Identification of salicylic acid binding proteins in the transcriptome of *Citrus latifolia* infected with *Candidatus* Liberibacter asiaticus

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Received: June 02, 2022. **Accepted**: November 05, 2022.

Santillán-Mendoza R, Rodríguez-Peralta ED, Estrella-Maldonado HJ, Rodríguez-Quibrera CG, Matilde-Hernández C, Adame-García J, González-Oviedo NA and Flores-de la Rosa FR. 2022. Identification of salicylic acid binding proteins in the *Candidatus* Liberibacter asiaticus infected *Citrus latifolia* transcriptome. Mexican Journal of Phytopathology 40(4).

DOI: https://doi.org/10.18781/R.MEX.FIT.2022-1

Abstract. Persian lime (PL) is one of the most economically important citrus crops in the state of Veracruz, Mexico. However, it is affected by the presence of Huanglongbing (HLB) disease, caused by *Candidatus* Liberibacter asiaticus (*C*Las), an obligate biotrophic pathogen. Overall, PL shows a certain level of tolerance to HLB. Therefore, it is important to study the defense response mediated by salicylic acid (SA) against biotrophic pathogens in PL. Some genes with the capacity to participate in the SA response pathway, known as *NtSABP*, have been identified in *Nicotiana tabacum*, but the presence and activity of these genes in PL in response to HLB are unknown. The objective of this study was to identify homologues of SABP-like proteins in the PL transcriptome and to determine their differential expression level during *C*Las infection. SABP protein sequences from five different species, including *N. tabacum*, were used as model sequences in a tBLASTn search. A 3D model of the SABP protein was constructed and compared between *N. tabacum* and *C. latifolia*. We identified the direct homologous to each *NtSABP* gene in the PL using tBLASTn analysis, phylogenetic reconstruction, and tridimensional structure. Interestingly, the *ClSABP1, ClSABP2,* and *ClSABP3* genes showed repression in *C*Las infected plants. There is at least one homologous to each *NtSABP* gene in PL. During *C*Las infection, these genes are somewhat suppressed.

Keywords: Persian lime*,* Huanglongbing, Molecular response.

The Persian lime (*Citrus latifolia*) is one of the most economically important citrus fruits in Mexico, especially for the state of Veracruz, which is the main international exporter of this fruit (Fernández-Lambert *et al.,* 2015). However, in recent years, the presence of the bacterium *Candidatus* Liberibacter asiaticus (*C*Las) has been reported as the causative agent of Huanglongbing (HLB) in the main Persian lime-producing areas in Veracruz (Rodríguez-Quibrera and Mendoza-Herrera, 2014; Rodríguez-Quibrera *et al.,* 2019). This is of great importance because this disease is considered the most destructive for the citrus industry worldwide (Mora-Aguilera *et al.,* 2014).

It has been shown that different citrus species have different responses to *C*Las infection. For example, acid citrus species, such as the Mexican lemon (*Citrus aurantifolia*), have a higher level of tolerance to HLB compared to citrus species that are considered sweet, such as orange, mandarin or grapefruit (McCollum *et al.,* 2016; Alves *et al.,* 2021). Empirical evidence suggests that the level of tolerance of the Persian lemon to *C*Las infection is also higher than in sweet citrus species. However, the infection still has some physiological effects such as starch accumulation and low total chlorophyll concentration (Flores-de la Rosa *et al.,* 2021). This is why it is very important to understand the molecular response of the Persian lemon to infection.

