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Transgenic *Citrus sinensis* Expressing the Pepper *Bs2* *R*-Gene Shows Broad Transcriptional Activation of Defense Responses to Citrus Canker

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Abstract

The pepper *Bs2* resistance gene confers resistance to susceptible *Solanaceae* plants against pathogenic strains of *Xanthomonas campestris* pv. *vesicatoria* carrying the *avrBs2* avirulence gene. Previously, we generated *Bs2*-transgenic *Citrus sinensis* plants that exhibited enhanced resistance to citrus canker caused by *Xanthomonas citri* subsp. *citri* (*Xcc*), although the underlying mechanisms remained unknown. To elucidate the molecular basis of the early defense response, we performed a comparative transcriptomic analysis of *Bs2*-expressing and non-transgenic plants 48 hours after *Xcc* inoculation. A total of 2,022 differentially expressed genes (DEGs) were identified, including 1,356 up-regulated and 666 down-regulated genes. In *Bs2*-plants, 36.8% of the up-regulated DEGs were associated with defense responses and biotic stress. Functional annotation revealed major changes in genes encoding receptor-like kinases, transcription factors, hormone biosynthesis enzymes, pathogenesis-related proteins, secondary metabolism, and cell wall modification. Among hormone-related pathways, genes linked to ethylene biosynthesis and signaling were the most strongly regulated. Consistently, endogenous ethylene levels increased in *Bs2*-plants following *Xcc* infection, and treatment with an ethylene-releasing compound enhanced resistance in non-transgenic plants. Overall, our results indicate the *Bs2* expression activates a complex defense network in citrus and may represent a valuable strategy for controlling canker and other *Xanthomonas*-induced diseases.

Keywords: citrus canker; citrus breeding; transgenic plants

1. Introduction

Citrus canker, caused by *Xanthomonas citri* pv. *citri* (*Xcc*), remains one of the most significant phytosanitary threats to the global citrus industry. Its management is particularly challenging, relying on early detection, eradication of infected trees, and strict quarantine measures that rarely achieve complete control [1,2]. Given the limitations of conventional control strategies, increasing efforts have focused on developing biotechnological approaches to enhance *Citrus* resistance. Among these, *Agrobacterium tumefaciens*–mediated genetic transformation has become an important tool in citrus improvement programs, offering multiple opportunities to complement the development of

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resistant cultivars [3]. Such approaches are especially relevant for the sustainable management of diseases in perennial crops like *Citrus* sp.

Several genetic strategies have been explored to mitigate citrus canker, including the expression of antimicrobial peptides [4–6], genes that enhance broad-spectrum defense mechanisms [7–9], and the introduction of heterologous resistance (R) genes [10,11]. R genes typically act as race-specific pathogen receptors and most belong to the large nucleotide-binding site–leucine-rich repeat (NBS-LRR) family [12]. Many R genes have been successfully transferred across genera through genetic engineering, resulting in effective disease control. For instance, constitutive expression of the rice *Xa21* gene in banana (*Musa* × *paradisiaca*) conferred resistance to *Xanthomonas campestris* pv. *musacearum* (*Xcm*) [13], and expression of *Xa21* gene in transgenic *C. sinensis* enhanced resistance to *X. citri* pv. *citri* [10]. Similarly, the maize *Rxo1* gene conferred resistance to *Xanthomonas oryzae* pv. *oryzicola* in rice [14]. However, resistance mediated by race-specific receptors is often short-lived, as pathogens can evolve mechanisms to overcome it [13].

In contrast, some R genes recognize conserved, nonredundant effectors that are essential for pathogen virulence and thus represent potentially durable sources of resistance [13]. One example is the *Bs2* gene from pepper (*Capsicum annuum* cv. Early Calwonder), which encodes an NBS-LRR resistance protein that recognizes the corresponding *avrBs2* avirulence gene and confers resistance to *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) [15]. Transgenic expression of *Bs2* enhances resistance to *Xcv* not only in susceptible pepper genotypes but also in other *Solanaceae* species, including tomato and tobacco [15]. Additionally, we previously demonstrated that the *avrBs2* gene is highly conserved among *Xanthomonas* species, including *X. citri* pv. *citri* (*Xcc*), whose *avrBs2* sequence shares 96% identity with that of *Xcv* [16]. Based on this finding, transgenic *C. sinensis* cv. Pineapple (sweet orange) plants expressing the *Bs2* gene under the control of a pathogen-inducible glutathione S-transferase (*gst1*) promoter from potato were generated, exhibiting enhanced resistance to citrus canker [11]. These *Bs2*-transgenic plants showed higher production of reactive oxygen species and increased expression of pathogenesis-related (PR) genes after *Xcc* inoculation compared with non-transgenic controls, suggesting that the reduced canker symptoms were the consequence of defense mechanisms triggered by recognition of the conserved *avrBs2* effector. Nevertheless, the downstream molecular and signaling pathways activated by *Bs2* in citrus remain poorly understood.

In the present study, we analysed early transcriptomic changes in *C. sinensis* plants expressing the pepper *Bs2* gene following *Xcc* inoculation to elucidate the molecular mechanisms induced by this gene. Because effective plant defense depends on rapid perception and early signaling, we focused our analysis on the early stages of infection. The transcriptome comparison revealed differentially expressed genes (DEGs) between *Bs2* and non-transgenic plants, which correlated with anatomical, biochemical, and molecular evidence consistent with the activation of defense responses in the transgenic plants. This study provides insights into the defense mechanisms triggered by the heterologous *Bs2* gene in citrus, offering valuable implications for improving citrus canker management through direct deployment of *Bs2* or by leveraging candidate genes identified in this work.

2. Materials and Methods

Plant material and bacterial culture

Seven-month-old *Bs2*-transgenic (*Bs2*-plants) [11] and non-transgenic (NT) *C. sinensis* cv. Pineapple were grown in 10-L pots containing GrowMix® Multipro commercial substrate (Terrafertil S.A., Argentina) under controlled environmental conditions (28–30 °C, 16-h photoperiod).

Xanthomonas citri pv. *citri* (*Xcc*) expressing GFP [17] was cultured at 28 °C with shaking at 200 rpm in PYM medium [19]. Bacterial cells were harvested by centrifugation at 4,000 rpm for 15 min and resuspended in sterile 10 mM $MgCl_2$ to a final density of either 10^8 CFU mL^{-1} (for spray inoculation assays) or 10^4 CFU mL^{-1} (for infiltration assays).

Challenge assays with *Xcc*

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For the RNA-seq experiment, young leaves were inoculated by infiltration to ensure bacterial entry and minimize variability among biological replicates. Inoculated plants were maintained at 28–30 °C and 70% relative humidity. At 48 h post-inoculation (hpi), leaf samples were collected using a cork borer. From the inoculated area, two leaf discs (1 cm^2) were taken from each of five leaves per biological replicate (plant). A total of ten discs per treatment were pooled, immediately frozen in liquid nitrogen, and stored at –80 °C until RNA extraction. Three biological replicates were collected per treatment.

