-like, leucine aminopeptidase 1, serine endopeptidase and somatic embryogenesis receptor kinase (Fig. 6). The role of threonine deaminase in plant defense against herbivores has been previously described by Gonzales-Vigil et al.[59]. One of the possible functions of this enzyme in the apoplast could be in the metabolism of threonine (Thr). Scalschi et al. [25] observed that the level of threonine in plants is increased by the treatment with 1-MT. Interestingly, higher plants use threonine deaminase (TD) to catalyze the dehydration of Thr to α-ketobutyrate and ammonia as the first step in the biosynthesis of isoleucine (Ile), contributing to jasmonic acid-isoleucine-mediated defenses against insects[59] and necrotrophs, having a role in induced resistance via priming[21]. Previous work showed that 1-MT is also effective against *Botrytis cinerea* [24].

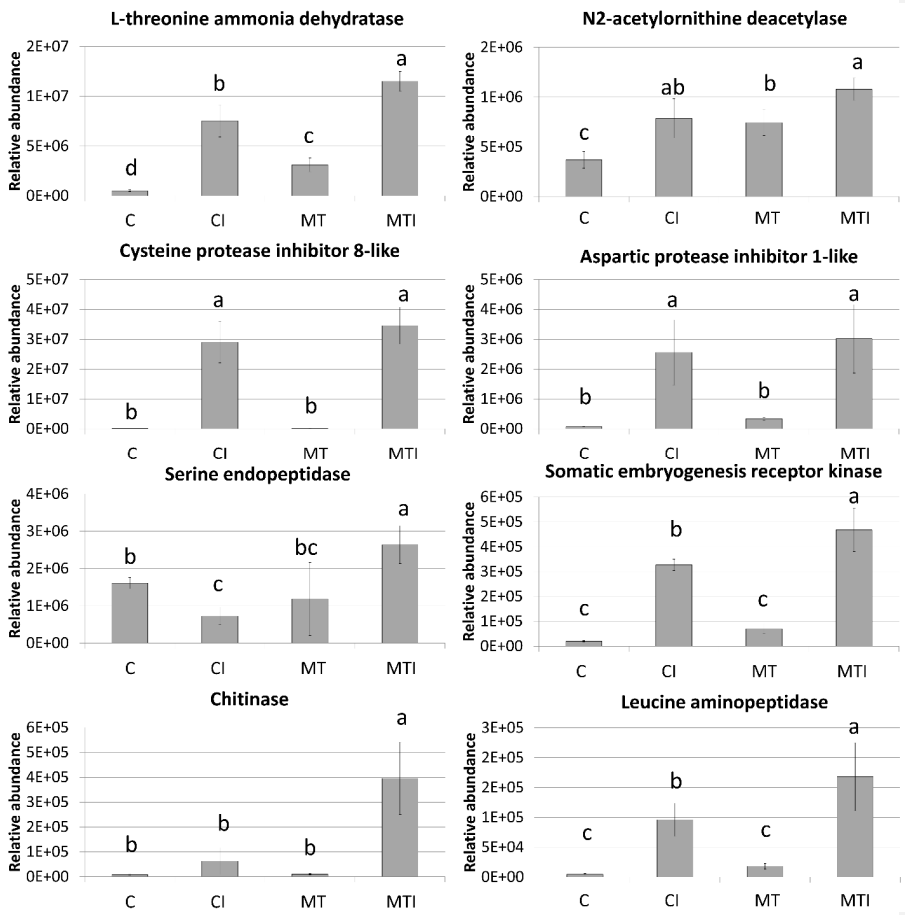

Figure 6. Relative peak area of representative differentially expressed proteins. Bars represent the relative abundance of the presented proteins in all four treatments: Control (C), Infected (CI), Treated with 1-MT (MT) and treated with 1-MT and infected (MTI).

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In the same way, it has been described that N2-acetylornithine deacetylase is involved in the synthesis of L-arginine, the precursor of polyamines and NO, from ornithine. The relation between the accumulation of polyamines and the resistance against *Pst* has been widely described[60,61]. As previously described, chitinase is one of the enzymes involved in defense against of fungal pathogens, but is also related with defenses against bacterial[42]. Cysteine proteinase 3-like is known as an extracellular protein that acts in the recognition of several pathogens[55]. Our results, showed significantly higher amounts in infected plants, being three times more abundant in treated and infected plants, suggesting a possible defensive role against *Pseudomonas*. In this way, previous studies showed that the paralogous protease *Phytophthora*-inhibited protease-1 have an important role in defense level of tomato plants against *Pseudomonas* [62].

Altogether, the comparison of the four conditions showed that from 492 proteins detected, 20 of them are induced in CI, 20 are induced in MT and MTI and only 8 are induced in MTI plants, and all of them are related with plant defense.

Changes of bacterial proteins in the apoplast after 1-MT treatment.