The main defense pathway of plants against biotrophic organisms, such as *C*Las, is mediated by salicylic acid (SA) (Gao *et al.,* 2015). This pathway activates numerous molecular responses in the plant, most of them as a result of the overexpression of the *NPR1* gene (Nonexpressor of Pathogenesis-related protein 1) (Backer *et al.,* 2019). Salicylic acid interacts directly with NPR1 by modifying the redox potential of the cytoplasm, leading to NPR1 monomerization and subsequent entry into the cell nucleus (Zhang *et al.,* 2019). It also binds directly to NPR1 (Wu *et al.,* 2012). In the case of *C*Las infection in citrus, it has been determined that SA signaling is interrupted by this pathogen due to the presence of a functional salicylate hydroxylase enzyme that limits SA mobilization by metabolizing it and suppressing plant defenses (Li *et al.,* 2017). It has also been observed that the exogenous application of high concentrations of SA in citrus trees affected by HLB helps the plant to sustain its defense response and the expression of antioxidant genes during infection (Flores-de la Rosa *et al.,* 2021; Li *et al.,* 2021). Furthermore, the insertion and overexpression of the *AtNPR1* gene in susceptible citrus plants generates a higher degree of tolerance to HLB compared to wild genotypes (Dutt *et al.,* 2015; Qiu *et al.,* 2020), which suggests the pivotal role of SA in the tolerance response to HLB.

Despite its important role in the biotic stress response, *ΔNPR1* mutant plants still exhibit SA-mediated defense responses, which indicates that *NPR1* and its paralogs are not the only genes involved in SA binding (Backer *et al.,* 2019; Pokotylo *et al.,* 2019). In tobacco (*Nicotiana tabacum*), at least three SA binding proteins (SABPs) have been identified, purified and characterized, with different levels of affinity for SA and with activity during the response to biotrophic pathogens (Pokotylo *et al.,* 2019). Likewise, homologs to the *NtSABP1*, *NtSABP2* and *NtSABP3* genes have been identified in other plants such as *Arabidopsis thaliana* (Manohar *et al.,* 2015; Pokotylo *et al.,* 2019). However, to date there are no reports of homologous genes in Persian lime, nor of their involvement during the infection with *C*Las. Therefore, the present work aimed to identify the presence of salicylic acid-binding protein (SABP) homologues in the Persian lime transcriptome and to determine their relative expression during *C*Las infection.

MATERIALS AND METHODS

Obtaining biological material. Generation and sequencing of the transcriptome. The material used in the present study was collected from a five-year-old commercial Persian lime plantation naturally infected with *C*Las. The scale of Ribeiro et al. (2021) was used to identify leaves at stage of physiological maturity V6 with the characteristic symptoms of HLB. Persian lime trees with no apparent symptoms of the disease were also identified. The samples were collected in the field, immediately stored in liquid nitrogen and subsequently macerated, after which the leaves were divided for DNA and RNA extraction. To confirm whether the tree samples had the pathogen, DNA was extracted using the CTAB method (Rodríguez-Quibrera *et al.,* 2019) and the detection of *C*Las was carried out using the nested PCR protocol described by Lin et al. (2010). In addition, the genetic homogeneity of the pathogen was confirmed at five microsatellite loci (data not shown). The description of the sequencing, assembly and general analysis of the transcriptome are summarized in Estrella-Maldonado *et al.*, (in preparation).

Isolation of sequences of the *SABP1*, *SABP2* and *SABP3* genes from the reference species. Using the sequences of the SABP1, SABP2 and SABP3 proteins from *Nicotiana tabacum* as a model, a tBLASTn analysis was performed against the protein databases (NCBI database) of *Arabidopsis thaliana*, *Marchantia polymorpha*, and *Citrus sinensis*. With the amino acid sequences of the four species, a file was constructed for each gene and a tBLASTn analysis was performed against the assembled Persian lime transcriptome. All the transcript sequences for each protein were identified in the transcriptome and used for further analyses. The percentage of similarity between each SABP protein sequence and the *N. tabacum* sequences was determined using MEGA7 software (http://www.megasoftware.net/) (Kumar *et al*., 2016).

