Cloning and Expression Analysis of the Stable Antimicrobial Peptide Gene from *Citrus Junos* 'Pujang Xiangcheng'

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Keywords: Huanglongbing, Citrus Junos 'Pujiang Xiaocheng', Antimicrobial Peptide.

Abstract: Citrus Huanglongbing (HLB) is a devastating disease which threatens the worldwide citrus industry. No effective curable measurement could be taken to deal with the diseases to date. The recent exciting report discovered a novel stable antimicrobial peptide (SAMP) in the HLB resistant citrus material. This SAMP could provide protective effect as well as treatment effect in citrus materials. In the present study, we cloned the *CjSAMP* coding gene in the newly released citrus stocks variety 'Pujiang Xiangcheng' (*Citrus junos*) referring to the genetic information in the *C. sinensis* genome. Sequence analysis revealed that the gene produced three kinds of isoforms due to alternative splicing. These three transcripts varied in length and could produce three types of proteins. Structural analysis revealed that none of these proteins could produce the effective 'coiled coil' based pore-like structure that has anti-microbial activity, indicating that 'Pujiang Xiangcheng' was not a citrus type with extreme HLB tolerance. Expression analysis was further carried out in five commonly used local stock varieties in the production application, including *C. junos* 'Pujiang Xiangcheng', 'Ziyang Xiangcheng', *Poncirus trifoliata*, citrus hybrid (*P. trifoliata* × *Citrus sinensis*) and a *C. grandis*. Real time quantitative PCR results indicated that *C. grandis* was the one with most tolerant ability, while 'Pujiang Xiangcheng' was the least tolerant one, in consistent with the protein structure prediction results.

1 INTRODUCTION

Citrus Huanglongbing (HLB), or citrus greening, is one of the most devastating diseases threatening the worldwide citrus industry (Dala-Paula, 2018). It was acknowledged that species of *Candidatus Liberibacter* (CL), was the causal agent for this disaster (Achor, 2020; da Graça, 2016). A total of three species, *Ca. L.* africanus (CLaf), *Ca. L.* americanus (CLam) and *Ca. L.* asiaticus (CLas) were identified which causes the spread of diseases in different regions of citrus production worldwide. To date, no curation methods were found to deal with the trees once being infected. The main strategies in coping with these problems include wiping out all infected trees, controlling the diseases transmitting insect citrus psyllid with chemicals, and using diseases-free seedlings. However, none of these measures could reduce the loose of fruit growers. Moreover, antibiotics were used hoping to kill the bacterium (Hu, 2018) but raising the environmental and health concerns. To finding out resistance or extremely tolerant resources was among the most promising ways to fighting with this disease.

The recent reports suggested that the immune system disorder was the main reason of damaging effect of HLB diseases (Ma, 2022). The arm race between plants and the pathogen have triggered various plant immune system components. Antimicrobial peptide (AMP) was among one of them. Most of the time, these AMPs directly target the cell wall, plasma membrane or organelles inside the cell to prevent the invading (Huan, 2020). In 2021, Huang and colleagues from university of California reported the findings of an endogenous short peptide, named stable antimicrobial peptide (SAMP) in HLB tolerant citrus resources (Huang,

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Luo, S., Lin, X., Wang, X., Tang, H. and Chen, Q.
Cloning and Expression Analysis of the Stable Antimicrobial Peptide Gene from Citrus Junos 'Pujang Xiangcheng'.
DOI: 10.5220/0012020600003633
In *Proceedings of the 4th International Conference on Biotechnology and Biomedicine (ICBB 2022)*, pages 329-334
ISBN: 978-989-758-637-8

2021). This peptide coding gene expressed extremely higher in tolerant citrus materials than that in the HLB-sensitive resources. Further experiments confirmed that the produced SAMP could recover and maintain the resistance of sweet orange trees to the *CLas* infection. This positive effect was attributed to the second alpha helix formed in the SAMP protein, which could break the bacterial cells using its pore-like structure (Huang, 2021). Although the detail genetic information of SAMP in citrus was not thoroughly investigated so far, it was a very promising way to find out resistant citrus or related resources with similar mechanism.

