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Disease Control and Integrated Management

e-Xtra*

Induction of Defense Responses and Protection of Almond Plants Against *Xylella fastidiosa* by Endotherapy with a Bifunctional Peptide

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ABSTRACT

Xylella fastidiosa is a plant pathogenic bacterium that has been introduced in the European Union (EU), causing significant yield losses in economically important Mediterranean crops. Almond leaf scorch (ALS) is currently one of the most relevant diseases observed in Spain, and no cure has been found to be effective for this disease. In previous reports, the peptide BP178 has shown a strong bactericidal activity in vitro against *X. fastidiosa* and to other plant pathogens, and to trigger defense responses in tomato plants. In the present work, BP178 was applied by endotherapy to almond plants of cultivar Avijor using preventive and curative strategies. The capacity of BP178 to reduce the population levels of *X. fastidiosa* and to decrease disease symptoms and its persistence over time were demonstrated under greenhouse conditions. The most effective treatment consisted of a combination of preventive and curative applications, and the peptide was detected in the stem up to 60 days posttreatment. Priming plants with BP178 induced defense responses mainly through the salicylic acid pathway, but also overexpressed some genes of the jasmonic acid and ethylene pathways. It is concluded that the bifunctional peptide is a promising candidate to be further developed to manage ALS caused by *X. fastidiosa*.

Keywords: almond leaf scorch, bacterial pathogens, defense pathways, plant immune responses, *Prunus dulcis*, synthetic antimicrobial peptides, transcriptomic response, *Xylella fastidiosa*

Xylella fastidiosa is a plant pathogenic bacterium that causes significant yield losses in a large amount of economically important crops (Bucci 2018). Recently, it has been ranked as the pest with the highest potential impact on agricultural crops in the European Union (EU), estimating losses in the potential production and export, if it fully spreads across the continent, in €5.5 billion and €0.7 billion per year, respectively (Sanchez et al. 2019).

X. fastidiosa is a xylem-limited bacterium transmitted by specialized xylem fluid feeding sap insects, mostly from the groups of spittlebugs and sharpshooter leafhoppers (Almeida and Nunney 2015). Its wide plant host range, genomic plasticity, and high homologous recombination frequency are responsible for the emergence of new *X. fastidiosa* genotypes adapted to novel plant hosts and is one of the main reasons why the spread of *X. fastidiosa* is difficult to control (Baldi and La Porta 2017; Denancé et al. 2019).

Almond trees have been one of the economically important crops affected by *X. fastidiosa* since the 1950s in California, North America (Greco et al. 2021). The two subspecies of *X. fastidiosa* that have been identified as causal agents of almond leaf scorch (ALS) are subsp. *fastidiosa* and subsp. *multiplex* (Krugner and Ledbetter 2016). Recently, the detection of both subspecies in almond orchards of the Balearic Islands (Moralejo et al. 2019) and Alicante in Spain (Giampetruzzi et al. 2019) has expanded ALS to the EU continent, putting almond production at risk (EPPO 2021; Olmo et al. 2021).

No cure has been found to be effective for the control of *X. fastidiosa*, neither for ALS nor for the other diseases caused by this pathogenic bacterium (Kyrkou et al. 2018). Different strategies, such as chemical control using oxytetracycline or a Zn, Cu, and citric acid fertilizer (Amanifar et al. 2016; Bruno et al. 2021; Dongiovanni et al. 2017; Scortichini et al. 2018), biological control using endophytes (Baccari et al. 2019; Lacava et al. 2006, 2007), and agricultural practices such as high-grafting on peach rootstock limbs (Cao et al. 2013), have been studied in different host plants under greenhouse and field conditions. Nevertheless, the general conclusion is that some treatments revitalize trees and reduce the symptoms, but *X. fastidiosa* is still detectable, resulting in the reappearance of the disease when the treatment ceases (Bragard et al. 2019). In this context, the need to find novel strategies for the management of ALS caused by *X. fastidiosa* arises.

Antimicrobial peptides (AMPs) have been largely proposed as a new generation of compounds with great potential in plant disease control (Caravaca-Fuentes et al. 2021; Khademi et al. 2020; Montesinos 2007). AMPs are short sequences, usually less than 50 amino acids and are mainly cationic with an amphipathic character (Balouiri et al. 2016; Mishra and Wang 2012) that can display a wide spectrum of antibacterial and antifungal activity against several pathogens (Park et al. 2017; Petre 2020). Their main mechanism of action involves electrostatic interaction with the pathogen's cell membrane disrupting it (Baró et al. 2020b; Brogden 2005; Mookherjee et al. 2020). Several AMPs developed by our research group have shown antimicrobial and antifungal activity against several plant pathogens such as *Erwinia amylovora*, *Xanthomonas* spp. and *Pseudomonas* spp., *Fusarium oxysporum*, and *Penicillium expansum* (Badosa et al. 2009; Camó et al. 2019; Oliveras et al. 2021). Moreover, some AMPs have been tested in planta with promising results for the control of different plant diseases such as fire blight (Badosa et al. 2017; Caravaca-Fuentes et al. 2021), rubus stunt, and stolbur disease (Rufo et al. 2017), brown spot of pear (Puig et al. 2015), and bacterial canker and gray mold in tomato (Montesinos et al. 2021).

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Peptides have also been described to act as defense elicitors in plants (Malik et al. 2020; Zhang et al. 2019). They trigger the immune response mediated through complex pathways regulated by signaling molecules such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) (Derksen et al. 2013; Li et al. 2019; Lievens et al. 2017; Mur et al. 2013; Ruan et al. 2019). This can elicit a systemic response throughout the whole plant such as the systemic acquired resistance (SAR) (Métraux 2013). This results in a priming state, characterized by an accumulation of pathogenesis-related (PR) proteins in healthy tissues, causing a stronger and faster defense response when future infection occurs (Conrath et al. 2015). An example of an elicitor defense peptide would be the well-studied fragment of flagellin from *Pseudomonas aeruginosa* known as flg22 (Chang et al. 2017; Ciarroni et al. 2018).

Several AMPs such as cecropin B and 1036 have been identified in vitro as potential control agents of *X. fastidiosa* diseases (Baró et al. 2020b; Bleve et al. 2018; Fogaça et al. 2010; Kuzina et al. 2006; Moll et al. 2021). Previous research on AMPs active against different strains of *X. fastidiosa* in our group demonstrated the strong bactericidal and lytic activity of the peptide BP178 (KKLFKKILKYL-AGPA-GIGKFLHSAK-KDEL-OH) (Baró et al. 2020a, b). The peptide is a conjugate of the sequence of the amphipathic cationic linear undecapeptide BP100 (Badosa et al. 2007), linked through an AGPA hinge sequence to a magainin fragment (1-10) with a KDEL retention signal sequence at the C-terminus. BP178 was developed to be synthesized in plants and shows high antibacterial activity against several plant pathogenic bacteria, low hemolysis, and moderate phytotoxicity (Badosa et al. 2013). Moreover, BP178 has recently been reported to act as a bifunctional peptide that is able to induce the plant immune system of tomato plants, upregulating genes coding for several PR proteins and transcription factors involved in the SA-, JA-, and ET-signaling pathways (Montesinos et al. 2021). Therefore, it would be interesting to study the efficacy of BP178 in planta against infections by *X. fastidiosa*, and the effect of this peptide on almond plants transcriptome. In addition, one important issue of the peptides in plant protection is their stability in the plant environment (plant surface or internal tissues), which can affect their physicochemical properties and modify their activity (Ferre et al. 2006; Güell et al. 2011; Montesinos et al. 2012). Thus, its persistence and performance in planta should be studied.