Alignment, phylogeny and three-dimensional structure of Persian lime SABP proteins. Once the transcripts of the assembled transcriptome were recovered, the search for the open reading frames (ORFs) was performed. The largest available was translated into proteins using the tools available on the NCBI server. A sequence alignment was made for each protein of the five species using the ClustalW algorithm with the default parameters. Subsequently, a phylogenetic reconstruction was carried out using Maximum Parsimony (1000 bootstrap). Both the alignment and the phylogenetic analysis were performed with the MEGA7 software (http://www.megasoftware.net/) (Kumar *et al.,* 2016). The amino acid sequence of each SABP protein from *N. tabacum* and *C. latifolia* was used to build a 3D model of the protein structure. This was done with the Phyre2 software (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The result was visualized on the EzMol server (http://www.sbg.bio.ic.ac.uk/ezmol/).

RESULTS

Sequence isolation and SABP phylogeny. Among the sequences of the SABP proteins of *N. tabacum*, different homologous sequences of the species *M. polymorpha*, *A. thaliana* and *C. sinensis* were identified. With these sequences, a tBLASTn was performed against the assembled Persian lime transcriptome, from which transcripts potentially homologous to the NtSABP genes were identified. Table 1 summarizes the sequences used from each gene from each species, as well as the transcripts recovered from the Persian lime transcriptome.

Table 1. Identity percentage among SABP amino acids sequences from *N. tabacum* and identified homologous sequences in five different species. The sequence from *Citrus latifolia* with the higher identity percentage with *N. tabacum* sequences is marked with an asterisk.

Especies		*N. tabacum*		*N. tabacum*		*N. tabacum*
	SABP1 homólogos	**SABP1 Catalasa**	**SABP2 homólogos**	**SABP2 MeSA esterase**	**SABP3 Homólogos**	**SABP3 β-Carbonic anhydrase**
Arabidopsis thaliana	Q96528 Catalasa 1	77.73	Q8S8S9 MeSA1	56.75	P27140 β Carbonic anhydrase 1	73.75
	P25819 Catalasa 2	78.36	O80476 MeSA2	52.51	P42737 β Carbonic anhydrase 2	64.85
	Q42547 Catalasa 3	76.47	O80474 MeSA4	51.15		
			O80472 MeSA7	48.63		
			O23171 MeSA9	46.48		
Marchantia polymorpha	OAE31579.1	74.16	OAE21631.1	33.98	OAE34792.1	45.61
	OAE35247.1	69.95	PTQ33879.1	33.06	PTQ38106.1	44.32
	PTQ30274.1	69.95				
	PTQ30353.1	74.16				
Citrus sinensis	KDO85095.1	75.94	KDO79350.1	57.91	KDO66162.1	76.97
	KDO85096.1	78.99	KDO79351.1	58.68	KDO66164.1	77.12
	XP_006473789.1	77.10	KDO79352.1	58.68	KDO66166.1	76.97
	XP_006473790.1	77.10	NP_001275858.1	58.68	KDO66167.1	76.97
	XP_006473792.1	78.78	XP_006466659.1	58.30	KDO66170.1	76.89
	XP_006473794.1	78.78	XP_006466663.1	59.07	XP_006470326.1	76.97
			XP_006485914.1	56.92		
			XP_024957411.1	55.76		
Citrus latifolia	DN42 c0 g2 i4	77.31*	DN10091 c0 g2 i1	59.07*	DN502 c0 g1 i2	63.92
	DN42 c0 g2 i3	76.21	DN1788 c0 g1 i8	9.31	DN3559 c0 g1 i1	76.02*
			DN1788 c0 g1 i1	9.69		
			DN1788 c0 g1 i9	9.69		

The homology relationship between the Persian lime SABP gene transcripts and those identified in the other species under study was determined based on the phylogenetic reconstruction. Regarding the *NtSABP1* gene, it was determined that the DN42_c0_g2_i4 transcript is the corresponding homologue, for which it was named *ClSABP1* (Figure 1). With respect to the *NtSABP2* gene, it turned out that the DN10091_c0_g2_i1 transcript is the direct homologue, for which it was named *ClSABP2* (Figure 2). Regarding the *NtSABP3* gene, it was concluded that the homologue is the DN3559_c0_g1_i1 transcript, now named *ClSABP3* (Figure 3). The transcripts sequences were deposited in the NCBI Genbank with the following accession numbers: *ClSABP1* (OM719611), *ClSABP2* (OM719612), and *ClSABP3* (OM719613).

