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(RESEARCH ARTICLE)

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Identification and analysis of the differential expression of the NPR family in Persian lemon with HLB

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Abstract

The NPR1 gene (No expresor de PR Genes1) and its homologues are regulators of Systemic Adquired Resistance (SAR), genes through their interaction with transcription factors allows the expression of defense genes or proteins related to pathogenesis (PR) and thus the improvement of resistance and are also considered receptors of salicylic acid. To date there are no records on its identification and analysis in *Citrus latifolia*. The present work aimed to identify the presence of the NPR family in the HLB-infected *C. latifolia* transcriptome using Arabidosis thaliana and Citrus sinensis reference genes. When obtaining the *C. latifolia* transcriptome, bioinformatics programs (Uniprot, Phytozome, BLAST, Linux, BioEdit and TnT) were used to identify NPR genes expressed in *C. latifolia*. As a result, a total of 5 genes were found: 2 NPR1 genes, 1 NPR3, and 2 putative genes: NPR1 and NPR4, expressed positively and negatively without statistical significance. The identified genes proved to belong to the same subfamilies of *C. sinensis* sharing similar protein domain composition patterns, so it can be said that these homologs could be effective against the CLas pathogen, since they induce a defense response. These findings present the bases as a management measure to combat HLB.

Keywords: Systemic Acquired Resistance; Citrus greening; Tolerance; HLB

1. Introduction

Currently, citrus growing plays a role of great economic and social importance for Mexico, its production, processing and industrialization is an important source of employment in the rural sector. However, they are seriously threatened by the Huanglongbing (HLB) disease [1], also known as citrus greening, caused by the pathogenic bacterium Candidatus Liberibacter asiaticus (CLas) and is the most destructive disease of citrus, severely infected trees experience reduced yield and fruit quality, ultimately leading to plant decline and death [2]. To date, there is no definitive cure for HLB, however, antibiotic and antimicrobial treatments as well as injections of plant defense activators in the stem and plant defense inducers have been included to control the disease [3]. The premise behind these studies is that induced resistance, either locally or systemically disseminated through trees, could confer long-lasting protection against HLB by activating salicylic acid (SA) signaling or Systemic Resistance Acquired (SAR) pathways. SA, which induces the synthesis, mediates resistance and activation of the NPR1 gene [4], this gene regulates the defense reaction of the plant by detecting the signal molecule SAR and SA to induce the expression of genes related to pathogenicity (PR), that is, they create a defense mechanism that confers protection against pathogenic microorganisms. Several studies have shown that "NPR" works as a positive regulator of disease resistance in *Arabidopsis thaliana, Citrus sinensis*, *Cocos nucifera* L., bread wheat, among others [5]. That is why the objective of this work is to identify if the NPR family is present in *C.*

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latifolia infected with the Huanglongbing disease (HLB), thus allowing us to understand the defense mechanisms of the plant against the disease.

2. Material and methods

2.1. Persian Lemon Transcriptome

The transcriptome sequenced and assembled by Estrella-Maldonado et al. (in preparation), which comes from six genomic libraries obtained from Persian lemon trees (three infected with HLB and three without detectable infection) of five years of age.

2.2. NPR identification in database

NPRS 1,2,3 and 4 sequences were sought from Arabidopsis thaliana and *C. sinensis* in three different programs: UniProtKB (https://www.uniprot.org/uniprot/q9hyn5), Phytozome (https: //phytozome-next.jgi .Doe.gov/), and BLAST (https://blast.ncbi.nlm.nih.gov/blast.cgi),), the latter was used to corroborate results using the NPRs found in UniProtKB, by Blast to obtain sequences of good quality. Subsequently, verified sequences were selected and in case of not being presented, they will present a high structural and functional score. The NPRs obtained, were placed in a file and ordered by species and groups of NPR1, 2, 3 and 4, with the purpose of a better visualization and understanding.

2.3. In silico identification of NPRs genes

The sequences obtained from *A. thaliana* and *C. sinensis* were placed in a single file that was contrasted against the transcriptome of *C. latifolia* to obtain the transcripts of *C. latifolia* by tBLASTn in the Linux operating system. With the results obtained in the database of the *C. latifolia* transcriptome, each transcript NPR1,2,3 and 4 was sought and identified by the parameters of their codes and were placed in a file.