The main objective of the present work was to study the effect of the treatment of almond plants with the bifunctional peptide BP178, and particularly (i) to evaluate the effect of different treatment strategies in the population levels of *X. fastidiosa* and ALS severity, (ii) to determine the persistence of the peptide over time, and (iii) to study the effect of the treatment on gene expression of almond plants.

MATERIALS AND METHODS

Plant host and greenhouse conditions. One-year-old almond plants (*Prunus dulcis*) from the cultivar Avijor provided by Agromillora S.L.U. (Spain) were used for the experiments. All plants were maintained in 0.8-liter pots (sphagnum peat with wood fiber [10%], calcium carbonate [9 g/liter], NPK fertilizer [1 kg/m^3], and microelements) in an environmentally controlled greenhouse at 25 ± 2°C (day) and 18 ± 2°C (night), with a minimum relative humidity of 60%, and with a photoperiod of 16 h light and 8 h dark. Prior and during the experiments, plants were watered to saturation every 3 days, and fertilized with a 200 ppm solution of NPK (20:10:20) once a week. In addition, throughout the experiments, standard treatments with insecticide and acaricide were performed to avoid presence of insect vectors or pests, except in plants used for transcriptomic analysis. Infected plants were cultivated in a Biosafety level II+ quarantine greenhouse authorized by the Plant Health Services, according to EPPO recommended containment conditions (EPPO 2006) and were maintained taking into account the consideration of *X. fastidiosa* as a quarantine pathogen in the EU (EFSA PLH Panel 2018).

***X. fastidiosa* strain and growth conditions.** *X. fastidiosa* subsp. *fastidiosa* IVIA 5387.2 (Moralejo et al. 2019), isolated from almond trees in Mallorca (Spain) and kindly provided by the Instituto Valenciano de Investigaciones Agrarias (IVIA), was used in all experiments. The strain was stored in Pierce disease broth (PD2, Davis 1980) supplemented with glycerol (30%) and maintained at −80°C. When needed, aliquots were cultured in buffered charcoal yeast extract agar plates (Wells et al. 1981) and grown at 28°C for two passages of 5 days each. Cell suspensions were prepared using phosphate-buffered saline (PBS) and adjusted to 10^8 CFU/ml ($OD_{600} \cong 0.3$), confirmed by plate counting as previously described (Baró et al. 2021). Before plant inoculation and to ensure viability, cells were pelleted (10 min at 13,000 rpm) and resuspended in PD2 broth.

Peptide synthesis. BP178 (KKLFKKILKYL-AGPA-GIGKFLH-SAK-KDEL-OH) was prepared by solid-phase synthesis by the LIPPSO laboratory of the University of Girona, with purity above 95% and its identity was confirmed by electrospray ionization-mass spectrometry (ESI-MS) (Badosa et al. 2013; Montesinos et al. 2021). Before use, lyophilized BP178 was solubilized in sterile Milli-Q water to a stock concentration of 20 mM and filter sterilized through a 0.22-µm pore filter.

Effect of BP178 on population levels of *X. fastidiosa* and ALS severity in almond plants. The effect of the peptide on population levels of *X. fastidiosa* subsp. *fastidiosa* IVIA 5387.2 and ALS severity in inoculated almond plants of cultivar Avijor was assessed. The peptide was applied by endotherapy according to the procedure described in Baró et al. (2021) for the pathogen inoculation. Each application consisted of three shots of 10 µl of a stock solution of BP178 at 20 mM using a high precision microinjector (NanoJet, Chemyx, Stafford, TX, U.S.A.) provided with a Hamilton 250-µl syringe with a thin needle with bevel tip (Fisher Scientific, NH, U.S.A.) as shown in Figure 1. The needle end was introduced until approximately one half of the plant stem diameter to reach the vascular system. Nontreated controls were performed using water instead of the peptide solution.

The inoculation of *X. fastidiosa* was performed as described in Baró et al. (2021). Plants were inoculated with three injections of 10 µl of a suspension of *X. fastidiosa* at 10^8 CFU/ml, equivalent to a total of 3×10^6 CFU/plant. The injections were performed on the same side of the stem in a section of 3 cm at around 15 cm above the substrate level, as depicted in Figure 1.

The experimental design consisted of nine plants per each treatment, and two independent experiments were performed. Three different strategies of treatment were used that consisted of (i) preventive applied 1 day before the inoculation of *X. fastidiosa*, (ii) curative applied 3 and 7 days postinoculation (dpi), and (iii) a combination of the above. Samples were collected at 30 dpi, and the levels of viable *X. fastidiosa* cells in sap were analyzed in each of five plants per treatment (Baró et al. 2021). Briefly, to determine the movement of the pathogen from the inoculated area, 16 cm of shoot material was sampled above the inoculation points (upwards zone 1; upwards zone 2; 8 cm each zone), and below (downward zone; 8 cm) (Fig. 1). Sap was obtained from each 8-cm fragment by removing the bark from the stems to mostly retain vascular tissue, cutting the fragment into three parts and putting them in 2-ml centrifuge tubes with a hole at the bottom. The 2-ml tubes were inserted in 5-ml tubes, and the assembly was centrifuged at 13,000 rpm for 25 min. The population levels of *X. fastidiosa* in sap was analyzed by viability-qPCR (v-qPCR) (Baró et al. 2020a). Sap collected in the 5-ml tube was diluted to a final volume of 500 µl of PBS. For v-qPCR, an aliquot of 180 µl was treated with PMAxx (VWR, Barcelona, Spain). DNA extraction was performed using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Finally, a TaqMan-based qPCR was used as described previously (Baró et al. 2020b). The number of

viable cells in sap, expressed as $\log_{10}$ CFU/ml, was obtained by interpolating C_T values from samples of the experiment in a standard curve, CFU versus C_T values, made with sap from a healthy almond plant of cultivar Avijor fortified with known concentrations of *X. fastidiosa.*

The remaining four plants of each treatment within each experiment were kept in the greenhouse for ALS symptom evaluation at 60, 90, 120, and 180 dpi, following the severity scale previously described in the literature (Baró et al. 2021). Differences between preventive and curative BP178 treatments in the population levels of *X. fastidiosa* and disease severity in almond plants were tested performing a one-way analysis of variance (ANOVA). In all cases, means were separated according to Tukey's test at $P \leq 0.05$.