Differential expression of the *ClSABP1*, *ClSABP2* and *ClSABP3* genes in the transcriptome of *C*Las-infected Persian lime. The analysis of DESeq2 expression showed that the *SABP1*, *SABP2*

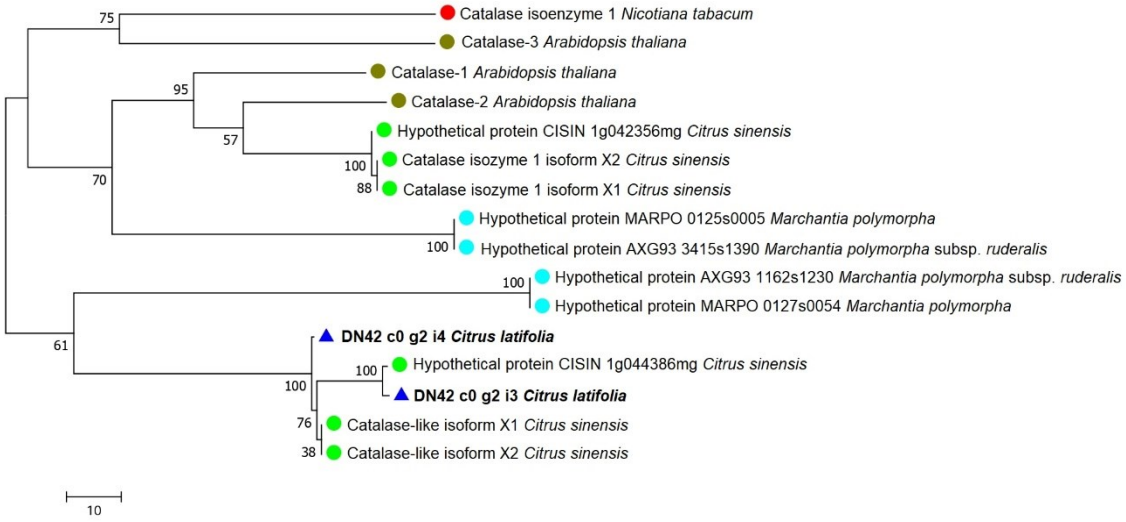


Figure 1. Maximum Parsimony phylogeny (1000 bootstraps) from tBLASTn sequences recovered homologous to the SABP1 gene. The sequences of the transcripts recovered from the Persian lime transcriptome are highlighted in bold.