In this study, we firstly cloned and identified the SAMP gene in a currently released citrus root stock 'Pujiang Xiangcheng' (*C.* junos) by our group (Xiaoke, 2017). Different transcripts of the same gene were found. Structure of each protein produced by each transcript was predicted. Lastly, the expression of the *SAMP* gene was analyzed in five commonly used root stock varieties.

2 MATERIALS AND METHODS

2.1 Plant Materials

The new citrus root strock 'Pujiang Xiangcheng' variety released by our group (Xiaoke, 2017) was used as starting materials for gene cloning. One year old seedlings from seeds were grown in plastic containers (5L in volume) with mixture of field soil and peat moss (3:1, v: v), irrigating at a half-month interval and fertilizing every month. Young leaves with light green color were collected for DNA and RNA isolation. The other widely used citrus root stock verities including *C. junos* 'Ziyang Xiangcheng', *Poncirus trifoliata*, citrus hybrid (*P. trifoliata* × *C. sinensis*) and *C. grandis* were grown in the field of the research center of Sichuan Agricultural University at Congzhou city of Sichuan Province. All leave materials for gene expression, including those from 'Pujiang Xiangcheng' were collected at the field. These resources were all grown in the second year after seeds sowing. To make the results comparable among species, fully expanded and hardy leaves at almost the same position from four sides of the tree canopy were collected from each species. Samples were snap-frozen in liquid nitrogen, transported to the lab and stored in a -80 °C refrigerator before use.

2.2 Gene Cloning and RT-qPCR

Genomic DNA of 'Pujiang Xiangcheng' was isolated using a modified CTAB method. Three percent of CTAB were used here instead of 2%, in combination with 1% beta-mercaptoethanol. Gene specific primers were designed using the *SAMP* gene of *C. sinensis* referring to the citrus genome (https://www.citrusgenomedb.org/). Amplification of the complete genomic sequence of the *CjSAMP* gene was carried out in a 20 μL PCR reaction system, consisting of 50 ng DNA, one pmol of both forward and revers primers (Table 1, P1 and P2) and 10 μL of CloneAmp HiFi PCR Premix (TaKaRa, Dalian, China). Thirty circles of regular three-step PCR reaction were carried out on a PTC-100 thermal cycler system (BioRad, US). Total RNA of the leaves was extracted using a commercial kit (TianGen, Beijing, China) following the manufactures' protocol. The first strand cDNA was synthesized by using the RevertAid™ H Minus Reverse Transcriptase (Thermo, US). Similar PCR reaction conditions were used as amplifying the genomic coding sequence except that 1 μL of cDNA was used as templates (Table 1). All amplicons were detected by electrophoresis in a 1% agarose gel. The specific bands on the gel were purified using the E.Z.N.A.® Gel Extraction Kit (OMEGA, US) as indicated by the instruction. The DNA fragment were ligated into the pEASY-blunt vector (TransGen, Beijing, China) and transformed into the competent cells of Trans-T1 (TransGen, Beijing, China) using a heat-shock method. Ten resistant clones were sequenced to identify the inserted fragments.

To compare the relative expression level of the *CjSAMP* gene in different citrus root stock varieties, RT-qPCR was employed. To make the results comparable to the previous reported results (Huang, 2021), the same specific gene primer pair were used (qSAMPF and qSAMPR in Table 1). This pair of primers could target to all three obtained transcripts, hence reflecting the overall expression level of the SAMP gene. The PCR reaction system were constructed by combining 1 μL of cDNA template, 1 μL of the forward and revers primer, and 10 μL of TB Green Premix Ex Taq II premixture (TaKaRa, Dalian, China). The qPCR reaction was done on a CFX96 system (BioRad, US) using a standard two step reaction protocol. The citrus ubiquitin gene (UBI, UbiF and UbiR in Table 1) were used as internal reference. The expression differences were expressed as the deferential threshold cycles between the SAMP gene and the UBI gene.

Table 1: Primers used in this study.