Analysis of synthetic BP178 in treated almond plants. The persistence over time of the peptide BP178 in healthy almond plants was determined. Twelve plants of cultivar Avijor were treated with 30 µl of the peptide (three applications of 10 µl in 3-cm fragment shown in Figure 1, equivalent to a total of 1.94 mg of peptide per plant) using a high precision microinjector as described above. Samples were taken at 18 h posttreatment (hpt), and 7, 15, 30, 45 and 60 days posttreatment (dpt). Samples were processed for western blot analysis as described previously (Montesinos 2014). Briefly, the treated fragment of the stem of two plants was cut (3 cm fragment of 0.2 g fresh weight for each plant) at each sampling time and the bark removed from the stems to mostly retain vascular tissue. Then, the tissue was treated with liquid nitrogen and homogenized in lysis buffer (10 mM Tris-HCl, pH 6.2, 50 mM KCl, 6 mM $MgCl_2$, 0.4 M NaCl, 1% (vol/vol) Triton X-100, and 10 mM ethylenediaminetetraacetic acid [EDTA]), and centrifuged at 16,000 × *g* for 15 min at 4°C. The proteins in the supernatant were separated by 16.5% (wt/vol) Tris-Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes using Mini Trans-Blot Cell Assembly (Bio-Rad, U.S.A.), according to the manufacturer's instructions.

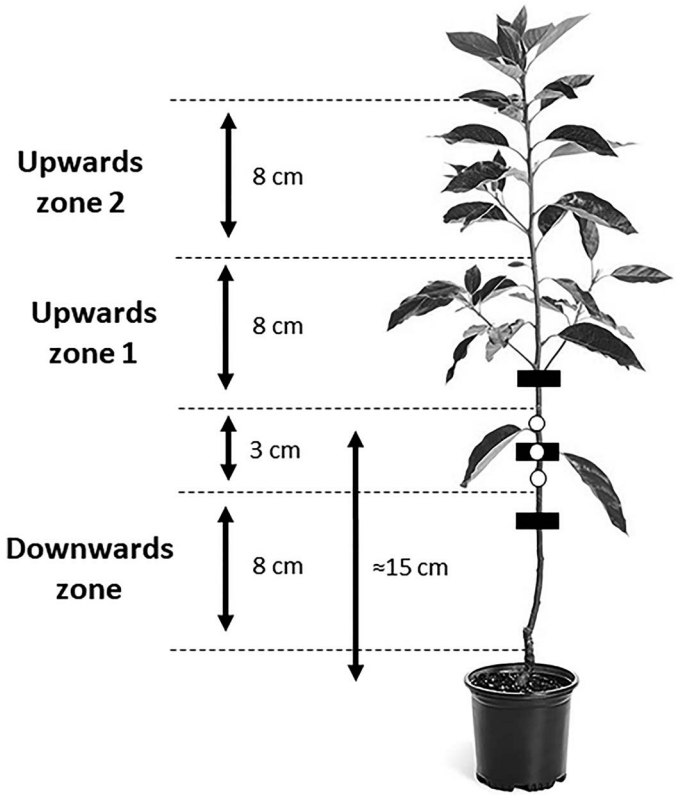

Fig. 1. Inoculation points for *Xylella fastidiosa* (white circles) and application points of the synthetic antimicrobial peptide (black rectangle) in almond plants. Sampled zones are also indicated as upwards zones 1 and 2 and downwards zone.

Membranes were washed twice in water and blocked in blocking buffer prior to the first incubation with the primary antibody against BP178 (GenScript Corp., Piscataway, NJ, U.S.A.). The primary antibody was used as a dilution of 1:2,000 in blocking solution, and the incubation was performed overnight at 4°C with shaking. Six washing steps of 5 min each were carried out in PBS plus 0.1% Tween 20 before the incubation of the membranes with the secondary antibody for 1 h at room temperature with shaking. A chromogenic method using an anti-rabbit IgG (Fc) alkaline phosphatase conjugate (Promega Biotech Iberica, SL, Madrid, Spain) as the secondary antibody (1:7,500 dilution in blocking solution) was used for the detection of anti-BP178. The Western BlueStabilized substrate served for the visualization of alkaline phosphatase activity (Promega). The signal of the BP178 peptide was quantified using the Quantity Tools Image Lab Software (Version 4.1) included in the ChemiDoc XRS+ System (Bio-Rad), by comparing the band intensity of the BP178 present in the stem to that of reference amounts of BP178 at known concentrations, run in the same gel.

Effect of BP178 on the transcriptome response of almond plants. The effect of the peptide BP178 on *P. dulcis* 'Avijor' transcriptome response was assessed by applying the peptide by two application systems, by spray and microinjection, to ensure a proper peptide acquirement by the plant. Each plant was injected with 10 µl of the peptide at 20 mM as described above, and subsequently sprayed with 2 ml of the peptide at 125 µM using an airbrush (Herkules, Nuair, Robassomero, Italy). The experimental design consisted of four replicates of five plants per treatment. Plants treated with water were used as untreated controls. Sampling was carried out 24 hpt and for each replicate 20 leaves were pooled (four leaves/plant) and they were immediately frozen in liquid nitrogen. The material was finely grounded and transferred to tubes with two glass beads and homogenized with a Tissue Lyser II (Qiagen, Hilden, Germany) at a frequency of 30 Hz for 1 min.

RNA was extracted from 100 mg of the grounded leaf material coming from each replicate using the PureLink Plant RNA Reagent (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) following the manufacturer's instructions. The remaining DNA was digested with the TURBO DNA-free Kit (Invitrogen Life Technologies). RNA concentration was estimated through absorbance at 260 nm using a NanoDrop ND1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, U.S.A.). RNA samples were stabilized at room temperature using the RNA Transport kit (Omega Bio-tek, Norcross, GA, U.S.A.) and sent to Sequentia Biotech (Barcelona, Spain) for RNA sequencing.

RNA TruSeq Stranded mRNA Sample Prep kit (Illumina, San Diego, CA) was used for library preparation following the manufacturer's instructions, starting with 1 to 2 µg of good quality RNA (RIN > 7) as input. The RNA was fragmented 3 min at 94°C and every purification step was performed using 0.81X Agencourt AMPure XP beads. Both RNA samples and final libraries were quantified using the Qubit 2.0 Fluorometer (Invitrogen) and quality tested by Agilent 2100 Bioanalyzer RNA Nano assay (Agilent technologies, Santa Clara, CA, U.S.A.). Libraries were then processed with Illumina cBot for cluster generation on the flowcell, following the manufacturer's instructions and sequenced on paired-end (2 × 150 bp, 30M reads per sample) at the multiplexing level requested on NovaSeq6000 (Illumina). The CASAVA 1.8.2 version of the Illumina pipeline was used to processed raw data for both format conversion and de-multiplexing.