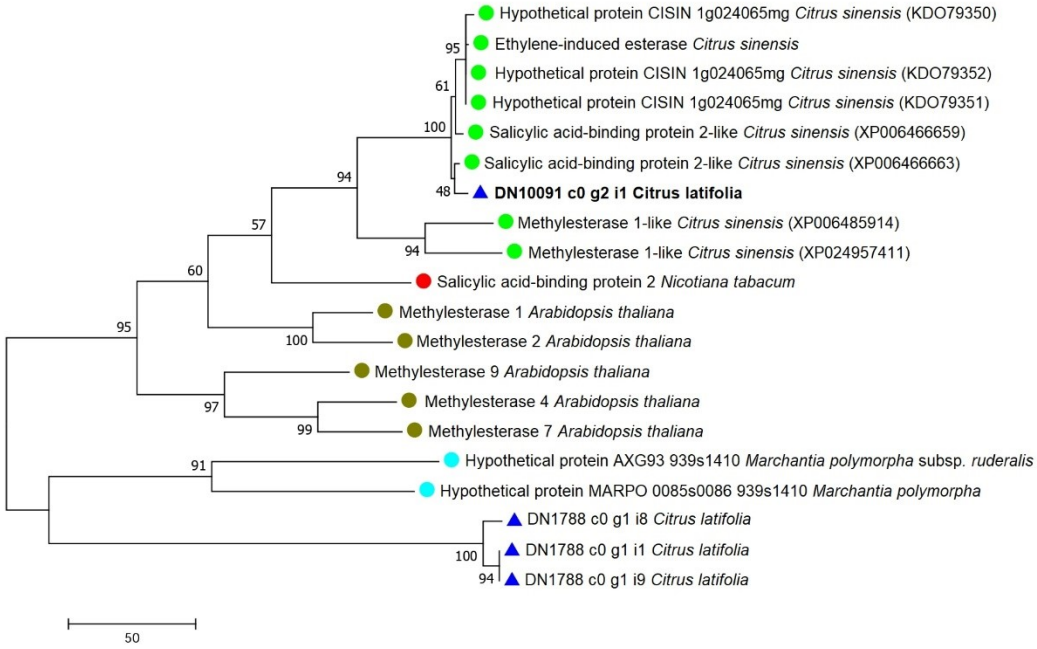


Figure 2. Maximum Parsimony phylogeny (1000 bootstraps) from tBLASTn sequences homologous to the *SABP2* gene. The sequences of the transcripts recovered from the Persian lime transcriptome are highlighted in bold.

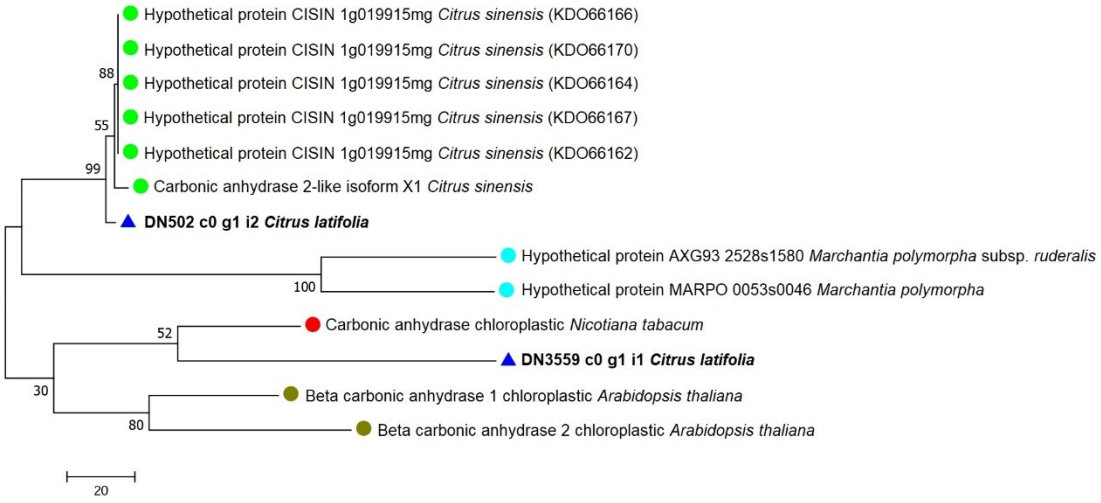


Figure 3. Maximum Parsimony phylogeny (1000 bootstraps) from tBLASTn sequences homologous to the *SABP3* gene. The sequences of the transcripts recovered from the Persian lime transcriptome are highlighted in bold.

and *SABP3* genes repress their relative expression when plants are infected by *C*Las. However, none of the three genes showed a statistically significant difference (Figure 4). Of the three genes identified, *ClSABP1* and *ClSABP2* caused greater repression, with fold change (log2) values higher than -0.5. *ClSABP3* induced lower repression, with a fold change value close to -0.3.