Primer Name	Sequence (5'- 3')
P1	ATGGAAGAAGCTAAAGGAGTGGTGAAG
P2	CCTACTAGTACAACTCAGACACGTACTG
P4	ATGCTCACATTCTTGCCCTTATACTTAACA
P3	GTTACTGGCTGTTAAGTATAAGGGCAAG
P5	GTCTTTCCAATGGTCTCCCATTTGC
P8	CAATAGTCAAGGGTGTATCCACTTGTGAT
P7	ATCACAAGTGGATACACCCTTGACTATTG
P6	GCAAATGGGAGACCATTGGAAAGAC
qSAMPF	AACAGGGGCAAGAATGTGAGCAT
qSAMPR	ACACGTACTGTTGTCGGTTTGTAGTCA
UbiF	ACCATCGACAATGTCAAGGC
UbiR	CCTTTTGGATGTTGTATTCGGC

2.3 Bioinformatic Analysis

The vector sequences were trimmed in the Geneious Prime suite (v2021.02). The splicing junction of the introns of the *CjSAMP* gene were obtained by aligning the cDNA sequences to the DNA sequences of the gene. The deduced protein sequences were searched against the non-redundant nucleotide database at NCBI using BLASTP with e-value 1e-10. Closely related gene sequences ranking in the top 10 hits were used to construct the phylogenetic tree. Protein sequences were aligned using the mafft software (v7.123b) and the tree was reconstructed using the IQTREE package (v1.6.8). The optimal protein substitution model 'LG+I' was selected during this process. Fast bootstrap procedures were repeated 10000 times to validate the branch topology of the tree.

3 RESULTS AND DISCUSSIONS

3.1 The Three Different SAMP Transcripts

In the *C. sinensis* genome, three different alternative splice products were annotated (Xu, 2013). Based on the results of Huang et al., (Huang, 2021), only the short SAMP could contribute to the resistance of HLB diseases. Since the genome sequences of the *C. junos* genome were still lacking, one pair of gene specific primer were designed flanking the start and stop codon of the longest SAMP transcript (Fig. 1A, P1 and P2). Amplification of the gene using DNA template presented us a unique and specific band corresponding to 1000 bp in length. When using cDNA as template, it produced a fragment about 340 bp (Fig. 1B lane 2). A DNA band with higher molecular could be observed as well. Sanger sequencing results indicated that the sharp and clear one was the longest transcripts coding for a protein of 109 amino acids (Fig. 1C). Two single nucleotide polymorphism sites were found when comparing with the SAMP gene in the *C. sinensis*, producing two different residuals correspondingly. Using the primer combination of P1 and P4, P3 and P2, we could still be able to amplify two products (Fig. B, lane 5 and 6). This indicated that an alternative transcript corresponding to the *SAMP-t2* was exist. Using the three pairs of primer (P5 and P2, P8 and P2, P1 and P7), we were able to amplify a corresponding product with expected size (Fig. 1, lane 3, lane 7 and lane 4). Sequencing results demonstrated that due to the intron retention, a premature termination codon (PTC) was introduced to the transcripts of *SAMP-t2*, leading to a production of a truncated protein with 55 amino acids in length (Fig. 1A). Likewise, the alternative 3' splicing site selection in the exon1 also brought a PTC mutation to the transcripts in *SAMP-t3*, producing a 66 amino-acid peptide.

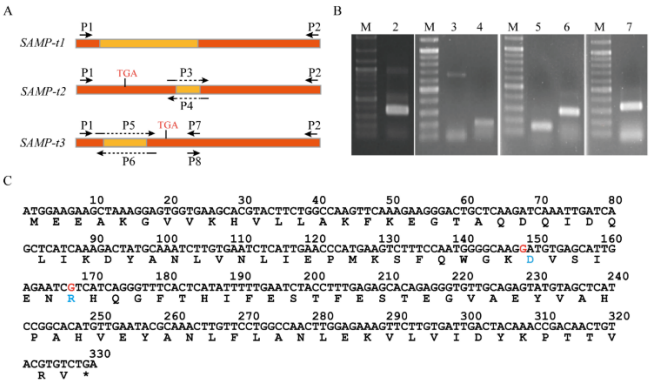

Figure 1: Amplification of the SAMP transcripts with specific primers and the coding sequences of CjSAMP1.

(Primer binding sites in the three transcripts (A) and the gel electrophoresis detection results of amplicons (B). The standard DNA marker was loaded in lanes M. Lane 2 to 7 were corresponding to products of the combination of primer pair P1+P2, P5+P2, P1+P7, P1+P4, P3+P2, P8+P2. The coding sequences of *SAMP1* and the deduced amino acids were arranged in accordance with their site number (C). The SNP sites and the alternative amino acids in *C. junos* comparing with *C. sinensis* were marked with red and blue colour respectively)

3.2 The SAMP Structure and Expression Analysis in Different Root Stocks

Using the obtained protein sequences, we searched the homologs of SAMP in the public protein database to have an overview of the conserveness of the protein. Several homologs with high sequence identity (>90%) were obtained in different species. The protein sequences with the top 10 hit score were downloaded. Multi-sequence alignment indicated several highly conserved amino acid residuals, especially at the C- terminal of the protein (Fig. 2B). Phylogenetic analysis revealed that the gene tree was in consistent to the species relationship (Fig. 2A), indicating an old origin of the gene in these investigated species (Salse, 2016). Genetic distances estimated among the proteins also supported that the proteins were highly conserved. The largest genetic distances were observed between the *Ziziphus jujuba* and *Fragaria vesca* subsp. *vesca* (0.402).

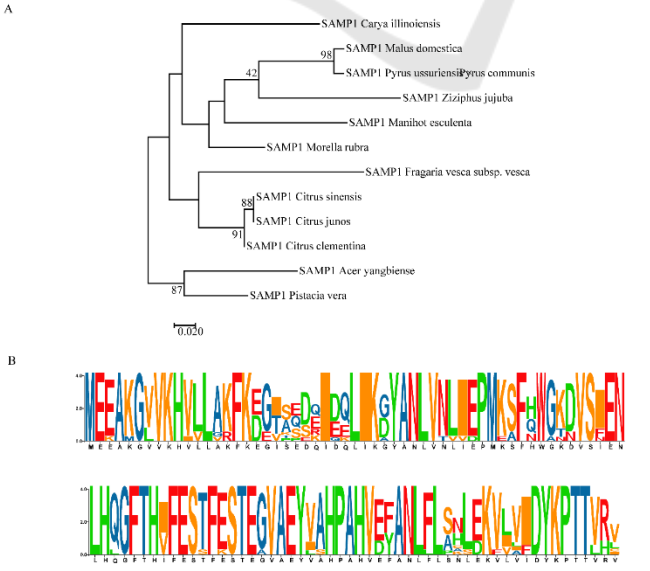

Figure 2: Phylogeny (A), conserved residuals (B) of the SAMP1 protein.

Since the active antibacterial peptide should form a pore-like structure (Huang, 2021), we used the AlphaFold system (Jumper, 2021) to predict the structure of all three proteins and compared to that reported in the functional SAMP in citrus resistant resources (Huang, 2021). Results indicated that the protein formed a ternary structure with three helix and four beta-sheet with high confidence (Fig. 3A). The second functional helix in the reported SAMP was corresponding to the third helix in this *SAMP1* protein. However, the special occupation of the first helix might hinder the formation of the pore-like structure from interacting with multiple monomers. In this regard, *C. junos* 'Pujiang Xiangcheng' does not code for an active antipathogenic SAMP peptide in the genome. Hence, it might not be highly tolerant with HLB, if it would use the same SAMP resistance mechanism. The other two proteins encoded by the alternative spliced transcripts truncated in the N terminal (Fig.3 A and B). They all lack the helix structures. In consistent to these results, the expression of the *SAMP* gene in this species was the lowest among the investigated five root stocks (Fig. 2B). The maximum expression level was observed in *C. grandis.*

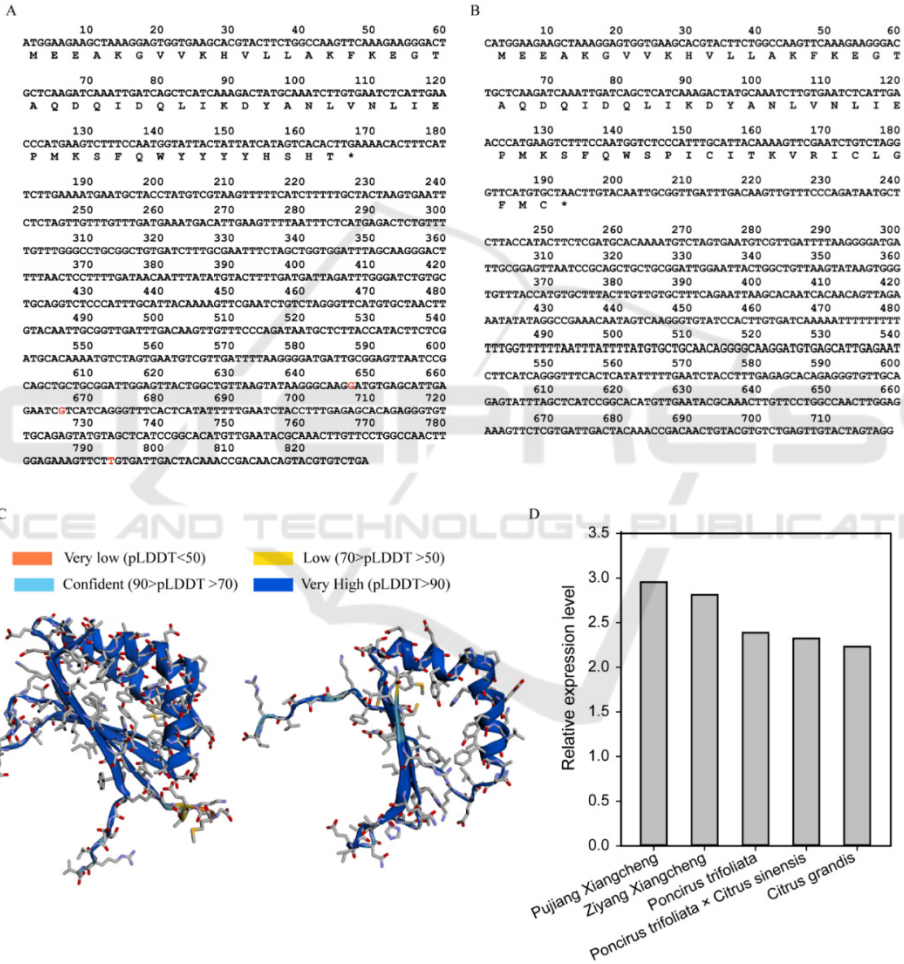

Figure 3: The truncated protein sequences of the transcript t2 and transcript t3 (A and B) of the same SAMP gene, SAMP1 protein structure (C) and the relative expression level of the gene in different citrus stock resources (D).

4 CONCLUSION

We cloned the *SAMP* coding gene from the *C. junos* root stock 'Pujiang Xiangcheng'. The gene produced three different splicing products. Structure of the identified *CjSAMP1* formed four beta-sheet and three alpha-helixes, most likely not being able to form a pore-like structure. The transcripts abundance was the lowest in the investigated citrus root stock resources

in 'Pujiang Xiangcheng'. It was not likely a HLB extreme tolerant material based on these results.

ACKNOWLEDGMENTS

This work was financially supported by the Key Research and Development Program of Science and Technology Project of Chengdu City, Sichuan Province (2019-YF05-00635-SN).

REFERENCES

Achor, D., Welker, S., Ben-Mahmoud, S., Wang, C., Folimonova, S.Y., Dutt, M., Gowda, S., Levy, A. (2020) Dynamics of *Candidatus Liberibacter* asiaticus movement and sieve-pore plugging in citrus sink cells. Plant Physiol., 182: 882-891.

Dala-Paula, B.M., Plotto, A., Bai, J., