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Functional Analysis of RMA3 in Response to *Xanthomonas citri* subsp. *citri* Infection in Citron C-05 (*Citrus medica*)

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Abstract: Citrus bacterial canker disease, caused by *Xanthomonas citri* subsp. *citri* (*Xcc*), poses a significant global threat to the citrus industry. *Lateral organ boundaries 1* (*Lob1*) is confirmed as a citrus susceptibility gene that induces pathogenesis by interaction with the PthA4 effector of *Xcc*. Citron C-05 (*Citrus medica*) is a *Citrus* genotype resistant to *Xcc*. However, there is little information available on the regulation of *Lob1* in resistant genotypes, which is important for the breeding of citrus cultivars resistant to canker disease. This study aimed to identify upstream regulatory factors of *Lob1* in Citron C-05 and to investigate its function in disease resistance. 'Bingtang' sweet orange (*C. sinensis*), a susceptible genotype, was utilized as the control. cDNA yeast libraries of *Xcc*-induced Citron C-05 and 'Bingtang' sweet orange were constructed. The capacities of 'Bingtang' and Citron C-05 were 1.896×10^7 and 2.154×10^7 CFU, respectively. The inserted fragments ranged from 500 to 2000 bp with a 100% recombination rate. The promoter of *Lob1* was segmented into two pieces and the P1 fragment from both genotypes was used to construct a bait yeast (PAbAi-*CsLob1*-P1; PAbAi-*CmLob1*-P1). Through library screening with the bait yeast, upstream regulators interacting with the *Lob1*-P1 promoter were identified and then validated using Y1H and dual-luciferase tests. The expression analysis of the three transcript factors indicated that RMA3 was upregulated by inoculation with *Xcc* in the resistant Citron C-05, but not in the susceptible sweet orange. The overexpression of *CsRMA3* in 'Bingtang' sweet orange led to reduced canker symptoms, with a significantly lower pathogen density in the leaves following *Xcc* inoculation. When *CmRMA3* was silenced by virus-induced gene silencing (VIGS) in Citron C-05, typical canker symptoms appeared on the *CmRMA3*-silenced leaves at 15 days post-inoculation with *Xcc*. Further expression analyses revealed that the CmRMA3 transcription factor suppressed the expression of *Lob1*. These results suggest that RMA3 participates in the resistant reaction of Citron C-05 to *Xcc* infection, and such a response might be in relation to its suppression of the expression of the pathogenic gene *Lob1*.

Keywords: citrus cDNA library; yeast one-hybrid system; citrus canker disease; *Lob1*; transcription factor; *Citrus medica*; disease resistance

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1. Introduction

Citrus is one of the most important fruit crops in the world [1]. However, citrus canker, caused by the bacterium *Xanthomonas citri* subsp. *citri* (*Xcc*), is one of the most destructive diseases in the citrus industry [2–4]. This disease is widely distributed in most citrus-producing areas except Australia and the Mediterranean region, and most commercial citrus varieties are susceptible to *Xcc* [5–8]. At present, the management of citrus canker is mainly based on copper bactericide sprays, which have limited effects or cause the resistance of the pathogen to these chemicals [9–13]. Therefore, the genetic improvement of citrus cultivars resistant to canker disease is essential to eliminate this threat.

Previous studies have confirmed that *CsLob1* is a key susceptibility gene of citrus canker [14]. A subcellular localization analysis showed that *CsLob1* was mainly located in the nucleus [15]. A single allele of *CsLob1* activated by PthA4, the *Xcc* pathogenicity effector, is sufficient to induce citrus canker [16]. Nonetheless, there have been scarce reports on the regulation of *Lob1* expression, which may provide a possible mechanism for the resistance to this disease.

After years of screening among citrus accessions, a *Citrus medica* genotype resistant to *Xcc* was identified in our laboratory, named 'Citron C-05' [17]. It showed high resistance to *Xcc* under field and indoor conditions with a necrosis reaction after being inoculated with *Xcc*. The serial spatiotemporal observation of the dynamics of *Xcc* infection indicated that Citron C-05 was able to initiate the defense response to the infection by effectively limiting the reproduction of *Xcc* in mesophyll cells [18].

In order to breed resistant citrus varieties, it is necessary to clarify the molecular mechanism of the response to *Xcc* infection in *Citrus*. Previously, the transcript factor RAP2-13 was identified to interact with BAK1 and its expression was upregulated by *Xcc* infection [19]. A better understanding of the interaction between the resistant genotype Citron C-05 and the susceptibility gene *CsLob1* would allow us to more closely clearly understand the molecular mechanism of resistance to *Xcc* infection.

Yeast one- and two-hybrid systems are often used to screen interactions between target proteins and bait molecules [20]. Based on high-throughput screening with strict screening pressure, many studies have obtained candidate prey proteins through yeast one- and two-hybrid systems, such as in wheat [21,22], *Arabidopsis thaliana* [23], rice [24], poplar [25], kiwifruit [26] and citrus [19].

In the present study, two cDNA libraries were constructed with *Xcc*-induced cDNA from the resistant Citron C-05 and the susceptible 'Bingtang' sweet orange (*C. sinensis*). The promoter of the pathogenetic gene *Lob1* from two genotypes was used as bait to screen the cDNA yeast library. The goal was to identify the upstream transcription factors that regulate the expression of the *Lob1* promoter in resistant and susceptible genotypes.

2. Materials and Methods

2.1. Inoculation of Citrus Leaves with Xcc

The resistant Citron C-05 and the susceptible 'Bingtang' sweet orange were chosen as test genotypes. Potted plants aged 2 years and grafted on trifoliate orange (*Poncirus trifoliata*) rootstock were used for inoculation with *Xcc*. All plants were grown in a greenhouse at 28 °C with a natural photoperiod.

The *Xcc* strain DL509, previously isolated in the laboratory, was prepared as described by Wu Qi [19]. The final concentration of the bacterial solution was determined by plate counting. According to the previous inoculation experiments, 10^4 CFU/mL was utilized as the pathogen inoculum concentration.

Three expanding young leaves from both citrus genotypes were inoculated with the *Xcc* inoculum by injection. According to the leaf size, 6–8 injections per leaf were performed. Canker symptom development was continuously observed at 1–2-day intervals until 17 days post-inoculation (dpi), when the disease symptoms had completely appeared.

No symptoms appeared at 2–6 dpi in both genotypes. Later, water-soaked spots began to appear on the leaves of 'Bingtang', and sunken tissue necrosis occurred in the leaves of Citron C-05. The disease symptoms continued to develop, and, at 17 dpi, typical canker occurred on 'Bingtang' leaves, while necrosis appeared on Citron C-05 leaves. Upon careful examination of symptom development, at 8 dpi, the symptoms started to show clear differences between the susceptible and resistant genotypes (Figure 1). The inoculated leaves of 'Bingtang' sweet orange and Citron C-05 at 8 dpi were sampled for library construction.