Three-dimensional structure analysis of ClSABP proteins. The 3D model of each SABP protein of *N. tabacum* and *C. latifolia* was reconstructed based on the sequence of translated amino acids. Figure 5

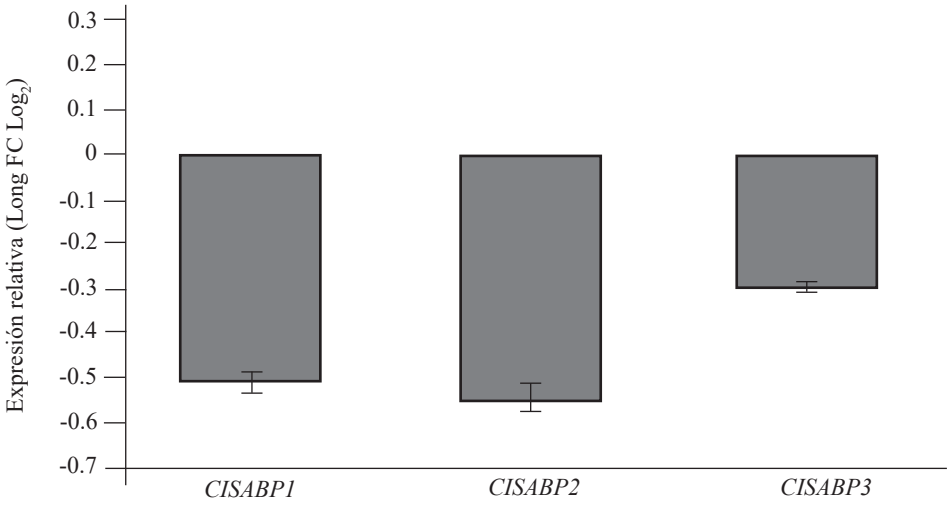


Figure 4. Relative expression of the *ClSABP1*, *ClSABP2* y *ClSABP3* genes in *C*Las-infected Persian lime plants compared with a healthy plant.

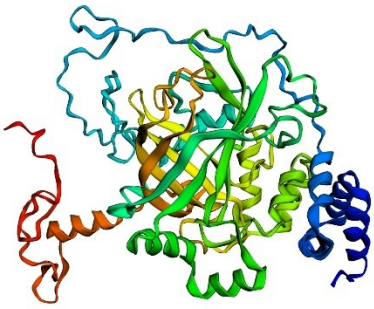
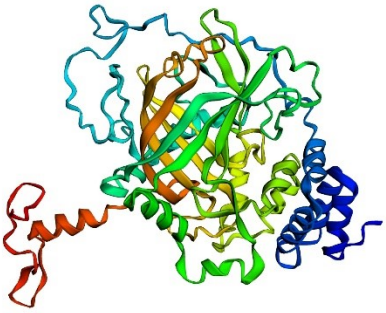
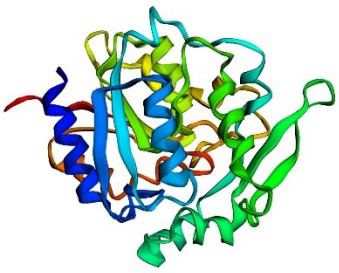
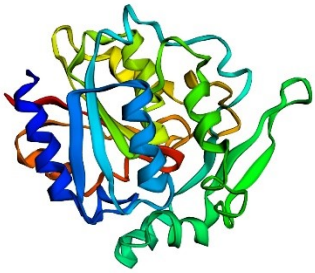
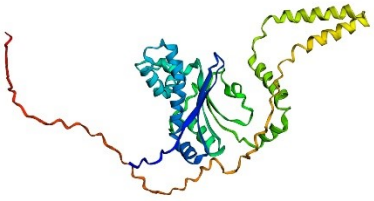
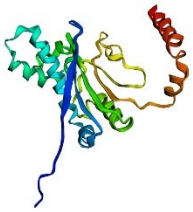
Proteína	*Nicotiana tabacum*	*Citrus latifolia*
SABP1		
SABP2		
SABP3		

