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Expression of the *RPM1-RIN4-RPS2* complex in two citrus species with contrasting response to Huanglongbing

Eric Ángel Mendoza-Pérez, Tecnológico Nacional de México Campus Úrsulo Galván Extensión Tlapacoyan. Calle Prol. Abasolo s/n, C.P. 93650 Tlapacoyan, Ver. México; **Ricardo Santillán-Mendoza, Humberto Estrella-Maldonado, Cristian Matilde-Hernández, Felipe Roberto Flores-de la Rosa*,** Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias. Centro de Investigación Regional Golfo Centro. Campo Experimental Ixtacuaco. Carretera Federal Martínez de la Torre–Tlapacoyan, Ver. km 4.5. Congregación Rojo Gómez, Tlapacoyan, Veracruz, C.P. 93650, México; **Jacel Adame-García,** Tecnológico Nacional de México Campus Úrsulo Galván. A El Paraíso S/N, Campestre, 91667 Úrsulo Galván, Ver. México.

***Corresponding Author**:
Felipe Roberto Flores-de la Rosa
flores.felipe@inifap.gob.mx

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ABSTRACT

Objetive/Antecedents. Persian lime (*Citrus latifolia*) shows a very high level of tolerance to Huanglongbing (HLB). A recent study suggests that genes from the *RPM1-RIN4-RPS2* complex could be partly responsible for HLB tolerance in Persian lime, unlike other highly susceptible species such as orange (*C. sinensis*). The objective of this study was to compare the expression of this gene complex between orange, highly susceptible to HLB, and Persian lime, a tolerant species.

Materials and Methods. Sequences of the three genes of the complex for orange and Persian lime were obtained from databases of previously published works, alignments and primer design for gene expression were performed using various bioinformatics tools. Subsequently, tissue samples from symptomatic HLB-infected orange and Persian lime were obtained and infection was confirmed. The expression of the *RPM*1-*RIN*4-*RPS*2 genes was compared using endpoint RT-PCR.

Results. The presence of all three genes of the complex was determined in both orange and Persian lime, and it was also determined that they are highly conserved between both species. Additionally, it was observed that there is no differential expression for the RPM1 gene in symptomatic HLB tissue; however, there is a difference in the expression of the RPS2 and RIN4 genes.

Conclusion. The results suggest that the contrasting response to HLB could be associated with the activity of the interaction of the RIN4 and RPS2 genes, thus, this could be of interest for citrus genetic improvement aiming at HLB control.

Keywords: PTI, ETI, HLB Tolerance, Transcriptomics

Introduction

Globally, citrus cultivation is suffering severe damage due to the effects of Huanglongbing (HLB) on all citrus species and varieties (Ghosh *et al*., 2022). HLB is caused by three different species of the genus *Candidatus* Liberibacter (*C*L): *C*L americanus (*C*Lam), which affects cultivars in Brazil (Teixeira *et al*., 2005), *C*L africanus (*C*Laf), found on the African continent (da Graça *et al*., 2022), and *C*L asiaticus (*C*Las), the most widespread species globally (Ajene *et al*., 2020) and present in citrus-growing areas of Mexico (Huang *et al*., 2022).

Although all agriculturally important citrus species are considered susceptible to the disease, there is a gradient of susceptibility among different species, with a notable difference between sour and sweet citrus (McCollum *et al.,* 2016; Gao *et al*., 2023). The Valencia orange (*C. sinensis*), considered a sweet citrus, is one of the most important citrus fruits in Mexico, both in terms of planted area and economic impact (SIAP, 2022); however, it is also among the species most susceptible to HLB. Numerous studies using omics tools have shown that *C*Las infection causes significant metabolic, physiological, and molecular alterations in *C. sinensis* (Fu *et al*., 2016; Chin *et al*., 2020; Curtolo *et al.*, 2020; Lally *et al*., 2021; Ribeiro *et al*., 2023), leading to significant economic losses (Li *et al*., 2020). On the other hand, some sour citrus species have been reported as less susceptible or tolerant to HLB, for example, rough lemon (*C. jambhiri*) (Yu *et al*., 2017), Australian finger lime (*C. australasica*) (Weber *et al.,* 2022), and Persian lime (*C. latifolia*) (Sivager *et al*., 2021).