2.4. NPRS transcript translation

The translation of nucleotide sequences of the recovered transcripts to amino acid sequences via MEGA X.

2.5. Alignment and phylogenetic analysis

The sequences of *A. thaliana*, *C. sinensis* and *C. latifolia* were cleaned in a single file, where the species name and NPR grade were placed. Subsequently, the sequences were aligned and edited in Clustal W in Bioedit software. The results were entered into the TNT program for phylogenetic analysis using a maximum parsimony approach, using the default parameters. The results were saved in Nex to visualize the maps in the Figtree program for better study.

2.6. Differential gene expression

Using the results of the phylogeny, we searched for homologs of *C. latiflolia* with *A. thaliana* and *C. sinensis*. The data obtained were placed in a new file to search and analyze their differential expression in the DESeq2 program, through the Idamex platform of the massive sequencing and bioinformatics unit (UUSMB). Adjusted p-values and Log2 FoldChange with parameters of 0.05 were taken as reference to evaluate the differential expression of *C. latifolia*.

2.7. 3D modeling of npr found in *C. LATIFOLIA*

3D modeling of the protein sequences obtained from the three species was performed using the online program Swill-Model https://swissmodel.expasy.org/interactive#sequence. Subsequently, protein structures were compared to identify the degree of NPR and possible differences and similarities between them.

3. Results and Discussion

3.1. Identification of transcripts

For NPR1, 2, 3 and 4 transcripts from *A. thaliana* and *C. sinensis*, 6 and 15 sequences were obtained, respectively, verified and under quality standards (data not shown).

3.2. Phylogenetic analysis

The phylogenetic analysis consisted of 5 sequences from *C. latifolia*, 6 from *A. thaliana* and 15 from *C. sinensis* with the purpose of identifying the NRP group to which they belonged. The 21 sequences belonging to the genus *Citrus* were

grouped into 4 main clades, distributed according to their homology. This analysis showed that *C. sinensis* and *C. latifolia* share a similarity percentage of more than 50%, suggesting that these homologous NPRs could be candidates for use against bacterial pathogens. In this regard, Us Camas (2013) points out that NPR1 is critical for the expression of PR genes, restricting pathogen growth at the site of infection. In this way, the overexpression of the NPRs of *C.latifolia* could be related to higher levels of resistance against the pathogen CLas. Phylogenetic analysis identified, by homology, sequences belonging to the NPR groups in *C. sinensis*: 2 in NPR1, 1 in NPR3, 1 putative NPR1 and 1 putative NPR4 (Figure 1).