Figure 5. Structural comparison of the 3D models of SABP proteins identified in the Persian lime transcriptome compared to those reported in *N. tabacum*.

summarizes the results. It can be clearly observed that there is a high structural similarity between the SABP1 and SABP2 proteins from both species, which supports the hypothesis that their encoding genes are homologous. However, in the case of the SABP3 protein, the structural similarity is lower, although most of the regions are conserved between both species. The ClSABP1 protein sequence was modeled with 100% confidence as a catalase based on 457 residues of it. Thirty-eight percent of the sequence formed α-helices and 22% formed β-sheet. The ClSABP2 sequence was modeled with 100% confidence as part of the hydrolase superfamily based on 258 residues, with 42% α-helices and 11% β-lamellae. Finally, the ClSABP3 sequence was modeled with 100% confidence based on 210 residues and associated with the superfamily of carbonic β-anhydrases, with 42% α-helices and 20% β-lamellae. Figure 5 shows the 3D models of the three protein sequences under study.

DISCUSSION

Due to the sessile nature of plants, their response mechanisms to different sources of stress are very complex and are only beginning to be fully understood (Zhang *et al.,* 2022). A special case of these response mechanisms is the defense against attack by disease-causing organisms, since plants do not have specialized immune systems like animals (Jones and Dangl, 2006). The SA-mediated pathogen response pathway has been shown to be the most important response mechanism to infection by biotrophic organisms (Yang *et al.,* 2015). However, recent studies have revealed that this pathway, which activates defenses through SA, is much more complex than previously thought (Huang *et al.,* 2020), involving proteins with a possible direct interaction with SA, such as SABP proteins (Pokotylo *et al.,* 2019).

SABP proteins have been identified in model species such as *N. tabacum* and *A. thaliana* (Pokotylo *et al.,* 2019). However, little is known regarding homologous genes encoding these proteins in species of agricultural importance, such as citrus plants. In the present research work, homologs to the genes NtSABP1, NtSABP2 and NtSABP3 were identified in the transcriptome of Persian lime plants infected with *C*Las. The results suggest that there is at least one direct homologue to each gene present in this fruit tree.

The phylogenetic analysis and the comparison of 3D structures suggest that the gene named in this work as *ClSABP1* has a high homology with *NtSABP1*. This gene has been characterized as a catalase 2 (CAT2) in *A. thaliana* (Chen *et al.,* 1993; Klessin *et al.,* 2018). It is related to the defense response. As the concentration of SA becomes higher, CAT2 activity is inhibited, then H_2O_2 levels increase and jasmonic acid synthesis and auxin accumulation stop. This, in turn, increases the defense response against biotrophic pathogens, while increasing susceptibility to necrotrophs (Yuan *et al.,* 2017). The results of the present study show that the relative expression of the *ClSABP1* gene was repressed (not significantly) in Persian lime plants infected with *C*Las. This could be explained by the ability of *C*Las to metabolize SA (Li *et al.,* 2017), preventing SA to reach the level necessary to amplify the defense response of the plant using the CAT2 pathway, as has been observed in *A. thaliana* (Yuan *et al.,* 2017). However, it has been found that during *C*Las infection, H_2O_2 levels increase and catalase activity is slightly repressed in citrus plants (Pitino *et al.,* 2017). Therefore, it is not possible to determine if the repression of the CAT2 gene (*ClSABP1*) is explained by the suppressive effect of *C*Las or by the response of the plant mediated by SA. However, the *ClSABP1* gene might play a key role in Persian lime plants infected with *C*Las.