To evaluate *in planta* bacterial growth, *Xcc* was inoculated using two different methods: infiltration with a needle (without syringe) and spraying. Inoculated plants were kept at 26 °C and 70% relative humidity. Mock inoculations were performed with 10 mM $MgCl_2$ solution as a control in all experiments. Each assay was repeated three times, using at least five leaves per plant.

Determination of *Xcc* population

Symptom development and disease progression were monitored phenotypically and documented using a Leica MZ6 stereomicroscope under both white and UV light (520 nm). The GFP-tagged *Xcc* strain enabled visualization of live bacterial colonies as bright green fluorescent *foci* under UV illumination, allowing accurate detection of infection sites and active bacterial proliferation. Representative images were taken at different time points, and bacterial growth was quantified as previously described [11]. Six leaf discs (1 cm^2) from inoculated leaves were ground in 0.2 mL of sterile distilled water, and serial dilutions (100 µL) of the homogenate were plated onto PYM nutrient medium [18]. The bacterial population was expressed as colony-forming units (CFU) per cm^2 and monitored up to 14 days post-inoculation (dpi).

RNA extraction and library preparation

Total RNA was extracted from three biological replicates per treatment: *Bs2*-transgenic (*Bs2*-plants) and non-transgenic (NT) plants, each either *Xcc*-inoculated or mock-treated. RNA samples were isolated and purified using the TURBO DNA-free™ Kit (Thermo Fisher Scientific). Four libraries were constructed for paired-end sequencing: (1) inoculated *Bs2*-plants (*Bs2*-*Xcc*); (2) mock-inoculated *Bs2*-plants (*Bs2*-Mock); (3) inoculated NT-plants (NT-*Xcc*); and (4) mock-inoculated NT-plants (NT-Mock).

RNA integrity was verified using a 2100 Bioanalyzer (Agilent Technologies). One microgram of total RNA was used to construct each library with the Illumina TruSeq Stranded mRNA Library Prep Kit (Illumina, RS-122-2102). Library construction was performed at the University of Florida ICBR Gene Expression and Genotyping Core Facility (RRID:SCR_019145). The resulting libraries displayed a broad fragment distribution (200–2000 bp) with a peak around 500 bp. Library quantification was performed by qPCR using the KAPA Library Quantification Kit (Kapa Biosystems, KK4824). The libraries were then pooled at equimolar concentrations and sequenced on an Illumina HiSeq 3000 platform (2 × 100 bp paired-end reads) at the University of Florida ICBR NextGen DNA Sequencing Core Facility (RRID:SCR_019152).

Sequencing read mapping and gene expression estimation

Raw reads were trimmed and filtered according to base quality and read length using BBDuk from the BBMap package, version 34.41 [19]. The trimmed paired-end reads were aligned to the *Citrus clementina* v1.0 reference genome using TopHat v2.0.09 with default parameters [20]. Reads from each biological replicate were mapped independently, and multi-mapped reads were discarded. Gene-level read counts were obtained using **htseq-count** with the union model. Expression levels were estimated as FPKM (Fragments Per Kilobase of transcript per Million mapped reads) using the DESeq2 package [21].

Identification of differentially expressed genes and functional annotation

Differential expression analysis between transgenic and non-transgenic plants, both inoculated and mock-treated, was conducted using the DESeq2 package (Bioconductor). Genes with an absolute $\log_2$ fold change ($|\log_2 FC| > 2$) and a false discovery rate (FDR) < 0.001 were considered significantly differentially expressed. Gene Ontology (GO) terms related to biological processes, molecular

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functions, and cellular components were assigned using the AgriGO toolkit [22]. Functional categorization of DEGs was carried out using MapMan 3.5.1R2 software [23].

To validate the RNA-seq data, fourteen DEGs were randomly selected for qRT–PCR analysis (Applied Biosystems). Gene-specific primers were designed based on coding sequences using IDT SciTools Web Tools (Supplementary Table S1). The *18S rRNA* gene was used as an internal reference [24]. Relative expression levels were calculated using the $2^{-}\Delta\Delta Ct$ method [25].

Treatments with exogenous ethephon

To evaluate the effect of ethylene on *Xanthomonas citri* pv. *citri* (*Xcc*) growth, exogenous ethephon (an ethylene-releasing compound) was added to PYM nutrient medium at concentrations ranging from 1000 to 2 µM. Each glass tube contained 5 mL of PYM medium supplemented with ethephon, and 100 µL of an *Xcc* culture (OD_{600} = 0.1) was inoculated into each tube. Cultures were incubated at 28 °C with shaking at 200 rpm for 24 h, and bacterial growth was measured by optical density at 600 nm.

To assess the effect of ethylene on plant defense, a sub-inhibitory concentration of ethephon (1 µM) was applied to *C. sinensis* NT-plants by spraying either 48 h before or 48 h after inoculation with *Xcc* (10^8 CFU mL^{-1} for spray inoculation and 10^4 CFU mL^{-1} for infiltration). Control plants were sprayed with sterile water. Disease symptoms were recorded at 14 days post-inoculation (dpi). Each treatment included three independent plants and three leaf replicates per plant.

Quantification of endogenous ethylene

Endogenous ethylene production was measured following Zou et al. [26]. After infiltration with 200 µL of *Xcc* suspension, leaves from *Bs2*- and NT-plants were placed in sealed 30 mL vials and incubated for 48 h in the dark at 25 °C. Gas samples (1 mL) were withdrawn using a gas-tight syringe and analyzed by gas chromatography (Agilent 7890A, USA). Each treatment included three biological replicates.

Antibacterial activity and phenolic compound content (PCC).

Leaf discs (0.1 g) from NT- and *Bs2*-plants were collected at 0, 24, 48, and 72 h post-inoculation (hpi) with *Xcc*. Samples were ground and extracted in 2 mL of 80% methanol:20% water for 24 h at 30 °C. Extracts were centrifuged, lyophilized, and re-suspended in distilled water to a final concentration of 0.1 g FW mL^{-1}.

Phenolic compound content (PCC) was quantified using the Folin–Ciocalteu method [27]. Gallic acid was used to construct a calibration curve, and results were expressed as milligrams of gallic acid equivalents per gram of fresh weight (mg GAE g^{-1} FW).

Antibacterial activity was evaluated by the agar well-diffusion method. *Xcc* suspension (OD_{600} = 1.0; 150 µL) was mixed with 10 mL of 0.7% PYM agar and poured into sterile Petri dishes. Eight wells (0.5 mm diameter) were made using a cork borer, and 50 µL of each methanolic extract (0.1 mg FW mL^{-1}) were added per well. Plates were incubated at 30 °C for 24 h, and inhibition zones were measured. All assays were performed by triplicate.

3. Results

3.1. Differentially Expressed Genes (DEGs) in Citrus Sinensis Bs2-Plants

To gain insight into the early molecular mechanisms involved in the response of *Bs2*-plants to *Xcc* infection, a global transcriptional analysis was conducted by comparing *Bs2*- and non-transgenic (NT) plants at 48 h post-inoculation (hpi) using RNA-seq technology. Four datasets of 100-bp paired-end raw reads were generated. Raw reads were subjected to quality control using SeqQC, with more than 95% of bases exhibiting a quality score above Q20 (data not shown). After mapping the high-quality reads to the *C. clementina* v1.0 reference genome, 82–88% of reads were uniquely aligned and used for downstream expression analyses.