Raw sequence files were first subjected to quality control analysis using FastQC v0.10.1 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) before trimming and removal of adapters with BBDuk (https://jgi.doe.gov/data-and-tools/software-tools/bbtools/), setting a minimum base quality of 25 and a minimum read length of 35 bp. Reads were then mapped against the *P. dulcis* genome (Sánchez-Pérez et al. 2019) with STAR v2.6 (Dobin et al. 2013). FeatureCounts v1.6.1 (Liao et al. 2014) was then used to obtain raw expression counts for each annotated gene using only uniquely mapping reads (MAPQ ≥ 30). The differential expression analysis

was conducted with the R package edgeR (Robinson et al. 2010) using the Trimmed mean of M-values normalization method and considering as significant the genes with a false discovery rate (FDR) ≤ 0.05. Fragments per kilobase million (FPKM) were obtained with edgeR. Gene ontology enrichment analysis (GOEA) was performed using in-house scripts based on the AgriGO publication (Tian et al. 2017).

Differently expressed genes with an FDR < 10^{-2} and a $\log_2$ fold change (FC) ≥ |1| were selected. The information of the selected genes was obtained from databases of *P. dulcis* genes (GenBank; https://www.ncbi.nlm.nih.gov/genbank/ and Uniprot; https://www.uniprot.org/). This information was complemented by correlating the selected genes with *Arabidopsis thaliana* genes by sequence identity (when available). Functional information was obtained from several databases, such as GenBank, Uniprot, and The Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org/). Using this information, genes were associated with a major defense pathway or with other general functions (when possible).

RNA-seq data has been deposited in the GEO-NCBI repository with the code number GSE198089.

RESULTS

Colonization and symptoms development in BP178-treated almond plants. The population levels of viable *X. fastidiosa* cells (v-qPCR) in almond plants of cultivar Avijor were determined after the treatment with BP178 by endotherapy, following the strategies including preventive (1 day before the pathogen inoculation), curative (3 and 7 dpi), and the combination of both treatments, and compared with a nontreated control (NTC). A two-way ANOVA was performed to assess if results obtained from the two independent experiments were significantly different. Because no significant differences were observed ($P > 0.1$), the data were pooled and are presented in Figure 2. The combined treatment was the most effective one in reducing the viable cells of *X. fastidiosa* in sap, with a decrease between 2 and 3 log in all plant parts analyzed, compared with NTC. The preventive or curative treatments when used independently did not significantly reduce the population levels.

The disease severity was also evaluated over a period of 180 dpi (Fig. 3). Symptoms started between 30 and 60 dpi. NTC plants were the most affected ones during the whole experiment, with almost half of the leaves showing marginal necrosis already at 60 dpi. Disease severity increased over time and plants showed leaf scorch symptoms and experienced a progressive decline at 180 dpi. In plants treated with BP178, ALS infection was reduced during the first 120 dpi compared with the NTC, being the group of plants preventively treated with BP178 (preventive and combination treatments) the ones showing the lowest disease intensity (60 to 65% of disease severity reduction compared with the NTC, at 60 and 90 dpi). Nevertheless, no significant differences were observed between treatments at 180 dpi.

Persistence of BP178 in treated almond plants over time. The vascular tissue of almond plants treated with BP178 (1.94 mg of peptide in a 3 cm fragment of 0.2 g fresh weight per plant) was analyzed at different times by western blot analysis using an anti-BP178 polyclonal antibody. The presence of BP178 was confirmed at all times analyzed until 60 dpt (Fig. 4; Supplementary Fig. S1). A decrease in the BP178 concentration was observed over time, with a maximum of 253.3 µg/g fresh weight of vascular tissue at 18 hpt to 71.1 µg/g fresh weight at 60 dpt.

Effect of BP178 on the transcriptome of almond plants. Eight mRNA libraries were sequenced from four replicates of *P. dulcis* 'Avijor' treated with BP178 and four replicates of NTC. Each library had approximately 15 million raw reads that, after filtering for high quality reads, 13 to 14 million sequences were kept. Reads were assigned to the *P. dulcis* reference genome (Sánchez-Pérez et al. 2019), and between 75 and 77% of them were uniquely mapped to genes (Supplementary Table S1). The quality of the experiment was assessed using a principal component (PC) analysis. PC2 accounted for 24.2% of the total variation in the dataset, which resulted in two clusters that clearly separated treated from nontreated plants (Supplementary Fig. S2). After bioinformatics analysis (Fig. 5; Supplementary Table S2), differentially expressed genes (DEGs) were identified, which after filtering (FDR ≤ 10^{-2} and a $\log_2$ FC ≥ |1|) resulted in 97 upregulated and 105 downregulated genes.

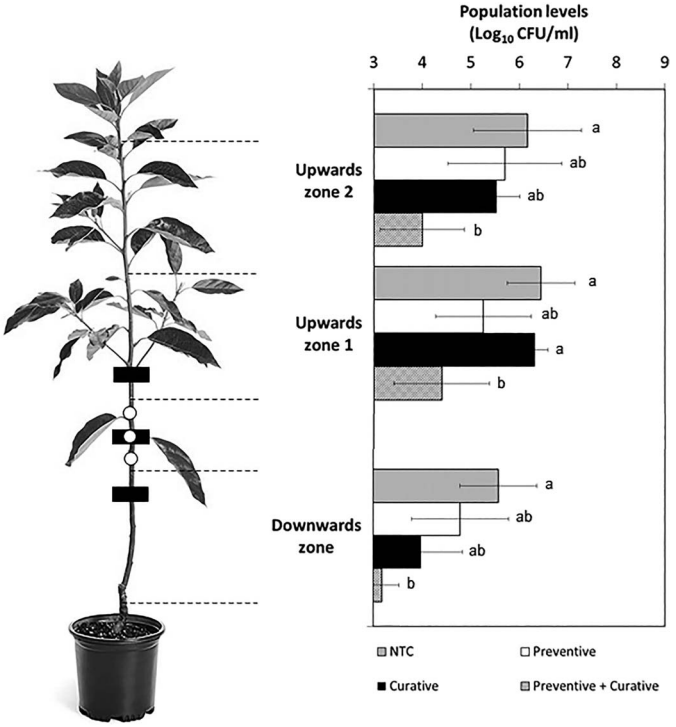

Fig. 2. Effect of BP178 treatments on viable *Xylella fastidiosa* subsp. *fastidiosa* IVIA 5387.2 population levels in sap of almond plants at 30 days postinoculation (dpi). The treatments were preventive (1 day before inoculation), curative (3 and 7 dpi), and a combination of both treatments. Values are the means of 10 plants (pooled results from two independent experiments), and error bars represent the standard deviation of the mean. Different letters between treatments at a specific plant part analyzed indicate significant differences between population levels of *X. fastidiosa* according to Tukey's test ($P \leq 0.05$).