A high structural similarity was observed between the SABP2 proteins of *N. tabacum* and *C. latifolia*. This protein has been characterized as a methyl-salicylate esterase associated with plant defense, since it converts methyl salicylate into SA (Forouhar *et al.,* 2005). A recent study showed that the overexpression of a gene encoding a carboxyl transferase in *C. sinensis* led to a greater accumulation of SA and therefore to the inhibition of the symptoms generated by *C*Las (Zou *et al.,* 2021). In the present research work, the *ClSABP2* gene was identified as a direct homologue to the *NtSABP2* gene. It can thus be inferred, based on functional homology analysis, that *ClSABP2* is a priority gene for the increase of SA in Persian lime plants. However, the repression of the relative expression of the *ClSABP2* gene suggests that *C*Las infection inhibits the defense response pathway not only by metabolizing SA, but also by suppressing its biosynthesis through the repression of genes such as *ClSABP2*. For this reason, further studies

are needed to determine the effect of the expression of the *ClSABP2* gene on the defense mechanisms of Persian lime plants against infection by *C*Las.

Regarding *ClSABP3*, the structural analysis showed highly conserved regions in the folds of the protein, characteristic of an homologous gene, although the protein sequence encoded by the *ClSABP3* gene does not present a C-terminal amino acid chain in *NtSABP3* (Figure 3). This protein has been described as a carbonic anhydrase located in the chloroplast, with antioxidant activity and important participation in the hypersensitive response (Slaymaker *et al.,* 2002). It has been confirmed that this protein accumulates to a lesser extent in *C. paradisi* as a result of *C*Las infection, which could be related to a deficiency in photosynthetic capacity, since this protein actively participates in CO_2 fixation (Nwugo *et al.,* 2013). It has also been observed that this gene is repressed in citrus plants susceptible to HLB during infection (Mafra *et al.,* 2013), which is interesting since the results of the present work show that the *ClSABP3* gene, although it is slightly repressed, is not significantly repressed compared to a healthy plant, which could be related to the greater tolerance of the Persian lime to *C*Las infection. Moreover, it is important to highlight that the *CLIBASIA05315* gene, which is present in the pathogen genome, contains a conserved domain related to carbonic anhydrases (Flores-de la Rosa *et al.,* 2020) that produces a protein that binds to chloroplasts in infected *N. benthamiana*, causing cell death (Pitino *et al.,* 2016). Therefore, we hypothesize that the similarity of the *SABP3* sequences between *N. tabacum* and *C. latifolia* could be associated with the similarity of the observable symptoms in these two species when infected with *C*Las.

The analysis shown in the present work lays the foundations for further study of the role of *ClSABP* genes in the SA-mediated defense response of Persian lime against HLB. The relative expression of these genes is repressed during infection with *C*Las. Thus, a functional analysis of them could help to understand why Persian lime plants show a more tolerant response to HLB compared to sweet citrus plants.

CONCLUSIONS

The transcriptome of healthy and infected Persian lime plants infected with *Candidatus* Liberibacter asiaticus contains the *ClSABP1*, *ClSABP2* and *ClSABP3* genes, which are homologous to the *NtSABP1*, *NtSABP2* and *NtSABP3* genes of *Nicotiana tabacum*, in which they code for salicylic acid-binding proteins.

The *ClSABP1*, *ClSABP2* and *ClSABP3* genes are slightly repressed in the transcriptome of HLB-affected Persian lime plants.

The *ClSABP1*, *ClSABP2* and *ClSABP3* proteins share a high structural similarity with *NtSABP1*, *NtSABP2* and *NtSABP3* from *N. tabacum*.

Acknowledgments

This study was supported by the Veracruz Council for Scientific Research and Technological Development (COVEICYDET) through funding for the project "Transcriptomics of Persian lime infected with HLB and genomic characterization of the causative agent, *Candidatus* Liberibacter asiaticus" (project number: 15 1906/ 2021). RPED is grateful for the scholarship granted to carry out this work (grant number 15 1906/2021-01). The technical support of Carlos González Cruz for data analysis is gratefully acknowledged.

CITED LITERATURE

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