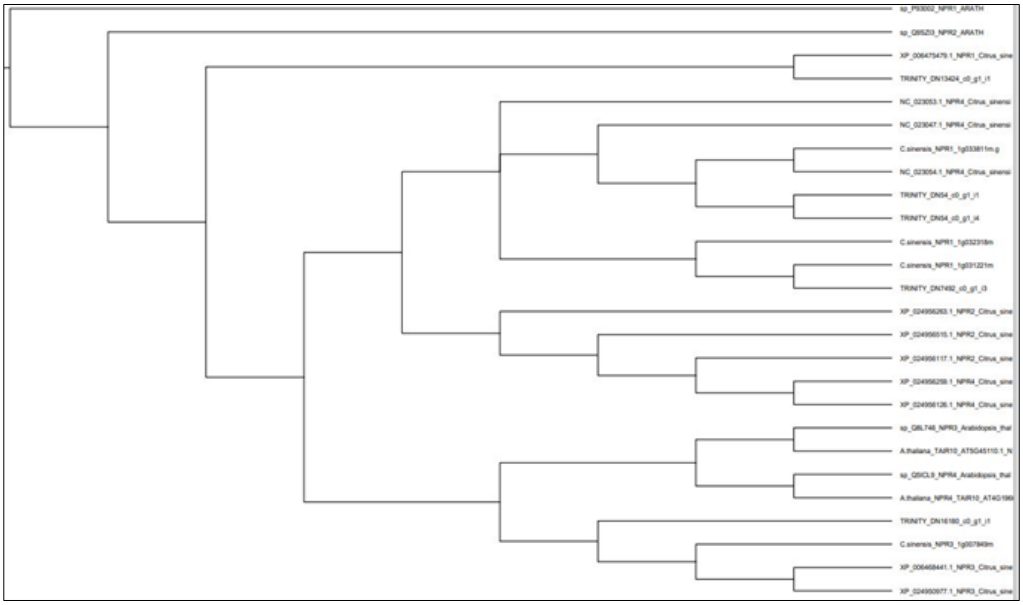

Figure 1 Phylogenetic tree obtained from the Figtree program (2022), of three different species (C. sinensis, A. thaliana and C. latifolia)

3.3. Differential expression

Differential expression analyses showed two genes belonging to *C. latifolia* negatively expressed (putative NPR1 and NPR1,4) and two genes positively expressed (NPR1 and NPR3) during infection with CLas (Figure 2).

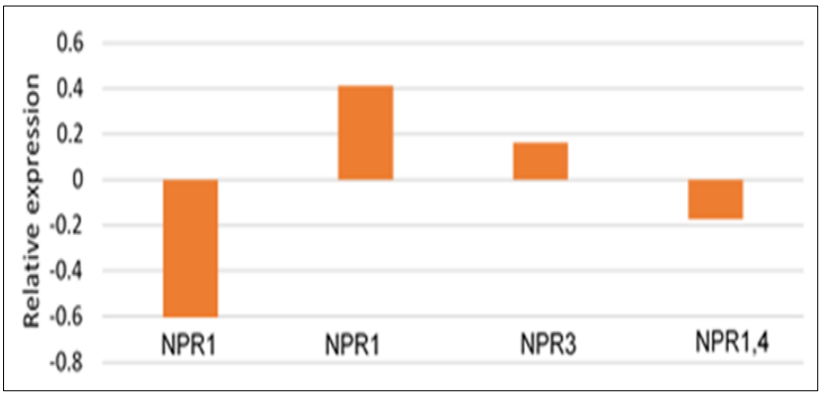

Figure 2 Differential expression of NPR genes of *C. latifolia* homologous to *C. cinensis*

However, the pvalue and padj values were different from 0.05, indicating that the results were not statistically significant (Table 1).

3.4. Protein modeling

The *C. latifolia* NPRs and their homologues identified in the phylogeny were modeled in 3D to corroborate their similarity (Figure 3).

Table 1 Relative expression values of the NPR genes in the Persian lemon transcriptome

Transcript	baseMean	log2FoldChange	lfcSE	stat	Pvalue	Padj
TRINITY_DN7492_c0_g1	340.5969216	-0.60452006	0.641795287	-0.94192037	0.346233408	0.999901738
TRINITY_DN13424_c0_g1	25.98782888	0.411180828	0.571909176	0.718961761	0.472164481	0.999901738
TRINITY_DN16180_c0_g1	116.6175921	0.161418235	0.519010881	0.311011274	0.755792051	0.999901738
TRINITY_DN54_c0_g1	639.8260885	-0.17206061	0.270227894	-0.63672408	0.524304585	0.999901738