Pairwise comparisons among biological replicates showed high consistency in estimated gene expression levels. Principal component analysis (PCA) revealed that *Bs2*-Mock and NT-Mock samples clustered closely together, indicating minimal transcriptional differences between them

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under non-inoculated conditions. In contrast, *Bs2*-*Xcc* and NT-*Xcc* samples were clearly separated from each other and from their respective mock treatments, demonstrating distinct transcriptional reprogramming induced by the presence of the *Bs2* gene (Figure 1a).

A volcano plot showing fold-change values versus the $-\log_{10}$ of adjusted p-values illustrated the overall differential expression patterns between treatments (Figure 1b). Under mock conditions, only 31 DEGs were detected between *Bs2*- and NT-plants (12 up-regulated and 19 down-regulated). In contrast, under *Xcc*-inoculated conditions, a total of 2,022 DEGs were identified, with the number of up-regulated genes (1,356) more than doubling the number of down-regulated ones (666). As expected, the *Bs2* gene was exclusively detected in *Bs2*-plants.

In NT-plants, comparison of NT-*Xcc* vs. NT-Mock revealed 2,037 DEGs, with similar proportions of up-regulated (958) and down-regulated (1,079) genes. Conversely, comparison of *Bs2*-*Xcc* vs. *Bs2*-Mock identified only 574 DEGs; however, the number of up-regulated genes (488) was nearly sixfold higher than that of down-regulated ones (86). These results indicate that expression of the *Bs2* gene reprograms the transcriptional response of *C. sinensis* to *Xcc* infection, biasing gene regulation toward activation rather than suppression—unlike the response observed in NT-plants (data not shown).

The accuracy of RNA-seq data was validated by quantitative real-time PCR (qRT-PCR) analysis of a randomly selected subset of 14 DEGs (Figure 1c). The expression trends obtained by both platforms were highly consistent, with a strong positive correlation (Spearman's $\varrho = 0.985$) between RNA-seq and qRT-PCR fold-change values, confirming the reliability of the transcriptomic results.

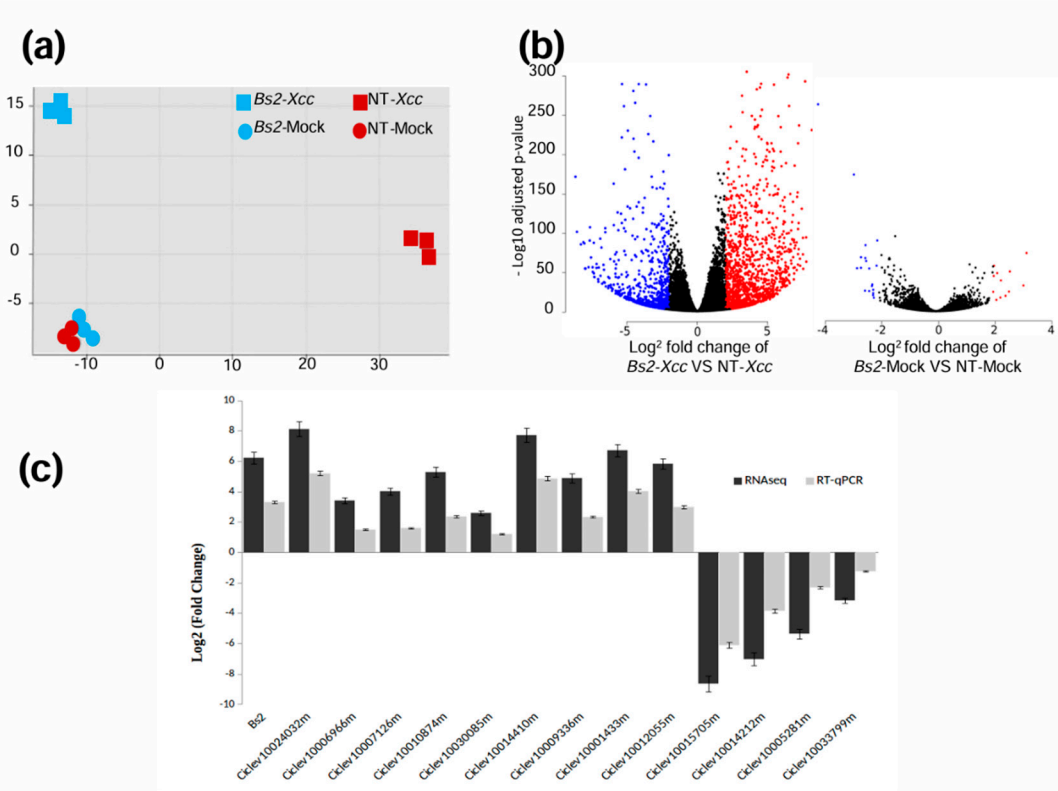

Figure 1. (a) Principal component analysis (PCA) of regularized log-transformed count data showing the first two principal components for *Citrus sinensis Bs2*- and non-transgenic (NT) plants at 48 h post-inoculation (hpi) with *Xanthomonas citri* pv. *citri* (*Xcc*) or mock-inoculated with 10 mM $MgCl_2$. **(b)** Volcano plots showing up-regulated (red) and down-regulated (blue) genes in *Bs2*-plants relative to NT-plants at 48 hpi under *Xcc*-inoculated (left) and mock (right) conditions. Coloured points represent differentially expressed genes (DEGs) with $\alpha = 0.05$ and $\log_2FC > 2$. The y-axis represents the negative $\log_{10}$ of the false discovery rate ($-\log_{10}$ FDR), and the x-axis shows the $\log_2$ fold change ($\log_2FC$) derived from RNA-seq data of three independent biological replicates. FC, fold change; FDR, false discovery rate. **(c)** Comparative analysis between qRT–PCR and RNA-seq expression profiles. Log_2-transformed relative mRNA levels of DEGs obtained by RNA-seq were validated by

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qRT–PCR for defense-related genes in *C. sinensis Bs2*-plants at 48 hpi with *Xcc*. The *Citrus* β-actin transcript was used as an internal reference gene, and non-inoculated *Bs2*-plants served as calibrators. Values represent means ± standard deviation (SD) from three independent biological replicates. Ciclev IDs and their corresponding annotations are listed in Supplementary Table S1.

3.2. Functional Analysis of DEGs

Gene Ontology (GO) classification and KEGG pathway enrichment analyses were conducted to gain insight into the biological functions of the differentially expressed genes (DEGs). GO terms were assigned to the predicted genes using the *C. clementina* GO annotation integrated into the DESeq2 analysis pipeline. Of the 2,022 DEGs identified between *Bs2*- and NT-plants, 1,717 were successfully annotated with at least one GO term.