Bacterial proteins were also detected in the apoplast of infected plants regardless of the treatment (Fig. 7). Interestingly, when comparing the level of proteins from bacteria, it was observed that, in treated plants, the amounts of these proteins were between 2 and 6 times lower than in untreated plants. This difference could be directly related to the smaller bacterial population observed in the treated plants. Nevertheless, the difference in the levels of flagellin detected is striking, since the level observed in MTI plants is 5 times lower than in CI plants. Flagellin is a component of the bacterial flagellum whose action as an elicitor in plants is widely

documented. The perception of this protein by the FLS2 receptor induces plant defense gene expression[63,64]. The lower amount of this protein detected in plants treated with MT and infected agree with previous studies showing lower expression of the gene *fliC,* which encodes flagellin, in the bacteria extracted from the 1-MT treated plants than in the bacteria extracted from the untreated ones[25]. Lipoproteins have been also detected in greater amounts in apoplast samples from untreated infected plants that in treated with 1-MT and infected. It is known that many of them have important functions of cellular recognition related to the pathogenesis. Moreover, some of the bacterial lipoproteins that function as extracellular toxins have been previously described[65].

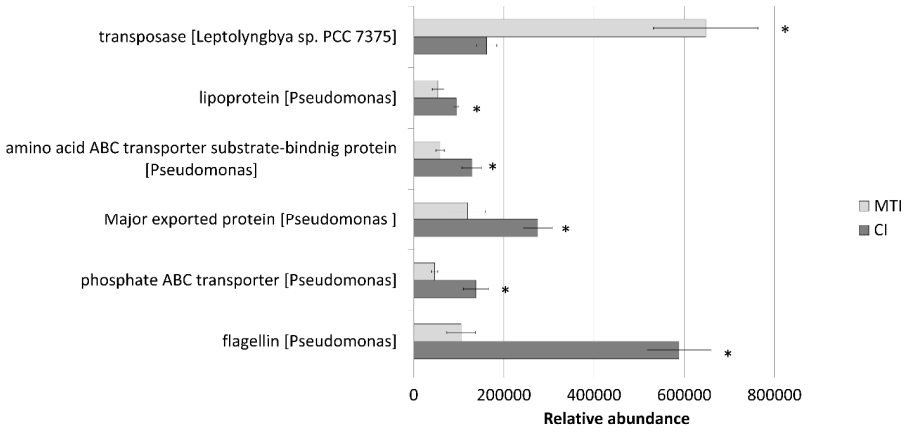

Figure 7. Relative peak area of differentially expressed proteins from bacteria in the apoplast obtained from Infected and treated with 1-MT and infected plants. Bars represent the relative abundance of the presented proteins in the treatments Control Infected (CI) and treated with 1-MT and infected (MTI).

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In addition, two binding proteins of the ABC transport system have also been detected namely phosphate ABC transporter substrate-binding protein and amino acids ABC transporter substrate-binding protein. These proteins play a major role in the unidirectional transport of components across the cellular membrane. These proteins showed high structural conservation between different species within the genus *Pseudomonas* which could point to the importance of their role in the survival of the bacteria. Several studies have related the *Pst* phosphate transport system with the virulence of the bacteria[66,67]. Likewise, the internalization of amino acids through amino acids ABC transporter substrate-binding protein is important for the survival of the bacteria and in some cases has also been related to virulence[68]. Therefore, the fact that these proteins are in smaller quantities in MT plants could be indicative either of a lower bacterial population or could be due to the treatment hinders the synthesis of these proteins by the bacteria, impairing its survival and virulence.

Moreover, a peptide corresponding to a transposase fragment was identified, and that, unlike other proteins from bacteria, is very abundant in plants treated with 1-MT. It is known that transposases help to overcome stress conditions in bacteria[69]. The high presence of this transposase in the apoplast may indicate that the treatment with 1-MT changes the apoplastic environment making it less suitable for the bacterial growth.

Antimicrobial effect of isolated proteins.

In our previous study we have showed that the growth of *Pst* in the apoplast extracted from 1-MT treated plants (with or without infection) was smaller than that one obtained from control plants (infected or not), which means that the treatment could induce the synthesis of certain

proteins that inhibit the growth of the bacteria[25]. To confirm the possible implication of the apoplastic proteins in the bacterial development, a cysteine protease induced by the treatment with 1-MT, both in the absence or presence of infection, was expressed in *E. coli* and purified. To characterize the effect of the protein CP3 on *Pst in vitro*, bacterial growth was measured for 72 h in M9 minimal medium amended with 70 ng/mL of the protein or with PBS. The protein at this concentration inhibited bacterial growth, as demonstrated in a reduction of the optical density by 57.14% after 72 hours of incubation (Fig. 8).