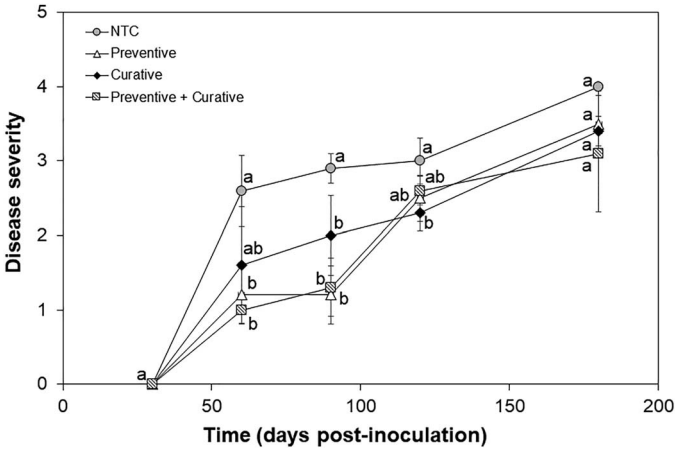

Fig. 3. Disease severity of almond leaf scorch in plants treated with BP178 using different strategies compared with a nontreated control over a period of 180 days postinoculation (dpi) of *Xylella fastidiosa*. The strategies were preventive (1 day before inoculation), curative (3 and 7 dpi), and a combination of both treatments. Values are the means of eight plants (pooled results from two independent experiments), and error bars represent the standard deviation of the mean. Different letters between treatments at specific time points indicate significant differences between disease severities according to Tukey's test ($P \leq 0.05$).

These genes were categorized manually into defense or nondefense pathways whenever enough functional information was available (GOEA and databases) (Fig. 6). A total of 74% upregulated (72 of the selected upregulated genes) and 43% downregulated genes (45 of the selected downregulated genes) were associated with defense functions. Specifically, 31 upregulated genes were associated with the SA pathway, 12 with the JA and ET pathways, 14 with the ABA, and 15 with general functions related to defense, such as transport of molecules or regulation of the pathways. Regarding the downregulated genes, 10 genes were associated with the SA pathway, 12 with the JA and ET pathways, 9 with ABA pathway, and 14 with other defense functions. A total of 63 genes related to nondefense pathways were also affected by BP178 (43 downregulated and 20 upregulated). These genes were related to development processes (shoot, leaf, and flower development and cell wall modifications) and metabolism processes (carbohydrate, lipid, and protein synthesis). It is interesting to highlight that the most affected category was the carbohydrate-related genes with nine downregulated genes.

Table 1 shows interesting DEGs associated with defense and nondefense pathways in order to have a holistic view of the effect of BP178 on the almond's transcriptome. The most overexpressed genes related to defense were PR proteins such as peroxidases (PR9), lipid transfer proteins (PR14), and polygalacturonase inhibitors (PR6) and genes related to the synthesis of terpenes. Some interesting downregulated genes were related to the biosynthesis of ET and JA such as 1-aminocyclopropane-1-carboxylate synthase (ACS) and allene oxide cyclase (AOC) and transcription factors

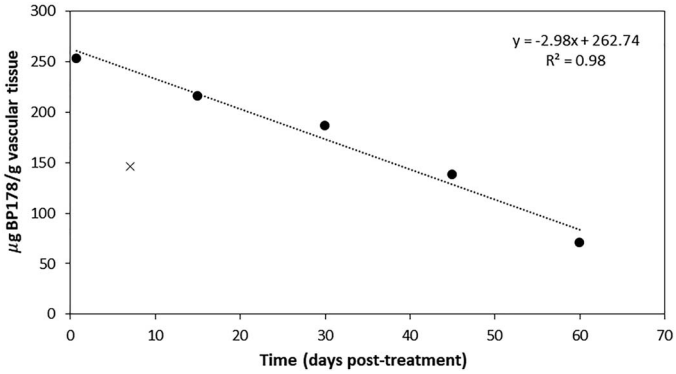

Fig. 4. Persistence of BP178 in the stem of almond plants of cultivar Avijor treated with the peptide solution (total of 1.94 mg per plant applied by endotherapy). Sampling was performed at 18 h posttreatment and 7, 14, 30, 45, and 60 days posttreatment. The concentration of peptide was calculated using the standards of BP178 shown in the Western-blot analysis (Supplementary Fig. S1) and the total amount of vascular tissue used to extract the peptide (two fragments of 3 cm each of 0.2 g fresh weight). The data point marked with an × doesn't follow the trend of the other ones and wasn't taken into consideration for the linear regression analysis.

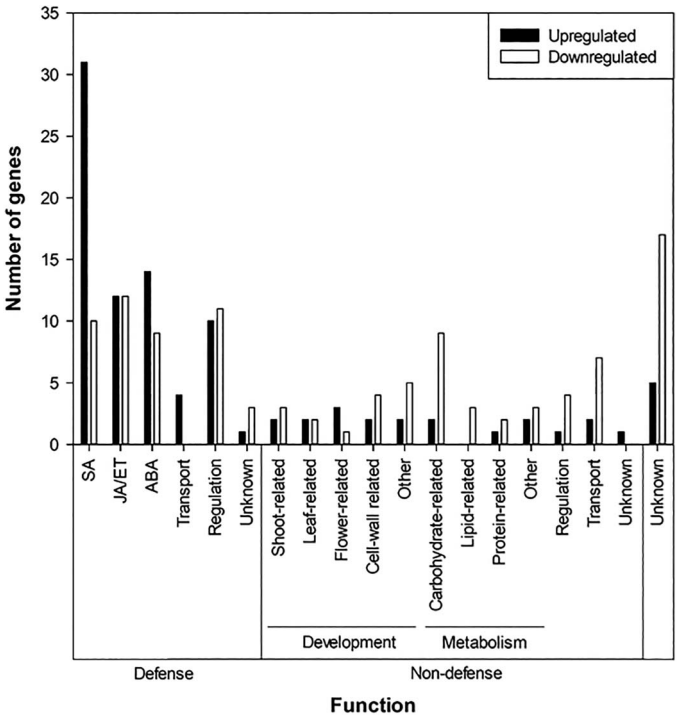

Fig. 6. Number of differentially expressed genes (upregulated and downregulated) in almond plants due to BP178 treatment classified by function (major defense and nondefense pathways).