The annotated DEGs were categorized according to GO Level 2 into 30 functional groups for up-regulated genes and 31 groups for down-regulated genes (Figure 2). Within the **Molecular Function** category, the most represented terms were *catalytic activity* (131 up-regulated and 64 down-regulated genes) and *binding* (126 up-regulated and 60 down-regulated genes). In the **Cellular Component** ontology, the predominant terms were *cell* and *cell part*, followed by *organelle*. Within the **Biological Process** category, the largest groups were *cellular process* and *metabolic process*, followed by *response to stimulus* and *regulation of biological process* (Figure 2).

These results indicate that *Bs2*-mediated responses in *C. sinensis* involve extensive transcriptional reprogramming of genes associated with core cellular metabolism and stress-related biological processes.

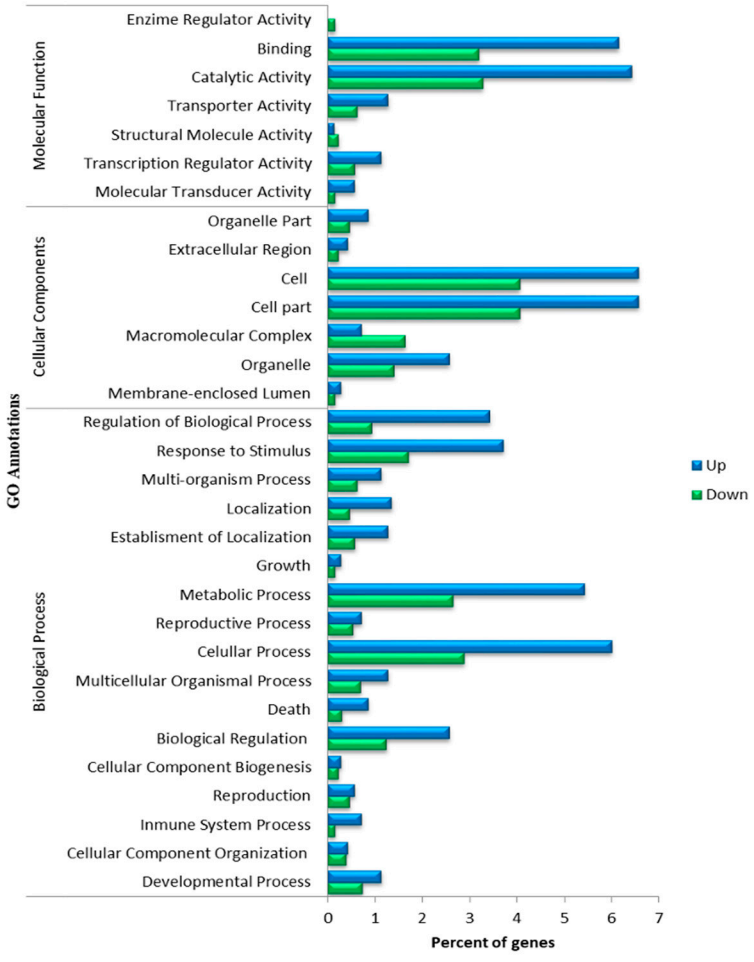

Figure 2. Gene Ontology (GO) annotation of differentially expressed genes (DEGs) in *Citrus sinensis Bs2*- and non-transgenic (NT) plants at 48 h post-inoculation (hpi) with *Xanthomonas citri* pv. *citri* (*Xcc*). Level 2 GO

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classification of up-regulated and down-regulated genes is shown. The DEGs were grouped into three major GO categories: Biological Process, Cellular Component, and Molecular Function. Blue and green bars represent the percentage of up-regulated and down-regulated genes, respectively.

3.3. Plant Defense Is Induced in Bs2-Plants After Xcc Inoculation

Functional classification and visualization of DEGs were performed using the MapMan software. The **biotic stress** category was the most enriched, comprising 752 DEGs (36.8%) in *Bs2-Xcc* plants (Figure 3). The principal subcategories included signaling, proteolysis, pathogenesis-related (PR) proteins, secondary metabolism, hormone signaling, transcription factors, and cell wall organization.

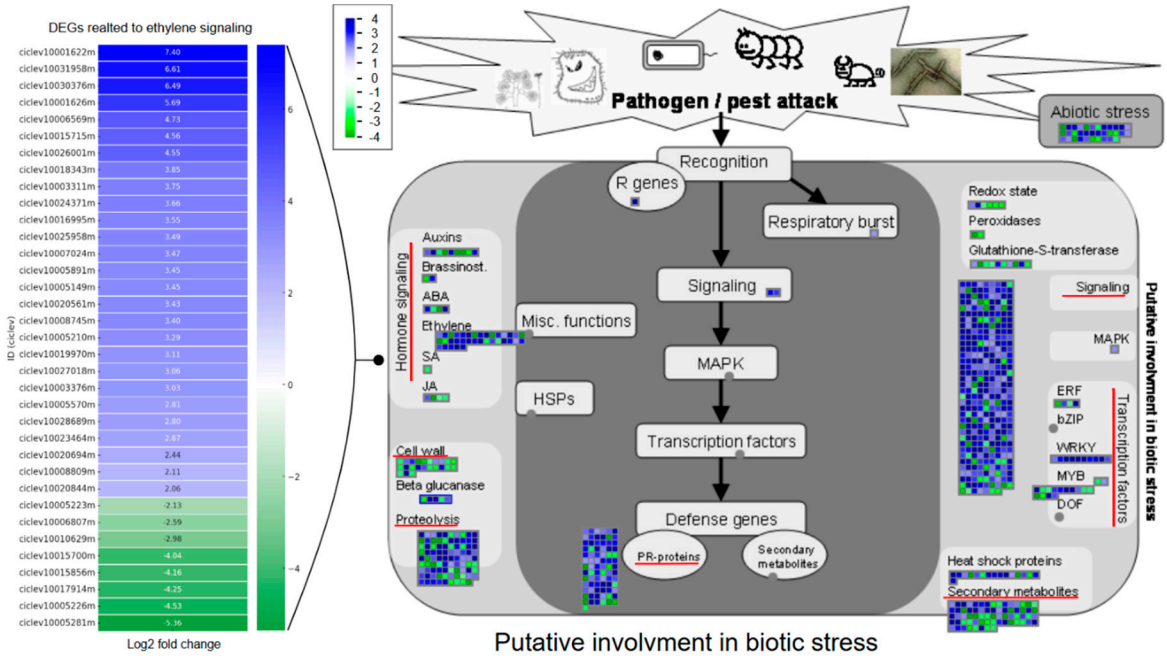

Figure 3. Biotic stress responses in *Citrus sinensis Bs2*-plants at 48 h post-inoculation (hpi) with *Xanthomonas citri* pv. *citri* (*Xcc*). Log_2 fold-change in gene expression was visualized using MapMan. Blue squares indicate up-regulated genes, green squares indicate down-regulated genes, and grey circles represent non-differentially expressed genes. Colour intensity corresponds to fold-change values >4 or <−4. On the left, a heat map displays the expression profiles of DEGs related to ethylene signaling.