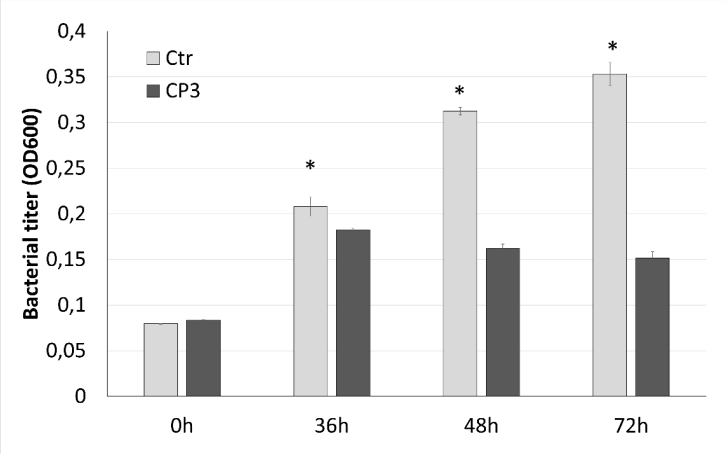

Figure 8. Inhibitory effect of protein CP3 on the growth of *Pseudomonas syringae*. *Pseudomonas syringae* growth was measured for 72 h in M9 minimal medium amended with 70 ng/mL of the protein CP3 or with PBS (control). Bacterial growth was measured at 600 nm.

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The bacteriostatic effect observed reinforce the hypothesis that the proteins secreted in the apoplast during the infection by pathogens or induced by priming treatments have a role in plant defense, and thus, are able to impair the bacterial growth. Related to this, Wang et al.[70] have demonstrated that two apoplastic proteins mediate resistance of *Arabidopsis thaliana* to *P. syringae* by cleaving the bacterial protein *MucD,* suppressing this way the growth of the bacterium in the leaf apoplast[71].

CONCLUDING REMARKS

In this study, we have analyzed the proteomic changes in the apoplast of plants treated with 1-MT in response to *Pseudomonas syringae*. To the best of our knowledge, this is the first report that shows changes in the apoplast induced by the application of a resistance inducer. Our results highlight the possible importance of the changes produced in the apoplast as a first layer of defense against pathogen infection, showing dramatic changes in the accumulation of proteins related to defense. Through the extensive comparisons between treated and untreated plants as well as inoculated and non-inoculated, we have shown proteins with functions in plant induced response to pathogen infection and we have identified a new list of proteins all of them related with plant defense. It is interesting to note that although infection provokes deep changes in the proteome of the apoplast, several unique proteins were found in the MT plants as well as in MTI plants, many of which are targets for future investigation. Preliminary results showed that the tested cysteine protease is able to reduce the bacterial growth in vitro, reinforcing the hypothesis of the accumulation of defensive proteins in the apoplast mechanism of plant defense. Moreover, apart from future work to elucidate the biological function of the selected proteins during plant-pathogen interactions, due to its extracellular nature, the proteins showed in this proteomics study may be a valuable source of new compounds for plant protection.

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ASSOCIATED CONTENT

Supporting Information.

Table S1. Complete list of proteins found in the apoplast

Table S2. Proteins in the apoplast altered by the treatment with 1-MT or by the infection with *Pst.*

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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The authors declare no competing financial interest.

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The mass spectrometry proteomics data have been deposited to the ProteomeXchange archive (http://www.proteomexchange.org/) via the MassIVE partner repository with the data set identifier PXD021316.

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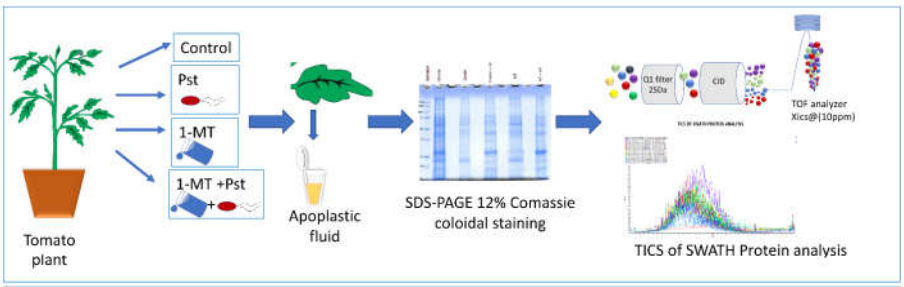
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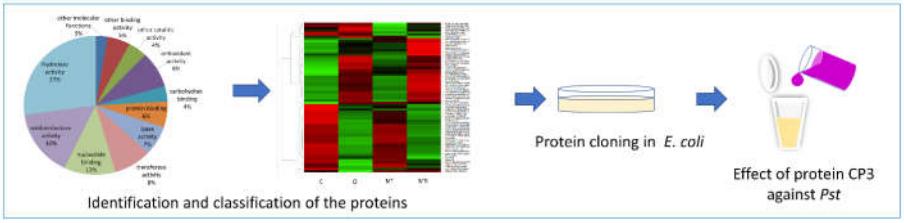
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Protein cloning in E. coli
Effect of protein CP3 against Pst

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