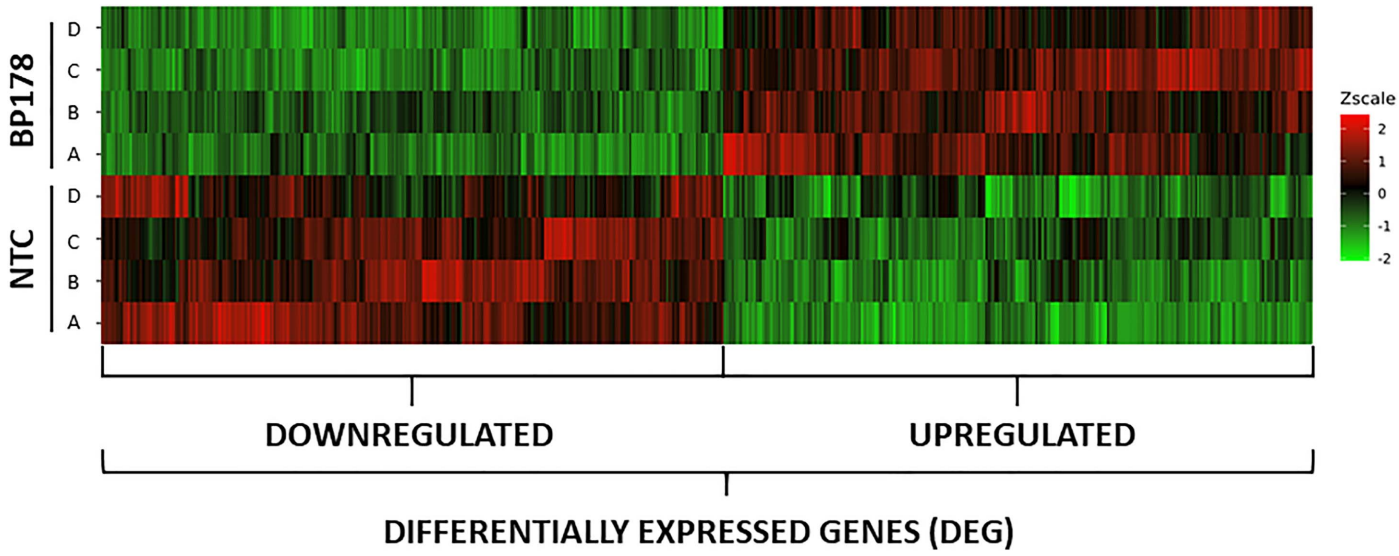

Fig. 5. Heat map of the expression patterns (Z-scaled fragments per kilobase million values) of the differently expressed genes (DEGs) in almond plants (replicates A, B, C, and D) that were treated with the peptide BP178 compared with the nontreated control (NTC). Columns correspond to the identified DEGs. Changes in expression levels are displayed from green (underexpressed) to red (overexpressed). The order of genes was established after hierarchical clustering using the Euclidean distance.

TABLE 1. Showcase of the most interesting differentially expressed genes (DEGs) in almond plants treated with BP178 associated with defense or nondefense pathways[a]

ID[b]	Log_2 FC[c]	FDR[d]	Protein[e]
Upregulated genes (97)			
Defense (72)/shown (18)			
Prudu.06G057900	1.05	2.17E-06	Thioesterase superfamily protein connected with phenylalanine-dependent pathways
Prudu.393S000300	1.44	4.35E-09	F-box/kelch-repeat protein connected with Cullin3 (CUL3) E3 ligase
Prudu.07G152500	1.06	2.29E-04	Thioredoxin H1, which can participate in NPR1 monomerization
Prudu.07G057700	1.12	7.03E-04	Thaumatin-like protein (TLP) (PR5)
Prudu.07G075800	1.47	4.50E-03	Polygalacturonase inhibitor (PR6)
Prudu.07G075200	1.54	5.01E-11	Polygalacturonase inhibitor (PR6)
Prudu.02G250900	1.39	8.18E-06	Peroxidase superfamily protein (PR9)
Prudu.06G232300	2.87	1.69E-05	Peroxidase superfamily protein (PR9)
Prudu.06G040900	2.69	8.89E-09	Lipid transfer protein (PR14)
Prudu.06G040800	2.89	1.02E-06	Lipid transfer protein (PR14)
Prudu.95S000400	1.41	6.42E-13	Lipoxygenase 2 (LOX2) required for JA synthesis
Prudu.04G026800	1.81	2.88E-10	Terpene synthetase related to response against biological stresses
Prudu.01G417600	1.06	8.07E-06	Probable WRKY transcription factor protein
Prudu.01G195400	1.14	9.53E-11	S-methylmethionine cycle and biosynthetic process
Prudu.08G098400	1.36	9.43E-05	Methyltransferases superfamily protein required for MeSA or MeJA
Prudu.01G111400	1.06	1.42E-07	MLP-like protein 423 related to ABA mediated processes like stomatal closure
Prudu.01G347900	1.22	7.38E-23	Bifunctional nuclease in basal defense response related to callose deposition
Prudu.07G122200	1.11	1.57E-07	CHY-type/CTCHY-type/RING-type Zinc finger protein related to stomatal opening/closing regulated by ABA and JA
Other (20)/shown (4)			
Prudu.1169S003200	1.26	3.77E-08	TCP family transcription factor involved in heterochronic regulation of leaf differentiation
Prudu.02G050900	1.31	3.13E-03	Microtubule-associated protein
Prudu.01G205100	1.55	2.19E-11	CCR-like protein related to the circadian rhythm
Prudu.06G052000	1.23	2.25E-04	Asparagine synthetase
Unknown (5)			
Downregulated genes (105)			
Defense (45)/shown (9)			
Prudu.07G223300	−1.93	5.61E-04	Ethylene-responsive transcription factor (ERF) WIN1, which belongs to the ET signaling pathway
Prudu.05G109600	−1.16	5.69E-03	Encodes a member of the 1-aminocyclopropane-1-carboxylate synthase (ACS), which belongs to the ET biosynthesis process
Prudu.03G217000	−1.11	3.68E-06	Encodes allene oxide cyclase (AOC), which catalyzes an essential step in JA biosynthesis
Prudu.1302S000100	−1.46	1.15E-04	Transcription factor MYB82, which belongs to the MCY branch of the JA signaling pathway
Prudu.06G334000	−1.25	6.25E-03	GDSL esterase/lipase related to lipid metabolic processes
Prudu.02G247100	−1.23	1.64E-06	Ankyrin repeat family protein related with the hypersensitive response
Prudu.01G306400	−1.60	3.24E-03	MAC/Perforin domain-containing protein related with the hypersensitive response
Prudu.06G228500	−1.17	3.37E-16	PHE ammonia lyase (PAL), which participates in the synthesis of SA
Prudu.04G156000	−1.18	1.37E-08	Basic-leucine zipper (bZIP) transcription factor family protein, which could interact with NPR1 related to the SA pathway
Other (43)/shown (9)			
Prudu.03G087800	−1.73	2.48E-06	Protein with boron transporter activity that directs boron to young developing tissues in the shoot such as immature leaves
Prudu.06G191500	−1.32	2.40E-05	Protein with an N-terminal BTB/POZ domain and a C-terminal NPH3 family domain required for correct primary shoot and root growth
Prudu.04G050800	−1.25	2.49E-05	Tetratricopeptide repeat (TPR)-like superfamily protein related to petal morphogenesis
Prudu.05G129600	−1.43	2.62E-05	Alpha/beta-hydrolases superfamily protein required for normal cuticle formation
Prudu.02G200300	−1.45	4.02E-04	NAC domain containing protein 86 related to sieve tube element development
Prudu.08G130200	−1.11	2.15E-03	Cyclin-D3-1 involved in the switch from cell proliferation to the final stages of differentiation
Prudu.05G161600	−1.77	2.60E-03	Beta-glucosidase related to carbohydrate metabolism
Prudu.03G012200	−1.45	1.43E-06	Sucrose synthase related to carbohydrate metabolism
Prudu.07G054200	−1.43	9.38E-04	Encodes a gibberellin-regulated GASA/GAST/Snakin family protein
Unknown (17)			