A more detailed analysis (Supplementary Table S2) revealed that approximately 43% of DEGs were related to signal perception and transduction, including receptor-like kinases (RLKs), calcium signaling, and phytohormone-related pathways. A total of 324 DEGs corresponded to RLKs and wall-associated kinases (WAKs), which are transmembrane receptors located in the cell wall that participate in broad-spectrum, elicitor-induced defense responses [28]. Among these, 170 DEGs belonged to the leucine-rich repeat receptor-like kinase (LRR-RLK) subfamily, of which 150 (88%) were up-regulated. Additionally, 79 DEGs (68 up-regulated) encoded receptor-like kinases containing a Domain of Unknown Function 26 (DUF26), also known as cysteine-rich receptor-like kinases (CRKs), which are known to regulate defense signaling and programmed cell death. A smaller group of 11 DEGs encoded wall-associated kinases (WAKs), 10 of which were up-regulated.

Following pathogen recognition, a signaling cascade is triggered in which calcium ions (Ca^{2+}) act as key secondary messengers. In *Bs2*-plants, 19 of the 22 Ca^{2+}-related DEGs were up-regulated, including 12 genes encoding calmodulin or calmodulin-like proteins, which function as intracellular calcium sensors. Consistent with these results, two DEGs encoding ABC-2-type domain-containing proteins were notably induced (6.89- and 6.22-fold, respectively) in *Bs2*-plants. Certain ABC

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transporters have been reported to interact with calmodulin and other Ca^{2+}-binding proteins to modulate signal transduction [29].

Intracellular nucleotide-binding site–leucine-rich repeat (NBS-LRR) genes play central roles in the recognition of pathogen effectors, mediating effector-triggered immunity (ETI). Most of these genes constitute a class of plant resistance (R) genes [30]. In the present study, 79 DEGs encoding NBS-LRR proteins were identified in *Bs2*-plants, 62 of which were up-regulated (Supplementary Table S2). Pathogenesis-related (PR) proteins are hallmark components of plant defense that accumulate following pathogen perception [30]. Although most PR genes exhibit low basal expression under normal growth conditions, they are rapidly induced after infection through signaling pathways mediated by salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), contributing to systemic acquired resistance (SAR) [31]. In *Bs2*-plants, 10 PR-related DEGs were identified, eight of which were up-regulated at 48 hpi.

Transcription factors (TFs) play key regulatory roles in orchestrating the transcriptional reprogramming that underlies plant defense responses. In this study, 30 DEGs corresponding to three major TF families associated with biotic stress—WRKY, MYB, and ERF—were differentially expressed in *Bs2*-plants, of which 21 were up-regulated. Notably, all WRKY TFs were up-regulated, with *WRKY70* (*ciclev10012055*) showing a 5.84-fold induction in *Bs2*-plants.

3.4. Bs2 Induces Major Regulation of the Ethylene Pathway

Within the *Plant hormone signaling* subcategory, 56 genes were differentially expressed in *Bs2*-plants, with 35 up-regulated and 20 down-regulated (Supplementary Table S2). The most abundant and differentially expressed hormone-related genes were associated with the biosynthesis, degradation, or signal transduction of ethylene. This group included 35 DEGs (27 up-regulated), such as *senescence-related gene 1* (*SRG1*), oxygenase family proteins, ethylene-responsive element binding proteins (EREBPs), and ethylene-responsive factors (ERFs). In contrast, only one gene related to salicylic acid (SA) synthesis or degradation (*ciclev10017993m*) was identified, and it was down-regulated. Additionally, two DEGs corresponding to *NPR1* were down-regulated (*ciclev10033799m* and *ciclev10030929m*, with fold changes of 3.17 and 2.36, respectively), whereas one *NPR1* suppressor gene was up-regulated (2.89-fold change) in *Bs2*-plants at 48 hpi. Regarding jasmonic acid (JA), three DEGs were down-regulated and only one was up-regulated. Moreover, one *methyl esterase 1* (*ciclev10033393m*), an enzyme involved in the methylation of compounds such as SA and JA and associated with systemic acquired resistance (SAR) [32], was significantly induced (5.3-fold) in *Bs2*-plants.

The high proportion of DEGs related to the ethylene (ET) pathway compared with those associated with SA or JA suggests a predominant role of ET signaling, at least during the early defense stages triggered by the *Bs2* gene. To confirm this hypothesis, ethylene content was quantified in *Bs2*- and NT-plants at 48 hpi. *Bs2*-plants showed significantly higher ET levels than NT-plants (5.4 ± 0.32 μg g^{-1} FW vs. 3.1 ± 0.12 μg g^{-1} FW, respectively).

To determine whether ethylene directly affects *Xcc* growth, an in vitro assay using ethephon (an ethylene-releasing compound) revealed that *Xcc* growth was inhibited at concentrations ranging from 1000 to 10 μM (Figure 4A). Subsequently, to assess whether ethylene modulates plant defense against *Xcc*, a subinhibitory concentration of ethephon (1 μM) was applied to NT-plants either 48 h before or after *Xcc* inoculation, using both infiltration (10^4 CFU mL^{-1}) and wound + spray (10^8 CFU mL^{-1}) methods. At 14 dpi, ethephon-treated plants exhibited fewer canker symptoms than untreated controls, with the greatest reduction observed when ethephon was applied prior to bacterial inoculation, regardless of the inoculation method (Figure 4B). Quantification of bacterial populations at 14 dpi was consistent with the observed symptom reduction (Figure 4C).

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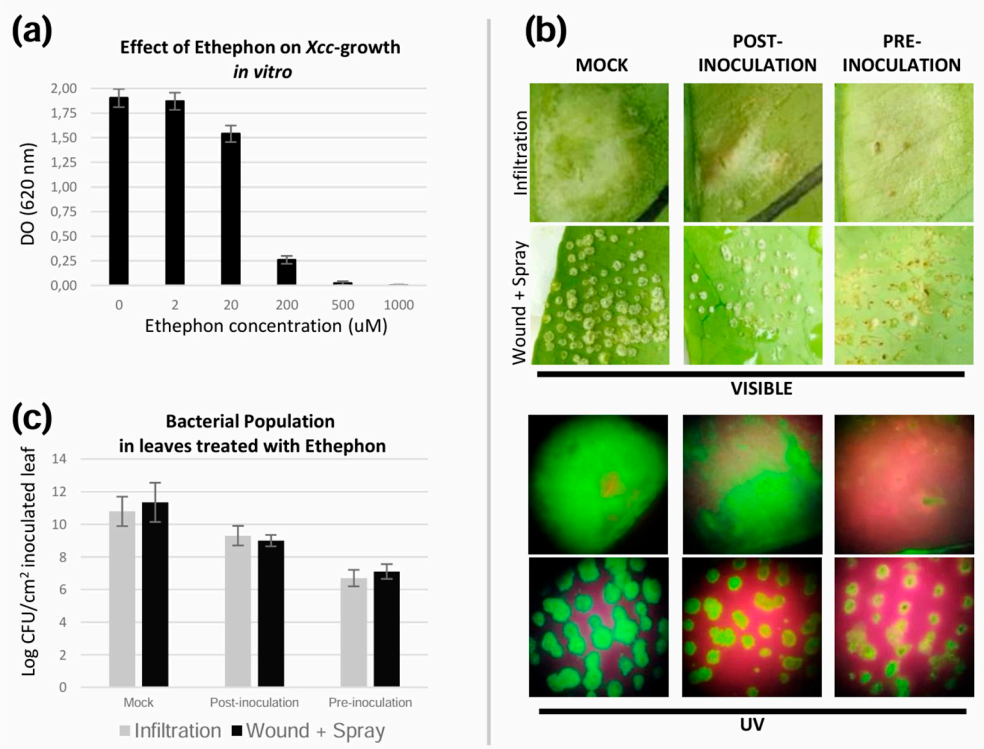