The case of Persian lime is of high importance for Mexico, with Veracruz being the leading producer and exporter of this fruit worldwide. Therefore, understanding the mechanisms involved in HLB tolerance is crucial. Initially, this tolerance was associated with a greater ability to maintain unaltered physiological functions such as photosynthesis, stomatal conductance, and transpiration (Sivager *et al*., 2021). Later, it was observed that, unlike in *Poncirus trifoliata* (Rawat *et al*., 2017), the *CDR* gene family is not related to tolerance (Flores-de la Rosa *et al*., 2023). Recently, the transcriptome of Persian lime infected with *C*Las was sequenced and assembled, revealing differentially expressed genes, notably those related to effector-triggered immunity (ETI) showing increased expression, such as the *RPS2* gene (Estrella-Maldonado *et al*., 2023).

The *RPS2* gene is part of a complex comprised of the *RPM1-RIN4-RPS2* genes. The relationship among these genes is intricately linked to the plant's response to infection by certain pathogens. For instance, the *RPM1* gene acts as a receptor for

effectors from the bacterium *Pseudomonas syringae* and triggers effector-triggered immunity (ETI) in *Arabidopsis* (Rose *et al*., 2012), with this activation mediated by the phosphorylation of the *RIN4* gene (Lee *et al*., 2015); that is, *RIN4* induces the expression of *RPM1*. On the other hand, the *RPS2* gene can activate immunity against the aforementioned bacterium; however, its interaction with the *RIN4* gene is negatively regulated, meaning the expression of *RPS2* inhibits *RIN4* and vice versa (Alam *et al*., 2021).

Interestingly, experimental evidence showed that overexpression of the *RIN4* gene facilitates the colonization of *C*Las in the phloem of citrus and the generation of HLB symptoms (Cheng *et al*., 2022), suggesting that susceptibility to HLB might be associated with the level of activity of the *RIN4* gene. Therefore, based on the above, the aim of the present work was to compare the expression of the genes in the *RPM1-RIN4-RPS2* complex in two species with contrasting responses to HLB: Valencia orange, highly susceptible, and Persian lime, with high tolerance to *C*Las infection.

The transcript sequences of the *ClRPM1*, *ClRIN4*, and *ClRPS2* genes were obtained from the assembled Persian lime transcriptome by Estrella-Maldonado *et al*. (2023), using local BLAST analysis with the *CsRPM1*, *CsRIN4*, and *CsPS2* gene sequences retrieved from the Citrus Genome Database (https://www.citrusgenomedb.org/). Subsequently, an alignment between the homologs of each species was performed using the online T-COFFEE software (https://tcoffee.crg.eu/apps/tcoffee/index.html). Figure 1 presents the alignment of the *CsRIN*4 and *ClRIN*4 genes.

Once the genes were identified and obtained, primers were designed to measure their expression, which was carried out using the Eurofins online software (https://eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design/). The following primers were generated: for the *RPM1* gene, the primers RPM1-F (5′-GCCCTGGATTTGCTGAAG-3′) and RPM1-R (5′-GCAATATTCAACAACTCTGGGA-3′) were obtained, expected to yield a 130 bp product; for the *RIN4* gene, the primers RIN4-F (5′-GCGAGAGGAGAGAAACAGTGCAGG-3′) and RIN4-R (5′-GACGATGATGGGGTGTGGTGGA-3′) were designed, aimed to produce a 165 bp product; finally, for the *RPS2* gene, the primers RPS2-F (5′-TGGTTCGATATGTAGTGGGG-3′) and RPS2-R (5′-CTGCTTCACTGCTGTTAGAC-3′) were obtained to yield a 135 bp product. All amplification reactions were performed at 60 °C for the annealing temperature.

To conduct the expression assays for the complex, samples of physiologically mature leaves with HLB symptoms from Valencia orange and Persian lime (Figure 1) were collected. Tissues were gathered from three different trees of each species. The collection was carried out in an experimental plot naturally infected with

Figure 1. Alignment of *CsRIN4* and *ClRIN4* genes sequences.

*C*Las, located at the INIFAP Campo Experimental Ixtacuaco (CEIXTA), situated at an elevation of 112 meters above sea level (20° 02' 36" N, 97° 05' 52.5" W). The trees were seven years old and grafted onto Swingle rootstock. Samples were immediately frozen in liquid nitrogen and transported to the CEIXTA Phytosanitary Diagnosis laboratory. The tissue was macerated and divided into two parts, one for *C*Las detection and the other for the expression assay.

For *C*Las detection, DNA extraction was performed using the protocol described by Rodríguez-Quibrera *et al*. (2022), followed by DNA integrity and quality analysis through agarose gel electrophoresis (1%, 90 V for 60 min) and nanodrop spectrophotometry, respectively. Subsequently, a portion of the protocol described by Lin *et al.* (2010) was used, involving the amplification of a fragment of the 16S ribosomal gene with the primers Las-O-F (5′-CGGTGAATGTATTAAGCTGAGGCGTTCC-3′) and Las-O-R (5′-TACCCACAACAAAATGAGATACACCAACAACTTC-3′).

For the gene expression assays of the complex under study, RNA extraction was performed using a 2% CTAB protocol (Estrella-Maldonado *et al*., 2023). The RNA obtained was analyzed for integrity and quality using horizontal agarose gel electrophoresis (1%, 90 V for 60 min) and nanodrop spectrophotometry, respectively. High-quality RNAs were treated to eliminate residual DNA using the DNase RQ1 enzyme (Promega) according to the manufacturer's instructions. cDNA synthesis was carried out starting from a concentration of 500 ng µL-1 of DNase-treated RNA

using the M-MLV enzyme (Promega), following the manufacturer's instructions. Amplification of the genes of the complex under study was performed by RT-PCR. For this amplification, the reaction mixture contained 1X PCR buffer, 25 mM $MgCl_2$, 10 mM of each dNTP, 10 µM of each primer, 1 U of DNA Taq polymerase, and 300 ng of cDNA, resulting in a final reaction volume of 10 µL. The thermal cycling profile consisted of an initial denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 15 s, with a final extension at 72 °C for 3 min. The amplification product was analyzed on a 2% agarose gel, stained with 1.2% ethidium bromide, and visualized under UV light. The *F-box* gene was used as an endogenous control (Mafra *et al*., 2012). Each sample was amplified in triplicate as a technical replicate.

Regarding the detection of the HLB causal agent in the samples, a unique fragment of 470 bp (data not shown) was obtained in symptomatic samples (Figure 2) from Persian lime (three samples) and Valencia orange (three samples), corroborating the infection of the samples with *C*Las according to the protocol used.

According to the gene expression results, RT-PCR amplification showed that there is no observable difference between the Valencia orange and Persian lime samples for the *RPM1* gene. However, for the *RPS2* gene, a difference in the intensity of the amplified band was observed, being greater in Persian lime than in orange, suggesting a difference in the activity of this gene in response to *C*Las infection, consistent with previous observations by Estrella-Maldonado *et*

Figure 2. Symptoms of HLB present in Persian lime leaves (A), Valencia orange (B), Persian lemon (C) and Valencia orange (D) fruits.

al. (2023). However, the main difference was observed in the amplification of the *RIN4* gene, which is clearly amplified in Valencia orange, while amplification of this gene is not detectable in Persian lime. This suggests a higher activity of the *RIN4* gene in a species highly susceptible to HLB, in contrast to a species with a high level of tolerance such as Persian lime, similar to the proposed role of the *RIN4* gene in *C*Las infection (Cheng *et al*., 2022). The results are shown in Figure 3.