[a] DEGs obtained through RNA-seq were filtered by $\log_2$ fold change (FC) > |1| and false discovery rate (FDR) < 10^{-2}.
[b] GenBank accession number.
[c] Binary logarithm of the FC expression of each transcript.
[d] FDR of each transcript.
[e] Protein codified in each transcript.

such as ethylene-responsive transcription factor (ERF). In addition, interesting downregulated genes related to nondefense were a sucrose synthase and tetratricopeptide repeat (TPR)-like superfamily protein.

DISCUSSION

The peptide BP178 (a bifunctional peptide derivative of BP100 linked to a fragment of magainin) treatments (preventive, curative and a combination of both) applied to the stem by endotherapy protected almond plants against *X. fastidiosa* disease in greenhouse conditions. Endotherapy was used since it is the most effective way to access the vascular system, and because most of tree spraying and irrigation approaches to apply treatments have been unsuccessful (Bragard et al. 2019; Scortichini et al. 2021). In the present work, the ALS disease severity reduction was associated with a decrease in the population levels of *X. fastidiosa* due to BP178 treatment, but the pathogen was not completely eliminated. These results are similar to those reported with compounds such as oxytetracycline, NAC or the Zn-Cu-citric acid fertilizer, in which a reduction in disease severity was observed after their application to infected almond and olive trees, in combination with other agronomic measures, although no cure or prevention of tree infection was achieved (Amanifar et al. 2016; Dongiovanni et al. 2017; Scortichini et al. 2018).

The peptide BP178 was detected in the plant vascular system for at least 2 months, which is in agreement with the long persistence of BP178 in particular plant tissues like the rice seed endosperm (Montesinos et al. 2017). The concentration observed in the vascular system seems to be enough to retain activity against *X. fastidiosa*. The total amount of peptide per plant (1.94 mg) was equivalent to 3.61×10^{17} molecules and the cells of *X. fastidiosa* inoculated or detected per plant were in the range of 3 to 5×10^{6} CFU. We reported that the in vitro minimum threshold for bactericidal action of BP178 was around 10^{9} molecules of peptide per cell (Baró et al. 2020b). Therefore, a ratio of 7.22×10^{10} molecules of peptide per cell was used under our experimental conditions, which is consistent with significant bactericidal activity in plants. However, in the in vitro interaction experiments, the peptide was uniformly distributed in the solution, whereas we do not know the distribution in the vascular tissue of the plant. Also, the loss of activity in planta over time could be due to binding to nontarget plant components (e.g., plant cell envelope), adverse physicochemical conditions (e.g., pH, salts), or degradation (e.g., proteases). Binding to certain cell envelope structures (e.g., cellulose, pectins) or compounds found in planta (Zeitler et al. 2013) could likely be due to its cationic amphipathic properties. In addition, it has been reported for CECMEL11 peptides and derivatives like BP178, that their activity depends on physicochemical conditions or susceptibility to protease hydrolysis (Baró et al. 2020b; Cabrefiga and Montesinos 2017; Güell et al. 2011; Montesinos et al. 2012).

The peptide BP178 has not only been reported to possess bactericidal activity against several plant pathogenic bacteria, but it has also been demonstrated by means of microarray and RT-qPCR analysis to act as defense elicitor in tomato plants (Montesinos et al. 2021). In the present study, the priming effect of BP178 was also demonstrated on almond plants but using only RNA-seq analysis. Several authors consider that RNA-seq methods are robust enough and do not require validation by RT-qPCR or other approaches (Coenye 2021). According to Everaert et al. (2017), RNA samples that were analyzed by RNA-seq and compared with RT-qPCR showed little to zero nonconcordance (near 1.8%) in DEGs with FC values higher than 2. Therefore, in the present work, only DEGs with FC values higher than 2 were taken into consideration.

Most of the identified DEGs were related with functions in plant defense. Taking into account these DEGs, the interaction between the plants defense pathways (SA, JA, and ET) can be summarized as it is depicted in Figure 7 (Ali and Baek 2020; Derksen et al.