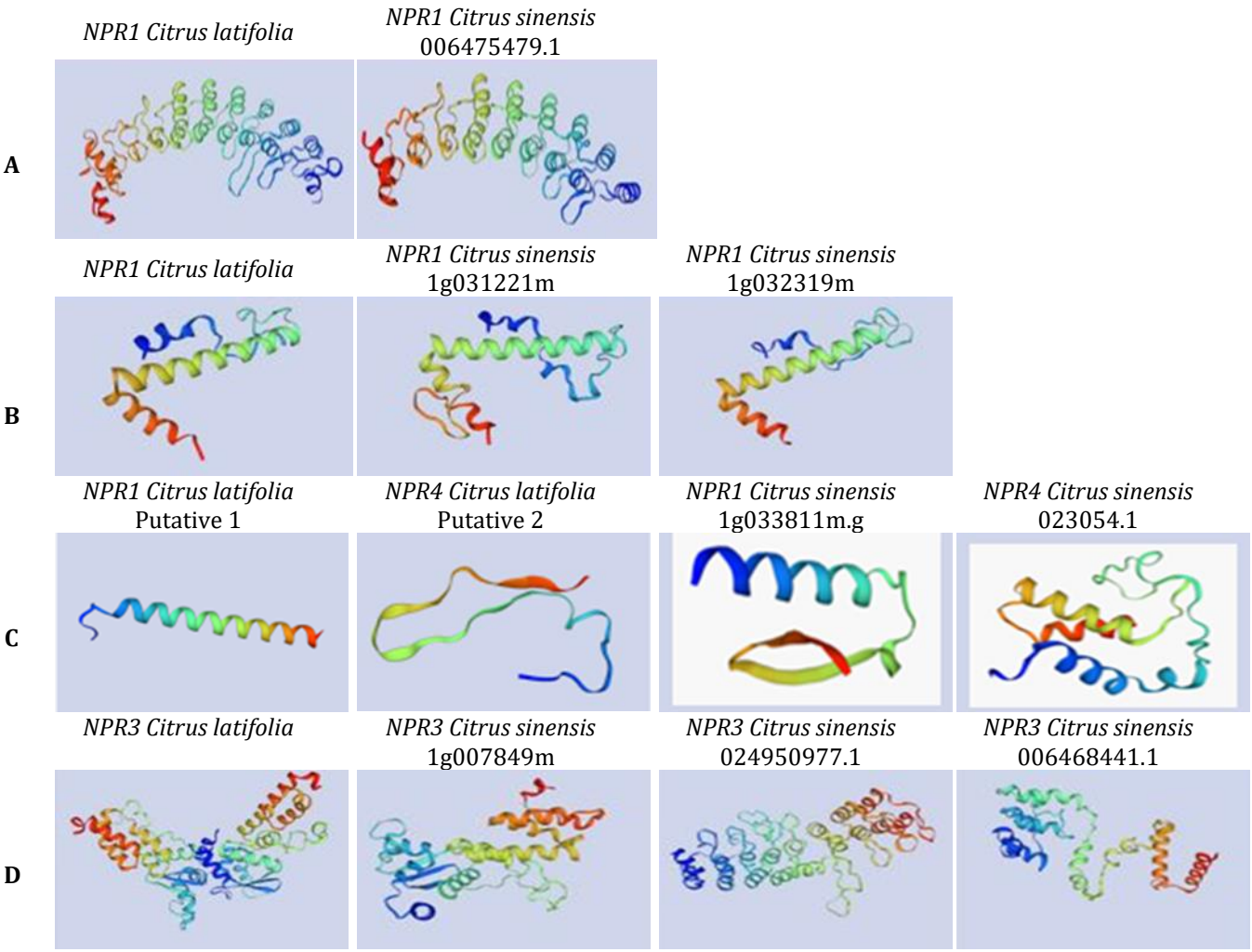

Figure 3 Protein modeling using Swiss-Model. A) Modeling of the ClNPR1 protein and comparison with CsNPR1, B) Modeling of an alternative *C. latifolia* NPR1 gene, C) Modeling of hypothetical NPR proteins and D) Modeling of the ClNPR3 protein and comparison with CsNPR3

The NPR1 proteins from *C. latifolia* and *C. sinensis* showed 90% similarity in their structures, confirming that they belong to NPR1 (Figure 3A and 3B). On the other hand, the proteins identified as NPR3 presented 80% similarity between them (Figure 3D). Finally, the putative structure 1 of C. latifolia is apparently homologous to NPR1 of C. sinensis with a similarity percentage of 90%, while the putative 2 presented a 74% similarity to NPR4 (Figure 3C).

4. Conclusion

The NPR family is present in the HLB-infected *C. latifolia* transcriptome, identifying the NPR1, NPR3 and NPR4 genes by homology with *C. sinensis.* The NPR genes are expressed both positively and negatively, although without statistical significance. Most of these identified genes share more than 50% similarity, thus considering them NPRs. Thus, it is

necessary to continue to explore the signaling pathways of these genes in the context of resistance, which could open up new alternatives to combat the citrus greening pathogen *Candidatus liberibacter asiaticus*.

Compliance with ethical standards

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Disclosure of conflict of interest

The author declares do not have conflict of interest.

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