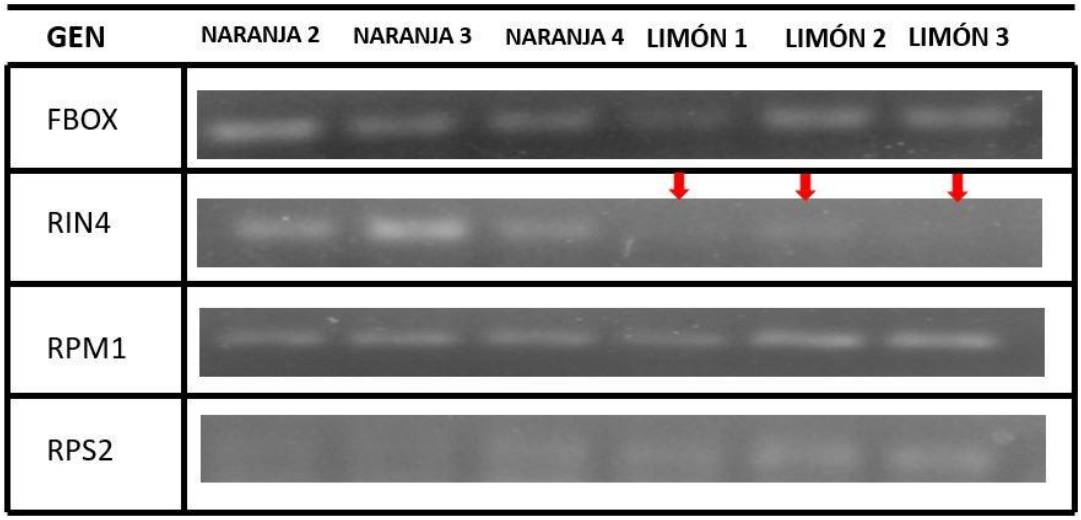

Figure 3. Expression of the *RPM1-RIN4-RPS2* complex by RT-PCR in Valencia orange, a species highly susceptible to HLB, and Persian lime, a species with a high level of tolerance to HLB.

It has recently been described that Huanglongbing (HLB) is more closely related to an exaggerated and uncontrolled defense response to *C*Las than to the damage caused directly by the pathogen itself (Ma *et al*., 2022). Therefore, understanding the mechanisms altered in plant defense is crucial for designing strategies to control this disease. The findings of this study provide evidence supporting the hypothesis that *C*Las infection and HLB development are facilitated by the expression of the *RIN4* gene. This phenomenon has been observed in other pathosystems, where *RIN4* acts as a repressor of Pattern-Triggered Immunity (PTI) (Ray *et al*., 2019), particularly by inactivating the response through *RPS2* (Belkhadir *et al*., 2004), while its phosphorylation induces Effector-Triggered Immunity (ETI) activation (Xu *et al*., 2017). It is significant that overexpression of *RIN4* has been shown to inhibit callose deposition in response to pathogens (Afzal *et al*., 2011; Ray *et al*., 2019), such inhibition of callose production being one of the mechanisms *C*Las

uses to translocate within the phloem (Bernardini *et al*., 2022), whereas the increase in callose in areas with low CLas presence is more due to the plant's uncontrolled response (Archor *et al*., 2020; Ma *et al*., 2022).

This study presents evidence of differential expression of the *RIN4* gene among citrus naturally infected with *C*Las (six samples analyzed), according to their susceptibility level to HLB. Further studies are required to confirm this hypothesis; however, our results suggest that the *RIN4* gene is an important molecular target for inducing HLB tolerance using biotechnological tools (Sun *et al*., 2019).

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