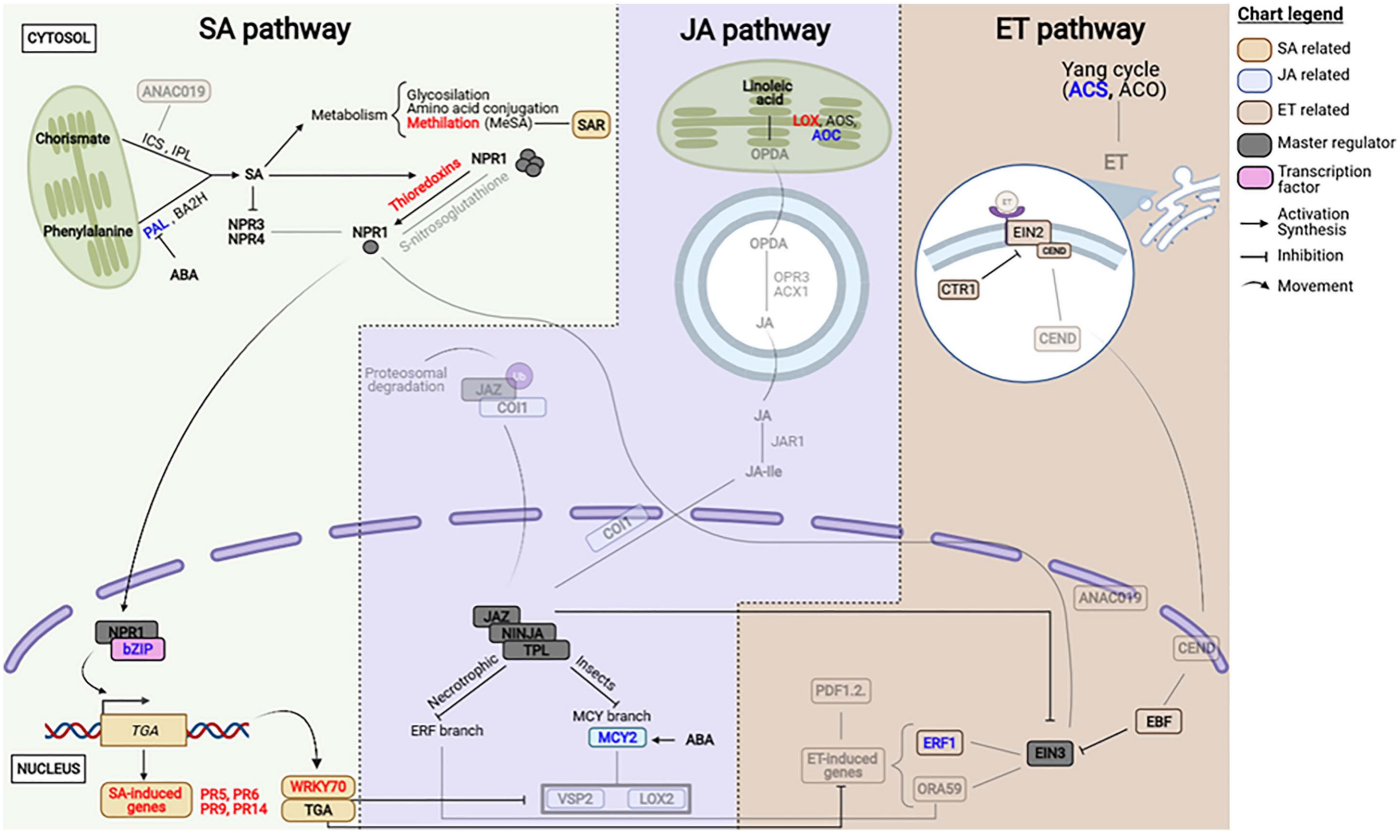

Fig. 7. Model of the major plant defense pathways. Expression of the proteins of each pathway that have been affected by the treatment of BP178 after 24 h are highlighted. Red names correspond to upregulated genes and blue names to downregulated genes. Names in black correspond to proteins that are participating in the pathway. Image created with BioRender (https://biorender.com).

2013; Lefevere et al. 2020; Lievens et al. 2017; Ruan et al. 2019). Many pathogenesis related (PR) proteins were identified such as thaumatin-like protein (PR5) considered a marker of the SA pathway (Qi et al. 2018), polygalacturonase inhibitors (PR6), lipid transfer proteins (PR14) (Moosa et al. 2017), and other proteins of this route such as thioredoxins and the WRKY transcription factor related to the SA pathway. Moreover, genes related to other major pathways were found to be downregulated such as the ethylene-responsive transcription factor WIN 1 (ERF1) and the 1-aminocyclopropane-1-carboxylate synthase (ACS) related to the ET pathway, and the allene oxide cyclase (AOC) related to the JA pathway. This goes in accordance with previous reports in the literature where it is stated that the SA pathway generally presents an antagonistic relationship with the JA and ET pathways (De Vleesschauwer et al. 2010; Derksen et al. 2013; Li et al. 2019). These observations are similar to the ones previously described in tomato plants where most of the upregulated genes by BP178 treatment were related to the SA pathway (Montesinos et al. 2021). Specifically, it was able to stimulate several PR proteins (PR5, PR6, PR9, and PR14), WRKY transcription factors, and genes related to the synthesis of secondary metabolites such as terpenoids (Montesinos et al. 2021). Nevertheless, some differences between the tomato and almond responses can be identified. For example, some genes that have not been upregulated in *P. dulcis* are PR1, PR2, PR3, PR7, and PR10 proteins, and ERF, MYB, and NAC-related transcription factors that are linked to the ET and JA pathways were upregulated in tomato. These differences could be explained by the fact that tomato is an herbaceous plant, whereas almond is a woody plant and that the application of BP178 treatment was different in the tomato from that in the almond experiments.

The activation of the SA pathway, which is the one that seems to be mainly triggered by BP178 in almond, could result in the activation of SAR which would cause a priming effect on the plants before *X. fastidiosa* inoculation. Priming grapevines with the endophytic bacteria *Paraburkholderia phytofirmans* PsJN, or with mutant cells of *X. fastidiosa* that lacked the O-antigen fragment of the lipopolysaccharide, activated the SA-mediated defense pathway and caused a reduction in *X. fastidiosa* population and Pierce's disease severity (Baccari et al. 2019; Rapicavoli et al. 2018). Moreover, the resistant olive cultivar Leccino also presents higher levels of SA when infected with *X. fastidiosa* (Novelli et al. 2019), in addition to an increase in reactive oxygen species (ROS), secondary metabolites (flavonoids and tannins) and lignin synthesis (Giampetruzzi et al. 2016). Also, the activation of SA was reported to result in a reduction of disease severity and pathogen population as it has been observed in other studies with different plant pathogens (Aranega-Bou et al. 2014; Frost et al. 2008; Malik et al. 2020; Westman et al. 2019). Therefore, the activation of the SA pathway triggered by BP178 could contribute to the reduction of *X. fastidiosa* population levels and disease severity of ALS.

Interestingly, several upregulated genes that were identified in our study through RNA-seq analysis play other significant roles, in addition to the SA pathway. The already mentioned upregulation of peroxidases may be associated with the production of reactive oxygen species, and the upregulation of the terpene synthetase and UDP-glycosyltransferase genes may be linked to secondary metabolites synthesis. Genes related to the synthesis of lignin, synthesis of suberin, and callose deposition were also upregulated, which would reinforce the cell wall structures. Moreover, the upregulation of the previously mentioned polygalacturonase inhibitors (namely PR6) could be inhibiting enzymes which are crucial to promote intervessel movement and allow vascular colonization and pathogenicity in *X. fastidiosa* (Ingel et al. 2019; Roper et al. 2007). Thus, BP178 treatment could restrict *X. fasidiosa*'s movement and colonization through adjacent xylem vessels since it is known that its horizontal movement between vessels is caused by the degradation of the interconnecting pit membranes (Ingel et al. 2019; Pérez-Donoso et al. 2010).

BP178 also slightly affected genes related to development and metabolism in almond. Most of these genes, which were downregulated, were related to carbohydrate synthesis, indicating that an activation of the plant immune system could cause a transitory effect on growth due to an optimization of resource use of the plant as reported in the literature (Karasov et al. 2017). Despite this transcriptomic response, no effect on plant growth performance was observed in the almond treated plants in the present study. Nevertheless, it has to be taken into consideration that all the information displayed so far related to the transcriptomic response was obtained at 24 h. Therefore, it would be necessary to perform further studies to elucidate the optimal conditions (concentration, method, and time of application) and possible nontarget effects of BP178 to be used in control strategies.

Conclusions. The peptide BP178 is able to decrease the *X. fastidiosa* population and reduce ALS severity in potted almond plants under greenhouse conditions, using endotherapy with preventive and curative applications. In addition, the activity in planta is not only due to the previously reported lytic activity, but it is reinforced by the activation of the plant immune system, mainly through the SA defense pathway. Therefore, BP178 can be considered a promising candidate to manage ALS caused by *X. fastidiosa*.

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