Figure 4. Effect of ethylene on *Xanthomonas citri* pv. *citri (Xcc)* population and symptom development in *Citrus sinensis* leaves. (a) *In vitro* growth of *Xcc* in PYM medium supplemented with different concentrations of ethephon (equivalent to 1000–0 µM of ethylene). Bacterial growth after 24 h was determined by optical density at 600 nm (OD_{600}). (b) Canker symptoms in a representative non-transgenic (NT) leaf treated with ethephon before (pre-inoculation) or after (post-inoculation) *Xcc* inoculation, or with 10 mM $MgCl_2$ as a control. The right and left halves of each leaf were inoculated by infiltration with an *Xcc* suspension (10^4 CFU mL^{-1}) and by spraying (10^8 CFU mL^{-1}), respectively. Symptoms were recorded at 14 days post-inoculation (dpi) under visible and UV light. (c) Viable *Xcc* cells were quantified from inoculated leaves of each treatment at 14 dpi and expressed as CFU cm^{-2}. Values represent the mean ± standard deviation (SD) of three independent biological replicates.

3.5. Changes in Secondary Metabolic Pathways and Cell Wall

A total of 70 DEGs were involved in the synthesis of secondary metabolites in *Bs2*-plants (Supplementary Table S2), of which 41 were up-regulated. One of the major enriched categories was *phenylpropanoid biosynthesis*, which includes numerous secondary metabolites associated with plant signaling and defense against biotic and abiotic stress [33]. Among the most highly up-regulated DEGs in *Bs2*-plants was *phenylalanine ammonia-lyase* (*PAL*, 5.28-fold change, *ciclev10010874m*), which catalyzes the first step in the phenylpropanoid pathway. In addition, *isoflavone-7-O-methyltransferase*, *cinnamyl-alcohol dehydrogenase*, *chalcone synthase*, and several *transferase family* genes were up-regulated.

The expression of genes related to phenolic compound biosynthesis was consistent with the increased total soluble phenolic content (Figure 5A) and with qRT–PCR validation of *PAL1* expression (Figure 5B), both of which were significantly higher in *Bs2*-plants. Histological analyses showed the accumulation of bright green autofluorescence corresponding to polyphenolic compounds, particularly on the abaxial side of *Bs2* leaves at 48 hpi (Figure 5C). Moreover, methanolic extracts from *Bs2* leaves exhibited higher phenolic compound content and *in vitro* antimicrobial activity against *Xcc* growth (Figure 5D), suggesting reinforcement of chemical defenses that restrict pathogen progression.

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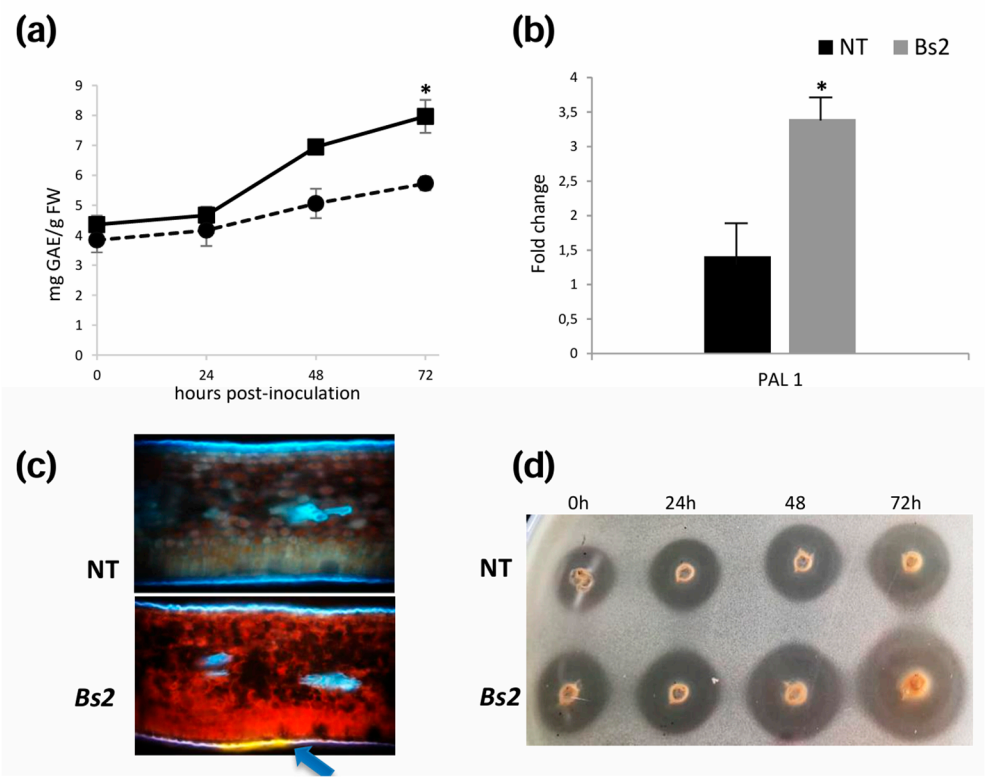

Figure 5. Evaluation of phenolic compound accumulation in the response of *Citrus sinensis Bs2*-plants to *Xanthomonas citri* pv. *citri (Xcc).* (a) Total phenolic compound content in Bs2 and non-transgenic (NT) leaves at 0, 24, 48, and 72 hours post-inoculation (hpi). Values represent means ± standard deviation (SD) of three independent biological replicates. (b) *qRT–PCR* analysis of *phenylalanine ammonia-lyase* (*PAL1*) mRNA levels in *Bs2*-plants at 48 hpi with *Xcc*. Relative gene expression was calculated using *Bs2* mock-inoculated plants (10 mM $MgCl_2$) as reference samples and normalized to *β-actin* expression. *Bs2*-plants without inoculation were used as calibrators. Data are presented as means ± SD from three independent biological replicates. Asterisks indicate significant differences according to Tukey's test ($P < 0.05$). (c) Light and UV microscopic images of *C. sinensis* (*Bs2* and NT) leaves inoculated with *Xcc*. Images were taken at 48 hpi under white and UV light. Bright green fluorescence indicates the accumulation of polyphenolic compounds (arrows), and red fluorescence corresponds to chlorophyll. Scale bar = 10 mm. (d) Representative inhibition zone assay showing the *in vitro* antibacterial activity of methanolic extracts from *Bs2* and NT leaves collected at different time points after inoculation with *Xcc*.

Additionally, 60 DEGs (43 up- and 17 down-regulated) were annotated as cytochrome P450 monooxygenases. This large enzyme family participates in multiple reactions, including secondary metabolite biosynthesis, and in higher plants plays crucial roles in plant–microbe interactions as well as in the biosynthesis of antioxidants, phytohormones, and callose [34].

In the *Cell Wall* category, 23 DEGs were identified in *Bs2*-plants (Supplementary Table S2), 14 of which were down-regulated. These included genes associated with cell wall modification, degradation, and loosening, such as *xyloglucan:xyloglucosyl transferase* and *pectinesterase*.

Other DEGs contributing to cell wall weakening, such as *cellulase*, *endoglucanase*, *polygalacturonase*, and *expansin*, were also down-regulated in *Bs2*-plants. Expansin promotes rapid tissue expansion and cell wall loosening, processes required for hypertrophy and hyperplasia during canker pustule formation [35]. Consistent with this, three DEGs encoding *SAUR* (Small Auxin Up RNA) auxin response proteins, which promote cell expansion [39], were also down-regulated in *Bs2*-plants.

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4. Discussion

The current study presents a comparative transcriptional analysis between *Bs2* transgenic and non-transgenic plants to identify differentially expressed genes after inoculation with *Xcc*, in an attempt to understand the nature of the defense mechanism induced by the pepper *Bs2* gene in *C. sinensis*. Our results indicate that the resistance of *Bs2*-plants to *Xcc* is linked to extensive transcriptional reprogramming across multiple functional pathways. We propose a regulatory model connecting key signaling networks with the expression of defense-associated genes (Figure 6).

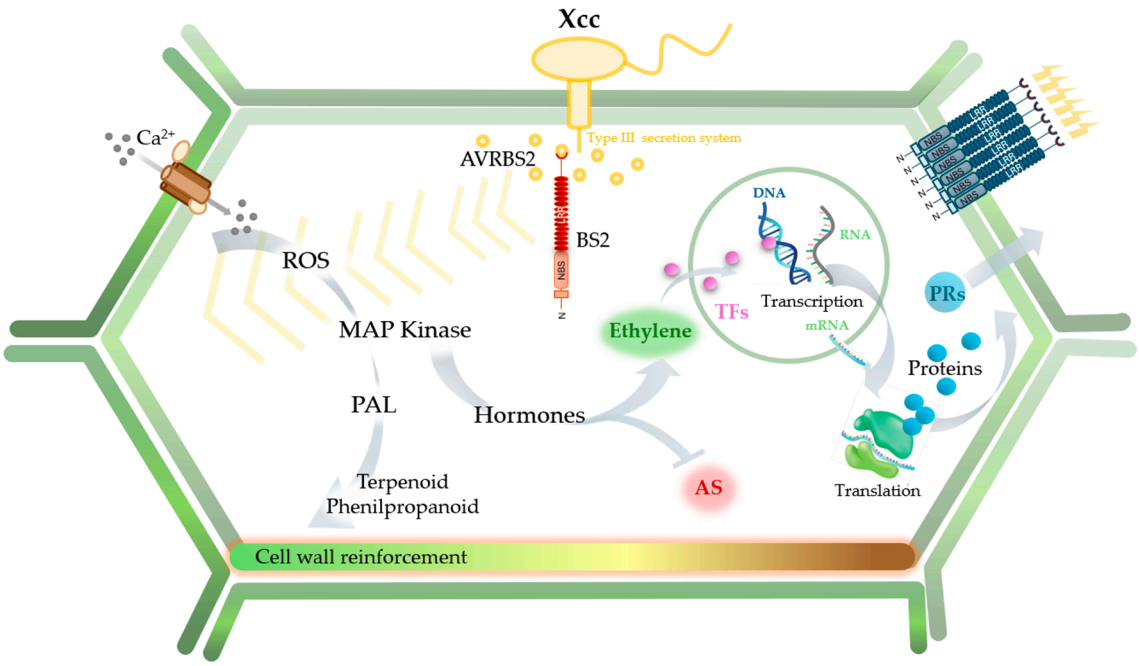

Figure 6. Diagrammatic representation of putative defense mechanisms in transgenic *Citrus sinensis* expressing the *Bs2* gene against *Xanthomonas citri* pv. *citri* (*Xcc*), based on the functional categories of defense-related genes identified in the RNA-seq analysis. The model integrates the following major defense responses: perception, signaling, transcription factors, defense regulators, hormone signaling, cell wall modification, and secondary metabolism.

A remarkable feature of the resistance response in *Bs2*-plants is that the number of up-regulated genes is approximately twice that observed in the susceptible response (NT–*Xcc*). A similar trend has been reported in canker-resistant transgenic *C. sinensis* overexpressing a spermidine synthase gene [36], in Meiwa kumquat (*Fortunella crassifolia*), which is immune to *Xcc* [37], and in *C. limon* following inoculation with an avirulent *Xcc* variant [38]. Therefore, the greater number of *up-regulated* genes is likely associated with resistance to *Xcc* infection. In susceptible genotypes, *Xanthomonas* pathogenesis involves the suppression of plant innate immune responses through molecular suppressors such as cyclic glucans and xanthan, resulting in a predominance of down-regulated genes [17,18].

Although the downstream signaling events following avr–R protein interactions are not yet fully understood, several characteristic responses have been described, including extensive transcriptomic reprogramming, strong activation of mitogen-activated protein kinases (MAPKs), increased production of reactive oxygen species (ROS), and localized programmed cell death or hypersensitive response (HR) [39]. Activation of the MAPK cascade subsequently triggers the regulation of downstream transcription [40]. In our study, *Bs2*-plants exhibited a stronger MAPK activation profile: 68.9% of the differentially expressed genes (DEGs) within the "Biotic Stress" MapMan category corresponded to enzymes, receptor kinases, and transcription factors (TFs), of which 70% were up-regulated. Moreover, several transcription factors belonging to four major families—MYB, WRKY, bHLH, and NAC—known as key regulators of defense responses, were induced. All MYB- and

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WRKY-related DEGs were up-regulated. Although they are multifunctional, both TFs orchestrate cellular strategies essential for plant defense against biotic stresses [41].

In previous work, we demonstrated that *Bs2*-transgenic plants exhibited maximum accumulation of H_2O_2 at 48 hours after *Xcc* inoculation. ROS are among the earliest and most important signaling molecules in plant defense, preceding HR and contributing to cell wall reinforcement, accumulation of phenolic compounds, and activation of defense-related genes [42]. Moreover, ROS and calcium have been proposed to act as interconnected messengers in systemic signal transduction, facilitating communication from local tissues to the entire plant [10]. In this context, several genes associated with calcium signaling were up-regulated, including calmodulin genes—whose proteins sense cytosolic Ca^{2+} fluctuations—as well as interacting partners such as ABC transporter proteins [29]. Consistently, two DEGs encoding ABC-2 type domain–containing proteins were highly up-regulated (6.89- and 6.22-fold, respectively) in *Bs2*–*Xcc* plants.

The phenylpropanoid pathway represents a key component of plant defense because it contributes to both mechanical barriers that restrict pathogen invasion and chemical defense through the production of antimicrobial metabolites [43]. Phenolic deposits have also been documented around HR lesions triggered by *Xcc* in resistant cultivars such as calamondin and kumquat [44]. In this context, the differential induction of key genes from the flavonoid and phenylpropanoid pathways, including PAL1 and CHS1, suggests their involvement in *Bs2*-activated defense responses in *C. sinensis*. Phenolic compounds additionally serve as precursors for important molecules such as phytohormones. For example, PAL1 participates in salicylic acid (SA) biosynthesis, a central component of R-gene-mediated resistance and effector-triggered immunity (ETI) [45]. In this study, however, no evidence of SA involvement was detected at 48 hpi, as no DEGs related to SA signaling were identified. Furthermore, two NPR1-encoding DEGs—NPR1 being a master regulator of SA signaling—were down-regulated, whereas one NPR1 suppressor gene was up-regulated. In contrast, Chiesa et al. [46] reported activation of the SA signaling pathway in *C. limon*, but in a nonhost interaction with *X. campestris* pv. *campestris* and at earlier time points (3 and 24 hpi).

By contrast, transcriptome analysis revealed that ethylene-responsive genes were distinctly induced in *Bs2*-plants upon *Xcc* infection. Ethylene is a multifunctional phytohormone involved in numerous plant processes, including responses to pathogen attack, where it can either promote resistance or susceptibility depending on the host–pathogen system [47]. Our results suggest that ethylene may contribute to *Bs2*-mediated resistance. Similar patterns have been reported in other plant–*Xanthomonas* interactions. For example, we previously observed induction of ACC oxidase transcripts—a key enzyme in ethylene biosynthesis—in *C. limon* at 48 hpi after inoculation with the avirulent variant *Xcc* AT [48]. Likewise, Cernada et al. [49] reported significant transcriptional changes associated with ethylene signaling during the interaction between *C. sinensis* and *X. axonopodis* pv. *aurantifolii* pathotype C (Xaa), which causes disease only in Mexican lime. In resistant *C. sinensis* plants, Xaa triggered a MAPK signaling cascade and activated WRKY and ethylene-responsive TFs at 6 and 48 hpi, leading the authors to propose a central role for ethylene in resistance.

Another major group of up-regulated genes in *Bs2*-plants consists of those involved in pathogen perception and signal transduction, including receptor-like protein kinases (RLKs), predominantly those containing leucine-rich repeat (LRR) domains, as well as lectin- and cysteine-rich RLKs. RLK genes function as membrane-localized receptors that recognize pathogen-associated molecular patterns (PAMPs) or damage signals and subsequently activate plant immune responses. Members of the RLK family, particularly LRR-RLKs, are key components of plant disease resistance. Their evolutionary expansion has broadened recognition specificity, enhancing the plant's ability to detect and respond to diverse pathogens [50]. Therefore, it is possible that *Bs2* may also contribute to expanding the plant's pathogen recognition capacity—an intriguing hypothesis for future investigation.

The reduced development of canker symptoms in *Bs2*-transgenic plants was also supported by the repression of 69% of DEGs related to cell wall modification, breakdown, or degradation. The down-regulation of genes associated with cell wall metabolism has also been observed in citrus plants

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challenged with an avirulent strain of *Xcc* [38] and in HLB-tolerant citrus trees [51]. Consistent with our results, one of the most strongly down-regulated DEGs corresponded to an expansin gene, whose suppression has been reported to promote pathogen resistance [10].

In conclusion, this study shows that the inducible expression of the pepper *Bs2* resistance gene in *C. sinensis* activates a complex defense program against *Xcc*. The results reveal the regulation of multiple resistance-related signaling pathways that together form an interconnected defense network, and they highlight ethylene signaling as a key component of this response. This work confirms the functionality of the *Bs2* gene from pepper in *C. sinensis*, thereby extending its taxonomic range of activity beyond the Solanaceae family. Overall, these findings broaden the potential applications of the *Bs2* gene for developing durable resistance against *Xanthomonas* spp. in citrus crops

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: Gene-specific primers to validate RNAseq; Table S2: DEGs details.

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Abbreviations

The following abbreviations are used in this manuscript:

avr*Bs2*	*avrBs2* avirulence gene
Bs2	*Bs2* resistance gene from *Capsicum annuum*
***Bs2*-plants**	Transgenic plants carrying the *Bs2* gene
Ca^{2+}	Calcium ion
CFU	Colony Forming Units
CHS1	Chalcone Synthase 1
CRKs	Cysteine-Rich Receptor-Like Kinases
CT / Ct	Cycle Threshold (ΔΔCt method)
DEGs	Differentially Expressed Genes
DESeq2	Differential expression analysis package
dpi	Days Post-Inoculation
DUF26	Domain of Unknown Function 26
ERFs	Ethylene-Responsive Factors
EREBPs	Ethylene-Responsive Element Binding Proteins
ET	Ethylene
ETI	Effector-Triggered Immunity
FDR	False Discovery Rate

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FPKM	Fragments Per Kilobase of transcript per Million mapped reads
FW	Fresh Weight
GAE	Gallic Acid Equivalents
GFP	Green Fluorescent Protein
GO	Gene Ontology
HLB	Huanglongbing
hpi	Hours Post-Inoculation
HR	Hypersensitive Response
ICBR	Interdisciplinary Center for Biotechnology Research
IDT	Integrated DNA Technologies
JA	Jasmonic Acid
LRR	Leucine-Rich Repeat
LRR-RLK	Leucine-Rich Repeat Receptor-Like Kinase
MAPK / MAPKs	Mitogen-Activated Protein Kinase(s)
MDPI	Multidisciplinary Digital Publishing Institute
$MgCl_2$	Magnesium Chloride
NBS-LRR	Nucleotide-Binding Site – Leucine-Rich Repeat
NPR1	Nonexpressor of Pathogenesis-Related Genes 1
NT	Non-Transgenic
OD600	Optical Density at 600 nm
PAL	Phenylalanine Ammonia-Lyase
PAL1	Phenylalanine Ammonia-Lyase 1
PAMPs	Pathogen-Associated Molecular Patterns
PCA	Principal Component Analysis
PCC	Phenolic Compound Content
PCR	Polymerase Chain Reaction
PR	Pathogenesis-Related
PYM	Peptone–Yeast–Malt Extract Medium
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
R	Resistance
RLKs	Receptor-Like Kinases
RNA-seq	RNA sequencing
ROS	Reactive Oxygen Species
rpm	Revolutions Per Minute
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SAUR	Small Auxin Up RNA
SD	Standard Deviation
SRG1	Senescence-Related Gene 1
TFs	Transcription Factors
UV	Ultraviolet
WAKs	Wall-Associated Kinases
Xaa	*Xanthomonas axonopodis* pv. *Aurantifolii*
Xcc	*Xanthomonas citri* pv. *Citri*
Xcm	*Xanthomonas campestris* pv. *Musacearum*
Xcv	*Xanthomonas campestris* pv. *vesicatoria*

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