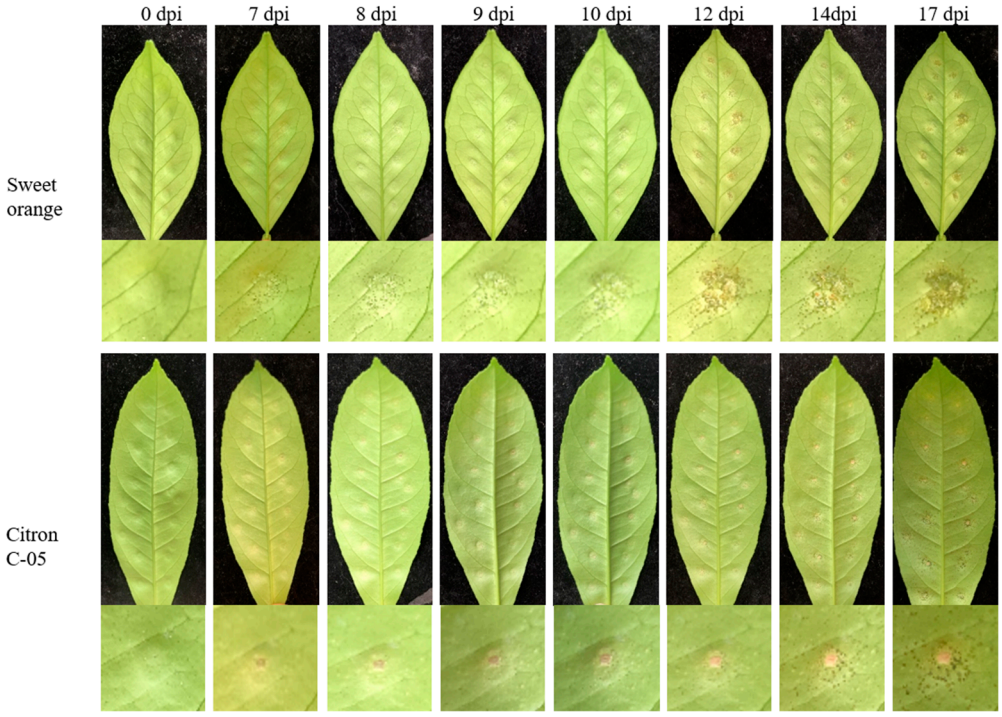

Figure 1. Leaf symptoms on the two citrus genotypes ('Bingtang' sweet orange = susceptible, Citron C-05 = resistant) after inoculation with 10^4 CFU/mL *Xanthomonas citri* subsp. *citri.* dpi = days post-inoculation.

2.2. Construction of cDNA Library

Total RNA was isolated from the sampled leaves of both genotypes using TRIzol reagent (Invitrogen, Grand Island, NY, USA), following the manufacturer's instructions. Subsequently, 1 μg of extracted total RNA was used for reverse transcription using the FastQuant RT kit (Tiangen biotech, Beijing, Co., Ltd., Beijing, China) to synthesize the first-strand cDNA, and the double-stranded cDNA (ds cDNA) was synthesized using the CloneMiner II cDNA Library Construction Kit (Invitrogen), according to the manufacturers' protocols.

The obtained ds cDNA was linked with the attB1 adapter in a mixture containing adapter buffer and DTT, which was incubated at 16 °C for 16–24 h. The cDNA with the attB1 adapter was collected from cDNA size fraction columns and cloned into the pDONR222 plasmid through a BP recombination reaction. The ligated product was then transferred into competent *Escherichia coli* cells and incubated at 37 °C on a shaker at 220 rpm for 1 h to obtain the cDNA library. The obtained *E. coli* solution was diluted 1000 times, and 50 μL was spread onto LB plates supplemented with the Amp (ampicillin) antibiotic to determine the recombination rate of the inserted fragments in the library. The number of colonies was recorded on the second day. Then, 24 clones were randomly picked from each library for PCR identification to assess the recombination rate.

2.3. Construction of Lob1 Promoter Bait and Detection of Autonomous Activation

The *Lob1* promoters of 'Bingtang' and Citron C-05 were amplified with pAbAi-*CsLob1*-P-F and pAbAi-*CmLob1*-P-F, respectively, with the pAbAi-*Lob1*-P-R primers (Appendix A Table A1), using Phanta Max Super Fidelity DNA Polymerase PCR (Vazyme, Nanjing, China). In order to prevent the full length of the Lob1 promoter from exhibiting auto-activation, the promoters of both genotypes were segmented into two pieces, i.e., P1 and P2. These two fragments were also amplified with the corresponding primers (Appendix A Table A1).

The PCR products of the full length and the P1 and P2 fragments of the *Lob1* promoter were recovered and cloned, respectively, into pAbAi, which had been digested with restriction endonucleases HindIII and KpnI at 37 °C for 25 min. The ligation was performed using

T4 DNA ligase and they were incubated overnight at 16 °C. Sequencing was performed using primers pAbAi seq-F/pAbAi seq-R (Appendix A Table A1) for bait verification. Plasmids containing the Lob1 promoter fragment were extracted.

PBait *Lob1* AbAi, PBait *Lob1*-P1 AbAi, and PBait *Lob1*-P2 AbAi, as well as p53 AbAi (positive control), were linearized and separated on 1.5% agarose gel at 37 °C. All plasmids were then purified from the gel and separately transferred to Y1H Gold. The transformed yeast cells were cultured on Synthetic Dextrose Minimal Medium without Uracil (SD/-Ura)-deficient plates for 3–5 days. The successfully transformed yeast clones were designated as bait yeast and subjected to subsequent autonomous activation analysis. Following validation, the positive clones were agitated and preserved in a −80 °C freezer.

The yeast bait strains were cultured on SD/-Ura culture dishes to assess their auto-activation according to the background expression levels of Aureobasidin A (AbA). The OD_{600} was adjusted to around 0.002 using an appropriate volume of 0.9% NaCl suspension, and 100 µL of the yeast suspension was applied to the SD/-Ura solid culture medium with varying AbA concentrations (0–1000 ng/mL) and incubated at 30 °C for 2–3 days. Yeast growth observation indicated that the full length and the P2 segment of the *Lob1* promoter possessed autonomous activation. Growth inhibition was observed for P1 from sweet orange (PAbAi-*CsLob1*-P1) on SD/-Ura medium containing 125 mg/mL of AbA and that from Citron C-05 (PAbAi-*CmLob1*-P1) on SD/-Ura medium containing 100 mg/L of AbA (Figure S1). Therefore, the P1 segment was utilized for successive library screening.

2.4. Screening Xcc-Induced Citrus cDNA Library with PAbAi-Lob1-P1

The bait yeast PAbAi-Lob1-P1 from sweet orange and Citron C-05 was further used for the screening of their corresponding cDNA libraries. Y1H Gold containing the suitable bait was adjusted to the yeast-receptive state; subsequently, the cDNA library plasmids of Citron C-05 and 'Bingtang' sweet orange were introduced into the Y1H Gold receptive cells for library screening. The transformation solution coated with SD/-Ura was incubated for 3–5 days, and positive clones were detected using PCR. Subsequently, 10 µL of the PCR amplification products was separated on the gel for size verification and the remaining PCR products were subjected to sequencing.

2.5. Verification of Candidate Interacting Proteins by Yeast One-Hybrid System

The full-length sequences of the identified positive clones from 'Bingtang' and Citron C-05 were amplified and then ligated into enzyme-cleaved prey vector pGADT7-AD. The constructed AD prey vector was introduced into the Y1H Gold yeast containing the *Lob1* promoter fragment in the receptive states for the identification of the positive yeast clone.

2.6. Validation of Interaction with Dual-Luciferase Enzyme Assay

By employing homologous recombination, the *CmLob1*-p1 promoter was cloned into the pGreen II 0800-LUC vector to generate the reporter construct pGreen II 0800-LUC-*CmLob1*-p1, which was then transformed into *Agrobacterium* GV3101 (pSoup). Simultaneously, the *CmRMA3* coding sequence (CDS) was cloned into the Pcambia1300-35S vector as the effector construct and transformed into *Agrobacterium* GV3101. Utilizing empty vectors pGreen II 0800-LUC and p1300-35S as the controls, a tobacco transient injection system was employed to co-inject the reporter and effector constructs into tobacco (*Nicotiana benthamiana*) leaves at a 1:5 volume ratio. After 2 days, the dual-luciferase enzyme activity was assessed, the LUC/REN ratio was calculated, and the relative LUC activity was determined.

2.7. Expression Analysis of Candidate Transcription Factors

Xcc was injected into both Citron C-05 and 'Bingtang' leaves as described in Section 2.1, and the inoculation concentration of *Xcc* was 10^5 CFU/mL, with sterile water as the control. The leaves at 0, 2, 4, 6, and 8 dpi were sampled and promptly transferred into pre-labeled 2 mL centrifuge tubes containing steel beads, and then they were immediately frozen in

liquid nitrogen. Subsequently, total RNA extraction and cDNA synthesis were carried out as described in Section 2.2. The cDNA products were subjected to quantitative real-time PCR analysis using a Bio-Rad CFX96 qPCR system. All qPCR assays were conducted in duplicate following the SYBR Green protocol using the qPCR primers for *RMA3*, *TFGTE*, and *NAC* (Appendix A Table A1).

2.8. RMA3 Cloning and Sequence Comparison in Different Citrus Genotypes

The *RMA3* sequences of American citron, Aiguo citron, Nanchuan citron, Danna citron, 'Shatian' pomelo, and 'Eureka' lemon were obtained through citrus genome query websites (http://citrus.hzau.edu.cn/index.php; https://www.citrusgenomedb.org/; and http://10.100.128.171:4567/, accessed on 20 August 2023).

2.9. Subcellular Localization of Citrus RMA3

The *E. coli* strain carrying the pCambia1300 vector was streaked on LB solid medium containing 50 mg/L of kanamycin for activation. The pCambia1300 plasmid was extracted and digested with a KpnI restriction enzyme. Then, *CmRMA3* was cloned into the plasmid through a homologous recombination enzyme at 37 °C for 45 min. Single positive colonies were picked, and the desired plasmid 35S::*CmRMA3*::eYFP was extracted. After verification by sequencing, the target plasmids were introduced into the *Agrobacterium* GV3101 strain. Simultaneously, the original pCambia1300 vector was introduced into the GV3101 strain as an empty vector control for subsequent experiments. Positive clones were identified and bacterial cultures were preserved. The activated cultures were grown at 28 °C to maintain an OD_{600} of 0.5–0.8. Bacterial suspensions were harvested in 50 mL centrifuge tubes and resuspended in an equal volume of injection medium: 1.98 g/L of $MgCl_2$, 0.98 g/L of 2-morpholinoethanesulphonic acid (MES), and 0.029 g/L of acetosyringone (AS). The adjusted bacterial suspension was injected into *Nicotiana benthamiana*, and, after 48 h, a fluorescence microscope was used for observation.

2.10. Transient Overexpression of Candidate Genes

The target plasmid was introduced into the *Agrobacterium* EHA105 strain via electroporation according to the procedures described in Section 2.9. Simultaneously, the original vector Pcambia1300 was also introduced into the EHA105 strain as an empty control. Citrus leaves were infiltrated with the transformed EHA105 and, 24 h later, inoculated with 10^5 CFU/mL of *Xcc*, as mentioned in Section 2.7. Leaf symptoms were regularly monitored and the quantity of bacteria in the leaf was recorded.

2.11. RMA3 Gene Silencing in Citron C-05 through VIGS

Citron C-05 seedlings at 8 days after seed germination were utilized as virus-induced gene silencing (VIGS) test plants. A 300 bp fragment from the conserved region of the *RMA3* CDS was ligated to EcoRI and the BamHI enzyme-cleaved TRV2 vector. The *RMA3*-TRV2 plasmid was transformed into *Agrobacterium* EHA105 using the electroporation method. Positive monoclonal colonies were preserved in 50% sterile glycerol at −80 °C. The bacterial suspensions of TRV1, *RMA3*-TRV2, and Empty-TRV2 were cultured on LB solid medium at 28 °C for 2 days. Colonies resistant to both kanamycin (50 μg/mL) and rifampicin (20 μg/mL) were selected and cultured in LB liquid medium until an OD_{600} of 1.2 was reached. The resuspended bacterial cells were mixed at a 1:1 ratio of TRV1 and *RMA3*-TRV2 suspensions in an osmotic solution (10 mM $MgCl_2$, 10 mM MES, 150 μM AS) and incubated at 24 °C for 3 h. The bacterial solution was then applied to 8-day-old seedlings of 'Bingtang' and Citron C-05 under a vacuum for 3 min to facilitate penetration. Subsequently, the seedlings were washed, transferred to MT medium, and kept in the dark for 3 days before being moved to a controlled environment and transplanted into soil. After 2–3 weeks of growth, RNA was isolated and reverse transcription was conducted on the leaves of the silenced 'Bingtang' and Citron C-05 clones. The expression of relevant genes was analyzed.

Xcc inoculation was performed as described in Section 2.7 to observe leaf symptoms at regular intervals for the evaluation of the resistance to *Xcc* infection.

2.12. Data Analysis

The Microsoft Excel 2013 software was used for data processing, and all of the presented data are shown as average values and standard errors. Differences between the means were evaluated using Tukey's Honest Significant Difference test.

3. Results

3.1. Screening Xcc-Induced Citrus cDNA Library

3.1.1. Quality Determination of *Xcc*-Induced cDNA Library of Yeast Hybridization

pGADT7-DEST containing *Xcc*-induced Citron C-05 and 'Bingtang' sweet orange cDNA fragments was transformed into *E. coli*. After being diluted 1000 times, the transformed *E. coli* solution was plated on Amp + LB plates and the number of grown clones was recorded. The total library capacity for 'Bingtang' was estimated at about 1.896×10^7 CFU, and that for Citron C-05 was 2.154×10^7 CFU. Twenty-four clones of *E. coli* containing both Citron C-05 and 'Bingtang' cDNA were randomly selected and identified by PCR amplification. The inserted fragments in the library ranged from 500 to 2000 bp, and the 24 checked colonies for each library showed a recombination rate of 100%.

3.1.2. Screening *Xcc*-Induced cDNA Library with PAbAi-*Lob1*-P1

The plasmids extracted from the cDNA library were transformed into Y1H yeast cells containing the P1 bait fragment of the *Lob1* promoter. The transformed yeast culture was diluted 10 times and spread on SD/-Leu plates for 3 days, and the library screening efficiency was calculated (Figure S2A,B). The remaining yeast culture solution was spread on SD/-Leu 100 ng/mL AbA plates and incubated at 30 °C for 3–5 days to screen candidate interacting proteins in yeast single colonies (Figure S2C). Each single yeast colony grown was separately resuspended in 0.9% NaCl solution and transferred to SD/-Leu plates with an increased AbA concentration of 50 ng/mL for repeated screening (Figure S2D). Colonies that continued to grow were identified by PCR (Figure S2E), and the amplified bands ranged from 500 to 1500 bp.

From the screening of the *Xcc*-induced 'Bingtang' library with PAbAi-*CsLob1*-P1, eight interacting proteins were detected, while, from the Citron C-05 library with PAbAi-*CmLob1*-P1, 11 interacting proteins were identified, among which CmNAC (CmLP1-11), CmTFGTE (CmLP1-7), and CmRMA3 (CmLP1-8) were transcription factors (Table 1).

Table 1. Interacting proteins obtained from the screening of cDNA libraries of 'Bingtang' sweet orange (susceptible) and Citron C-05 (resistant) induced by *Xanthomonas citri* subsp. *citri* with *Lob1-P1* promoter.

Bait Name	Library Genotype	Gene Code	Function
PAbAi-*CsLob1*-P1	'Bingtang' sweet orange	CsLP1-1	Bispecific inhibitors; lipid transfer proteins; seed storage 2S albumins.
		CsLP1-2	Oxygen-evolving enhancer protein 2; chloroplast.
		CsLP1-3	Composition of ribosomal protein S9; cytoplasmic 80S ribosome.
		CsLP1-4	E3 ubiquitin protein ligase RING1; RING finger protein 126; E3 ubiquitin ligase.
		CsLP1-5	Zinc finger protein and putative TFIIIA-like zinc finger protein.
		CsLP1-6	Oxygen-evolving enhancer protein 3; chloroplast.
		CsLP1-7	Wound-induced protein 1; putative unidentified protein.
		CsLP1-8	Uncharacterized protein At4g01150 in chloroplast.

Table 1. *Cont.*

Bait Name	Library Genotype	Gene Code	Function
PAbAi-*CmLob1*-P1	Citron C-05	CmLP1-1	Protein binding chlorophyll a and b.
		CmLP1-2	Alkaline chitinase A and lectin.
		CmLP1-3	Unidentified protein.
		CmLP1-4	Bifunctional dihydroflavonol 4-reductase plays crucial role in reduction of flavonoids.
		CmLP1-5	60S ribosomal protein L10, a fragment of the same.
		CmLP1-6	Nuclear transcription factor Y subunit C-1.
		CmLP1-7	Transcription factor GTE1; chloroplast transcription factor GTE3.
		CmLP1-8	The first variation could be 'E3 ubiquitin protein ligase RMA3, peroxisome generating factor 10, and helicase-like transcription factor are the proteins under study'.
		CmLP1-9	Receptor kinase 1 associated with brassinosteroid insensitivity 1.
		CmLP1-10	2-Acetamido-2-deoxy-d-galactose-bound seed agglutinin 2, also known as lectin.
		CmLP1-11	NAC domain referred to as Protein 29.

3.2. Verification of Candidate Interacting Proteins by Yeast One-Hybrid System

The three transcript factors (CmTFGTE, CmRMA3, and CmNAC) detected from the Citron C-05 cDNA library were chosen for further verification. The full-length DNA sequences coding for the three candidate interacting proteins were amplified and ligated into the prey vector pGADT7-AD. The constructed AD-prey vector plasmids were then transformed into Y1H-PAbAi-*CmLob1*-P1 bait yeast cells. The results revealed that all three candidate proteins exhibited interactions with PAbAi-*CmLob1*-P1 (Figure 2).

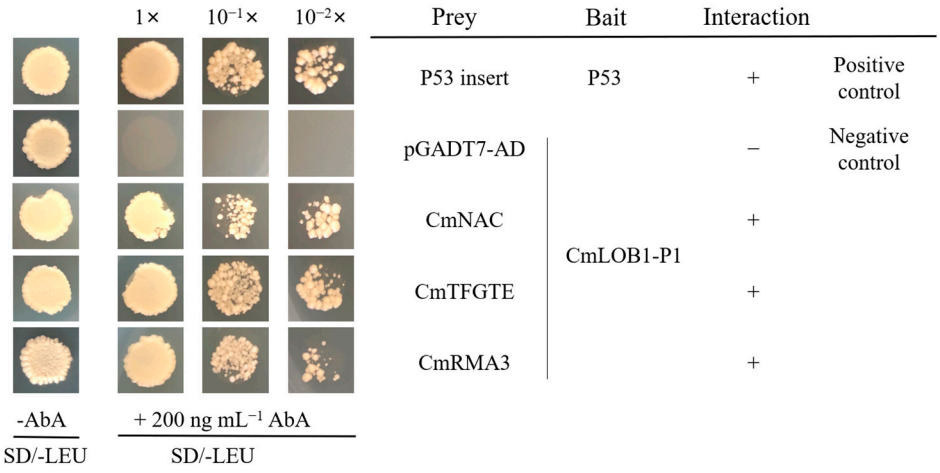

Figure 2. Validation of the interacting protein in Citron C-05 by *CmLob1*-P1 in Y1H system. pGADT7-AD: empty vector introduced into the same yeast cells as the negative control; pGADT7-p53: plasmid containing the p53 antibody introduced into yeast cells as the positive control; '+': yeast containing Citron C-05 cDNA able to grow normally in SD/-LEU medium, representing the candidate protein interacting with *CmLob1*-P1; '−': yeast did not grow in SD/-LEU medium, meaning that the protein had no interaction with *CmLob1*-P1. The concentration of AbA was 200 ng/mL.

The homologous genes from sweet orange with the three identified transcript factors in citron were introduced into sweet orange yeast cells (Y1H-PAbAi *CsLob1*-P1) to detect the possible interactions with PAbAi *CsLob1*-P1. CsNAC, CsTFGTE, and CsRMA3 demonstrated interactions with PAbAi *CsLob1*-P1 (Figure 3), even though their interactions had not been detected in the previous screening (Table 1).

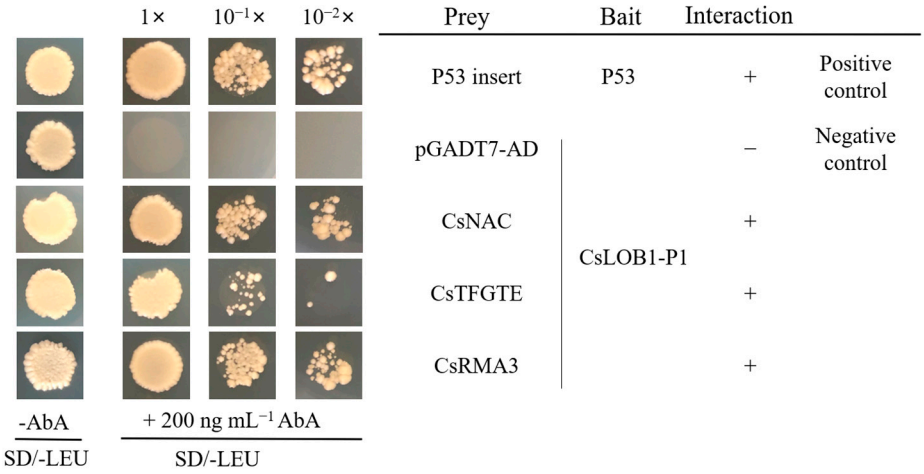

Figure 3. Identification of the interacting proteins in sweet orange and *CsLob1*-P1 in the Y1H system. pGADT7-AD: empty vector introduced into the same yeast cells as the negative control; pGADT7-p53: plasmid containing the p53 antibody introduced into yeast cells as the positive control. '+': yeast containing sweet orange cDNA able to grow normally in SD/-LEU medium, indicating that the candidate proteins interact with *CmLob1*-P1; '−': the same yeast did not grow in SD/-LEU medium, meaning that the protein had no interaction with *CmLob1*-P1. The concentration of AbA was 200 ng/mL.

3.3. Interaction Validation with Dual-Luciferase Enzyme Assay

The results of the dual-luciferase assay indicated that the CmNAC transcription factors had very low expression, even though they significantly reduced the expression of *Lob1*. CmTFGTE did not show the suppression of the expression of *Lob1*. Moreover, the CmRMA3 and CsRMA3 transcription factors bound to the promoter of *Lob1*, thereby significantly downregulating the expression of the *Lob1* gene (Figure 4).

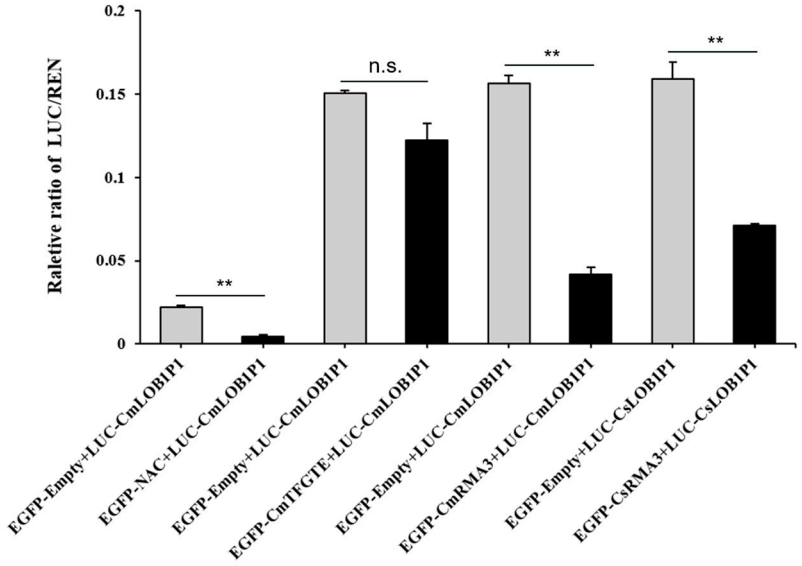

Figure 4. Identification of proteins interacting with the *Lob1-P1* promoter by dual-luciferase assay. The CmNAC transcription factors had very low expression, even though they significantly reduced the expression of Lob1. CmTFGTE did not show the suppression of the expression of Lob1. The CmRMA3 and CsRMA3 transcription factors bound to the promoter of *Lob1*, thereby significantly downregulating the expression of the *Lob1* gene. CsNAC and CsTFGTE were not analyzed for interactions with *CsLob1*-P1. '**' indicates a significant difference ($p < 0.01$). 'n.s.' indicates no significant difference.

3.4. Expression Analysis of Candidate Genes following Infection with Xcc

To investigate the expression profiles of the *NAC*, *TFGTE20*, and *RMA3* genes induced by *Xcc* in 'Bingtang' sweet orange and Citron C-05, qPCR validation was conducted using cDNA from the two genotypes inoculated with *Xcc* at different intervals following inoculation with 10^5 CFU/mL of *Xcc*. The expression of *RMA3* started to be upregulated at 2 dpi and peaked at 6 dpi, with significantly higher expression in Citron C-05 compared with 'Bingtang'. The expression levels of the *NAC* and *TFGTE20* genes were low, displaying higher expression levels in the susceptible 'Bingtang' than in the resistant Citron C-05 (Figure 5). The results clearly indicated that the expression of the *RMA3* gene was stimulated by *Xcc* infection; therefore, *RMA3* was considered the candidate transcript factor for the subsequent functional analysis.

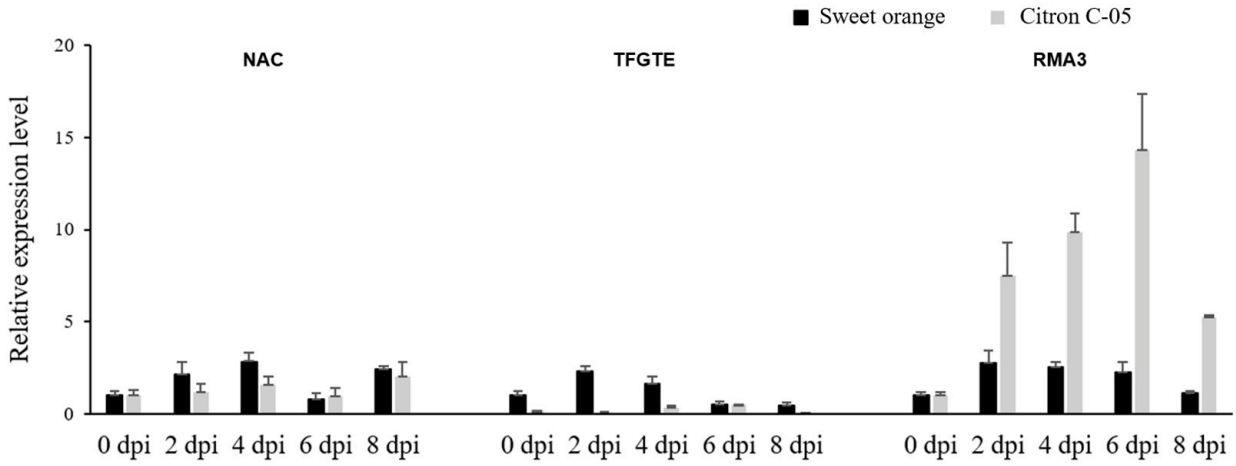

Figure 5. Relative expression of three candidate genes in 'Bingtang' sweet orange (susceptible) and Citron C-05 (resistant) following inoculation with 10^5 CFU/mL of *Xanthomonas citri* subsp. *citri*.

3.5. Comparison of RMA3 Sequences among Different Citrus Genotypes

The *RMA3* CDS was cloned from pomelo (*C. grandis*), sweet orange (*C. sinensis*), lemon (*C. limon*), and five citrons (*C. medica*) with different behaviors regarding *Xcc* infection. Sequence alignment revealed 91.17% homology among the eight genotypes with only 14 nucleotide mutations. The RMA3 amino acid sequences in the different citrus genotypes showed a high degree of homology (90.05%). Focusing on 'Bingtang' sweet orange and Citron C-05, both citrus genotypes contained 260 amino acid residues, with differences at six amino acid positions. The RMA3 proteins presented a C3HC4-RING finger domain that allowed them to function as E3 ubiquitin ligases (Figure S3).

3.6. Subcellular Localization of RMA3

The constructed 35S::CmRMA3::GFP vectors were transformed into GV3101 and subsequently infiltrated into *N. benthamiana* leaves. Fluorescence microscopy showed green fluorescence signals emanating from the cell nuclei and membranes, potentially suggesting the presence of RMA3 in both the nucleus and cell membrane (Figure 6).

3.7. Transient Overexpression of RMA3 in 'Bingtang' Sweet Orange

The 35S::*CsRMA3*::GFP vector was introduced into *Agrobacterium* EHA105, which was then injected into the susceptible 'Bingtang' leaves inoculated with *Xcc*. The transient overexpression of *RMA3* inhibited *Xcc* colonization in the 'Bingtang' leaves. The number of lesions per square centimeter area in the treatment group was 0.136 times that of the control group. Bacterial quantification recorded 2.31×10^5 CFU/cm^2 for RMA3-overexpressed leaves and 2.37×10^6 CFU/cm^2 for the control, thus confirming the significant inhibitory effects on *Xcc* by RMA (Figure 7).

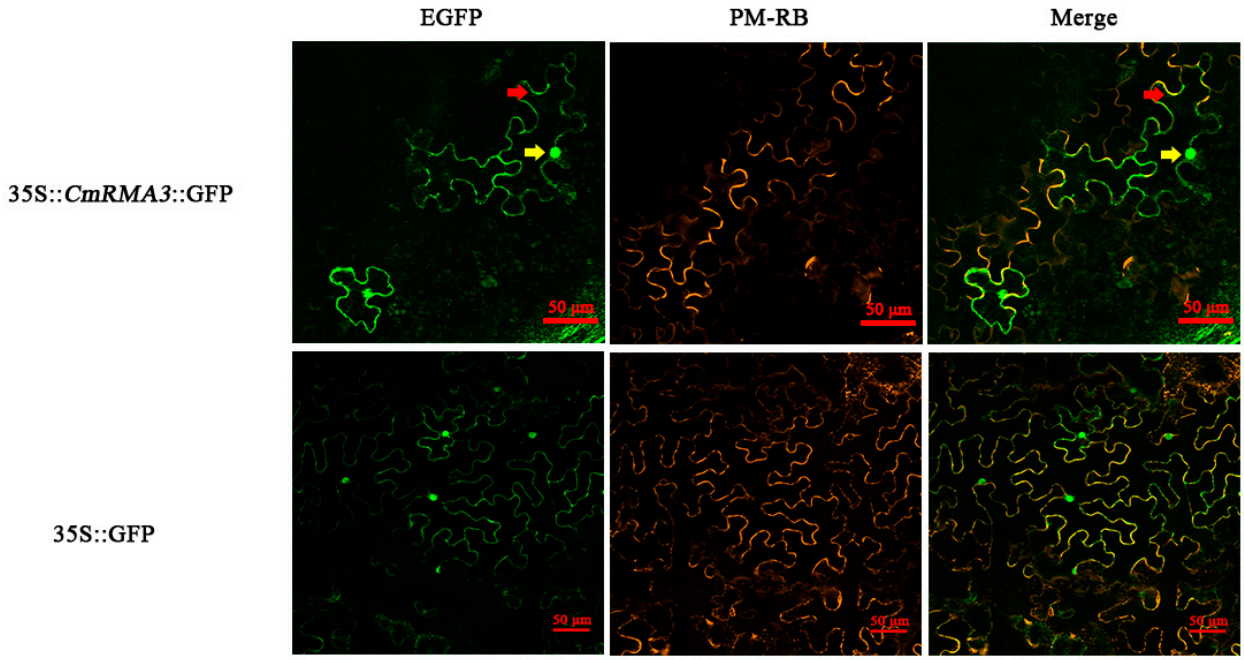

Figure 6. The subcellular localization of CmRMA3 of Citron C-05. Yellow arrows indicate the nucleus; the red arrow points to the cell membrane; PM-RB is a membrane-targeting mark; 35S::GFP: an empty control, localized to the cell membrane and nucleus.

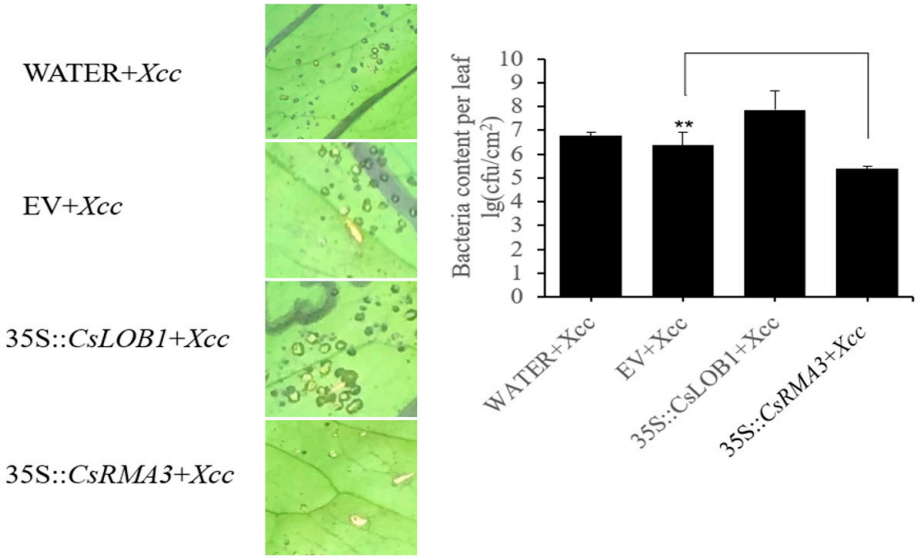

Figure 7. Effects on the inhibition of *Xanthomonas citri* subsp. *citri* (*Xcc*) infection following *RMA3* overexpression in susceptible 'Bingtang' sweet orange leaves. On the left, upon inoculation with *Xcc*, canker symptoms were observed in sweet orange plants with the transient overexpression of the *RMA3* gene; on the right, quantitative counting plate count data of *Xcc* bacteria. The concentration of *Xcc* was 10^5 CFU/mL. '**' indicates a significant difference between 'EV + *Xcc*' and '35S::*CsRMA3* + *Xcc*' ($p < 0.01$).

The expression analysis of *Lob1* demonstrated that the transient overexpression of *RMA3* suppressed the expression of the *Lob1* gene. At 3 days following inoculation with *Xcc* in both sweet orange and citron leaves overexpressing *RMA3*, the expression of *Lob1* in both genotypes was significantly reduced compared with the controls (Figure 8).

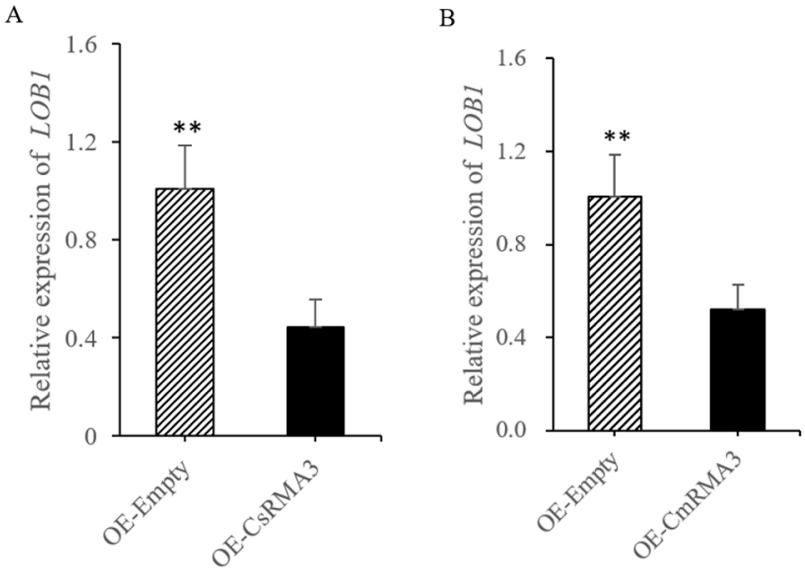

Figure 8. Relative expression of the *Lob1* gene in citrus leaves following the transient overexpression of *RMA3* at 3 dpi. (**A**) Leaf of 'Bingtang' sweet orange; (**B**) leaf of Citron C-05. The concentration of *Xanthomonas citri* subsp. *citri* was 10^5 CFU/mL. '**' indicates a significant difference($p < 0.01$) between the transient clone and the control.

3.8. Silencing RMA3 Gene in Citron C-05 Using VIGS

RMA3 was silenced by VIGS in Citron C-05 and the expression of *RMA3* was down-regulated in the six tested *RMA3*-silenced Citron C-05 clones (Figure 9). Additionally, the relative expression levels of *Lob1* in four out of six silenced clones were significantly lower than those in the control plants (Figure 10). Nonetheless, after being inoculated with *Xcc*, the silenced plants at 3 dpi increased the relative expression levels of *Lob1*, significantly higher than those of the control plants (Figure 10).

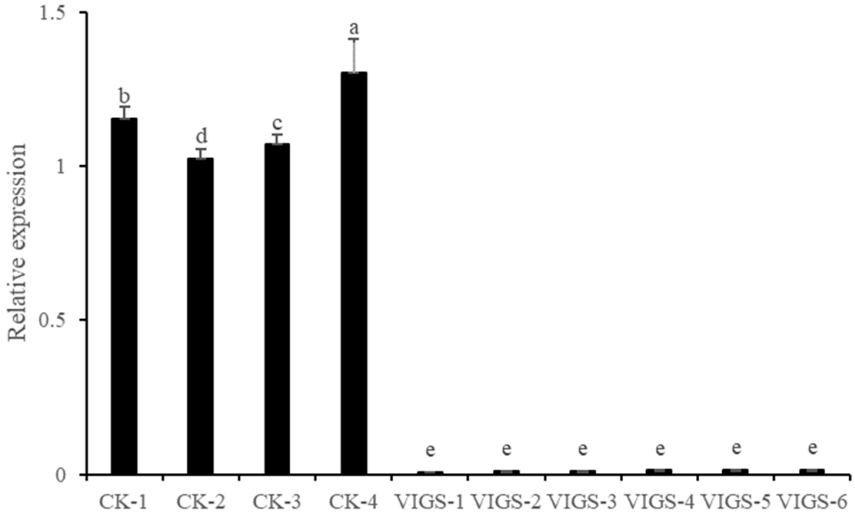

Figure 9. Relative expression of the *RMA3* gene in Citron C-05 with *RMA3* silenced by VIGS. CK-1 to CK-4 are clones transferred with the Empty-TRV2 plasmid as controls; VIGS-1 to VIGS-6 are *RMA3*-silenced plants transferred with *RMA3*-TRV2. The different letters in the columns indicate significant differences ($p < 0.05$).

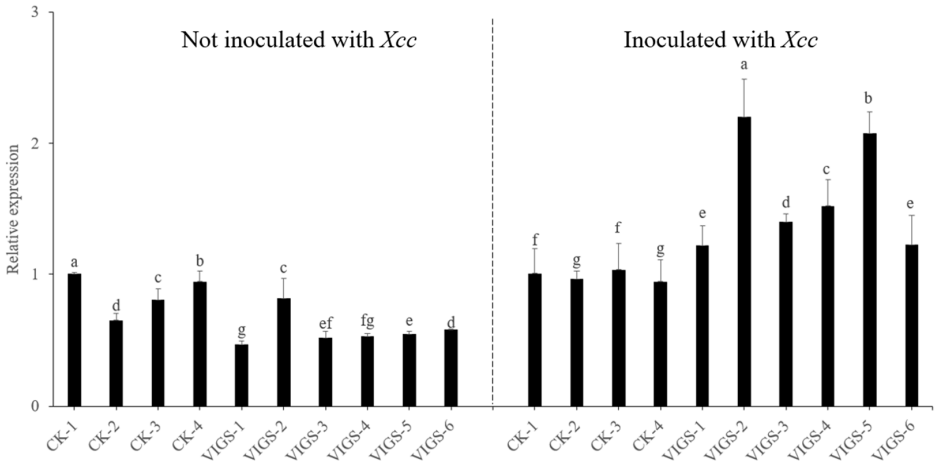

Figure 10. Relative expression of *Lob1* in Citron C-05 with *RMA3* silenced by VIGS. On the left, the plants were not inoculated with *Xanthomonas citri* subsp. *citri* sampled at the same time as the inoculated ones; on the right, the plants were inoculated with *Xcc* and evaluated at 3 days post-inoculation. The concentration of *Xcc* was 10^5 CFU/mL. CK-1 to CK-4 are clones transferred with the Empty-TRV2 plasmid as controls; VIGS-1 to VIGS-6 are *RMA3*-silenced plants. The different letters in the columns indicate significant differences ($p < 0.05$).

3.9. Canker Symptom Observation of RMA3-Silenced Plants following Inoculation with Xcc

To evaluate the resistance of RMA3-silenced plants to *Xcc* infection, the *RMA3*-silenced Citron C-05 clones were inoculated with 10^5 cfu/mL of *Xcc* by injection. At 7 dpi, symptoms began to manifest on the leaves, characterized by pinpoint raised lesions. Over time, the lesions increased in both their number and spot size. At 15 dpi, numerous typical canker symptoms occurred on both sides of the inoculated leaves. The sweet orange, used as a susceptible genotype for the positive control, exhibited the first callus symptoms (Figure 11). It seemed that the silencing of *RMA3* rendered the resistant Citron C-05 susceptible.

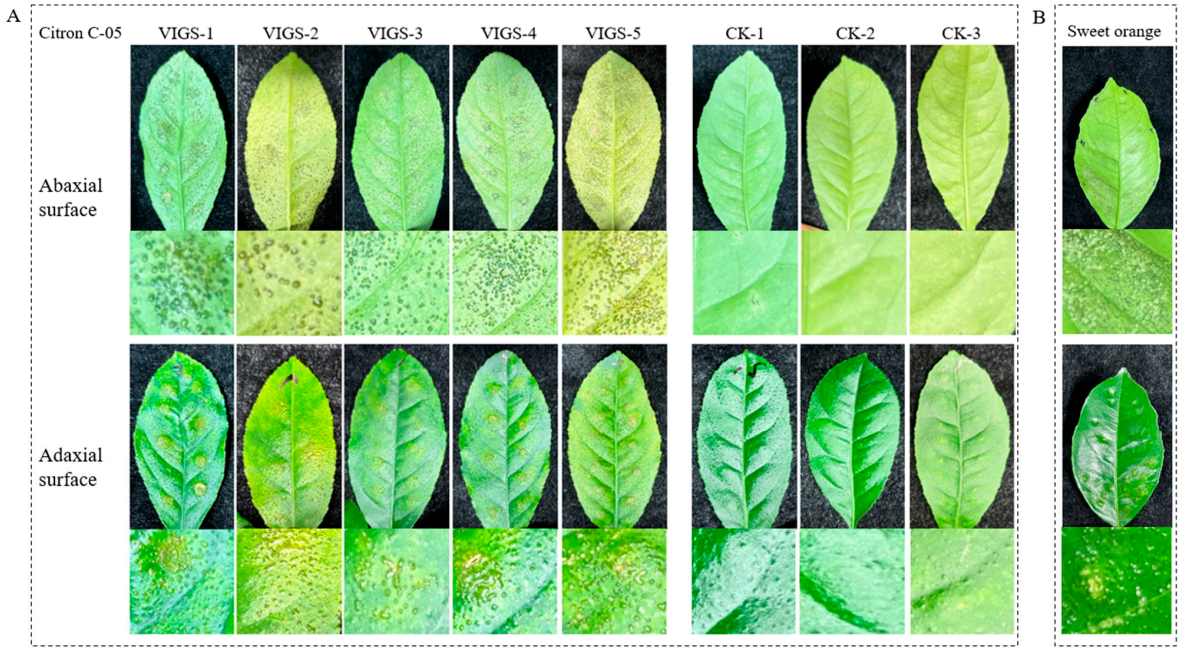

Figure 11. Leaf canker symptom observation of *RMA3*-silenced Citron C-05 at 15 days post-inoculation with *Xanthomonas citri* subsp. *citri*. (**A**) Leaf canker symptoms of *RMA3*-silenced Citron C-05. (**B**) Leaf symptoms of 'Bingtang' sweet orange as a positive control for disease susceptibility genotypes. The inoculation concentration of *Xcc* was 10^5 CFU/mL.

4. Discussion

The yeast hybrid system is effective for the identification of interacting proteins [27]. A high-quality cDNA library and an accurate bait protein are essential for the successful discovery of the target gene. The present study aimed to identify the transcription factors regulating the resistance to canker disease in Citron C-05. The cDNA library of resistant and susceptible genotypes was separately constructed using leaf samples collected when the *Xcc* symptoms started to exhibit a difference between the two tested citrus accessions, thus enabling us to identify resistance genes.

Lob1 has been confirmed as a pathogenic gene for citrus canker. We chose the promoter of *Lob1* as bait to screen the resistant and susceptible citrus cDNA libraries and to detect possible upstream regulatory factors in relation to resistance to canker disease. After the initial screening, eight interacting proteins were identified in the susceptible 'Bingtang' sweet orange library induced by *Xcc* with PAbAi-*CsLob1*-P1, but none of them were useful transcription factors. However, 11 interacting proteins were detected in the resistant Citron C-05 library with PAbAi-*CmLob1*-P1, among which three were transcription factors. In the yeast one-hybrid validation, not only did the three transcription factors confirm the interaction with *CmLob1*-P1 for Citron C-05, but the three homolog transcription factors in 'Bingtang' also revealed an interaction with *CsLob1*-P1. Such double confirmation indicated the interaction between the transcription factors and the *Lob1* promoter.

Among the three candidate transcription factors, *RMA3* solely exhibited significant upregulation in the resistant citrus genotype Citron C-05 upon *Xcc* induction, which was not observed in the susceptible sweet orange in the qPCR analysis. RMA3 is a helicase-like transcription factor (HLTF) with E3 ubiquitin ligase activity, containing the zinc finger C3HC4 ring 2 domain. Ubiquitination plays a crucial role in the degradation of abnormal proteins under metal stress [28]. It is also involved in the recognition and regulation of pathogen-associated molecular patterns (PAMPs) by cell membrane receptors during pattern-triggered immunity (PTI). Furthermore, ubiquitination modulates the levels of intracellular immune receptors to prevent the constitutive activation of effector-triggered immunity (ETI), which is typically associated with hypersensitive cell death (HR) at the infection site [29]. E3 ubiquitin ligase has been reported to play an important role in various stages of the plant immune pathway [30,31]. In the present study, the interaction between RMA3 and the *LOB1* promoter was confirmed, and it was observed that the overexpression of *RMA3* suppressed the expression of *LOB1,* thus inducing resistance in the susceptible sweet orange. Conversely, *RMA3* silencing in the resistant Citron C-05 caused canker symptoms. These results suggest that *RMA3* plays an important role in the resistance to citrus canker by inhibiting the expression of the pathogenic gene *Lob1*.

The expression analysis revealed that RMA3 was induced by *Xcc* infection, which indicated its involvement in the response to pathogen invasion. The reaction mechanism, however, is not clear and needs further investigation. The E3 ligases of the RING family are considered essential for fundamental defense mechanisms in plants [32,33]. It might be hypothesized that *RMA3* participates in the basic defense response of Citron C-05 against *Xcc*.

Citron C-05 is the first identified genotype resistant to canker disease in the *Citrus* genus [17]. Citron (*C. medica*), as one of the true citrus species [34], has a unique genetic background, differing from other citrus genotypes. All of the analyzed citrus genotypes have *RMA3* with high homology; however, they demonstrate quite different behaviors in response to *Xcc* infection. It seems that there may be other factors regulating RMA3's function. Therefore, more extensive studies are needed to clarify the functional mechanism of *RMA3* in resistance to canker disease in Citron C-05, and whether or not such a mechanism is also valid in other citrus genotypes.

5. Conclusions

Through yeast hybrid screening in the *Xcc*-induced Citron C-05 cDNA yeast library, transcription factor RMA3 was identified using the *Lob1* promoter P1 fragment as the

bait protein. The yeast one-hybrid assay and dual-luciferase tests further validated the binding of RMA3 to the *Lob1* promoter P1 fragment. The transient overexpression of *CsRMA3* in the susceptible 'Bingtang' sweet orange significantly inhibited *Xcc* growth and reduced canker symptoms, while the silencing of *CmRMA3* in the resistant Citron C-05 promoted symptom development. Furthermore, the expression analysis indicated that *RMA3* suppressed *Lob1* expression in both genotypes. Overall, these results suggest that RMA3 may participate in the resistance to citrus canker in Citron C-05 by inhibiting *Lob1* expression. Further study is necessary to clarify the mechanism of citrus' response to *Xcc* invasion. The overexpression of the RMA3 gene in susceptible citrus genotypes should be realized by genetic transformation in order to thoroughly comprehend its function in resistance to canker disease. The knock-out of the RMA3 gene in the resistant Citron C-05 using CRISPR/Cas9 would give a complete picture of RMA3 in response to invasion by the pathogen.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10070693/s1, Figure S1: Autonomous activation screening of P1 segment of *Lob1* promoter; Figure S2: Screening of *Xanthomonas citri* subsp. *citri*-induced cDNA library with PAbAi-*Lob1*-P1; Figure S3: Schematic illustration of the protein domains of RMA3 in 'Bingtang' sweet orange and Citron C-05.

Author Contributions: Conceptualization, X.M. and Z.D.; methodology and experimentation, M.Z., R.Y., Y.L., L.L. and H.S.; formal analysis, M.Z., R.Y. and Z.D.; writing—original draft preparation, M.Z. and Z.D.; writing—review and editing, Z.D.; supervision, X.M. and Z.D. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. Primers used in this study.

Primer Name	Sequence (5′-3′)
pAbAi-*CsLOB1*-P-F	GCTTGAATTCGAGCTGACCTCTGCCTACCCTATTGC
pAbAi-*CmLOB1*-P-F	GCTTGAATTCGAGCTGACCTCTGCCTACCCTATTAC
pAbAi-*LOB1*-P-R	TACAGAGCACATGCCCAAAGACAGTAAGGGATGAGG
pAbAi-*CsLOB1*-P1-F	GAAAAGCTTGAATTCGGACCTCTGCCTACCCTATTGCC
pAbAi-*CsLOB1*-P1-R	ATACAGAGCACATGCCGAACTTCGAGGTTCATTGCATTG
pAbAi-*CsLOB1*-P2-F	GAAAAGCTTGAATTCGCCAGGTTATAAGTAAACAGAACGAC
pAbAi-*CsLOB1*-P2-R	ATACAGAGCACATGCCCATCAAAGACAGTAAGGGATGAGG
pAbAi-*CmLOB1*-P1-F	GAAAAGCTTGAATTCGGACCTCTGCCTACCCTATTACC
pAbAi-*CmLOB1*-P1-R	ATACAGAGCACATGCCGAACTTCGAGGTTCATTGGATTG
pAbAi-*CmLOB1*-P2-F	GAAAAGCTTGAATTCGCCAGGTTATAAGTAAACAGCACGAC
pAbAi-*CmLOB1*-P2-R	ATACAGAGCACATGCCCAAAGACAGTAAGGGATGAGG
pGADT7-*RMA3*-F	GTACCAGATTACGCTCAATGGAACAGAACCTCTTTGAGCC
pGADT7-*RMA3*-R	ATGCCCACCCGGGTGGTCAGAACAAAAGAAGACACAGGACTAGG
35S *RMA3*-F	CACGGGGGACGAGCTCATGGAACAGAACCTCTTTGAGCC
35S *RMA*3-R	CCTTGCTCACCATGGTACCGAACAAAAGAAGACACAGGACTAGG
q*LOB1*-F	CTGCCAGAATCTCAACGAGC
q*LOB1*-R	TTGGCTAACTGAGCCTGAAGC

Table A1. *Cont.*

Primer Name	Sequence (5′-3′)
q *actin*-F	CATCCCTCAGCACCTTCC
q *actin*-R	CCAACCTTAGCACTTCTCC
q*RMA3*-F	TCAATCACAGGCACCAGCAT
q*RMA3*-R	TAGAAGACGCCAAAGCTGCA
q*NAC*-F	GATGTGGCGCAAGAGAGGTA
q*NAC*-R	TGGCCCTGTTCGATCTGTTC
q*TFGTE*-F	TTGGTGCAGCACTTACCAGA
q*TFGTE*-R	AGCCTGTGTTACTCTGAGCA
VIGS *RMA3*-F	TGAGTAAGGTTACCGAATTCGAACAGAACCTCTTTGAGCCTGAGA
VIGS *RMA3*-R	GTGAGCTCGGTACCGGATCCTTGGGCTTCTTGGAGTCGGA

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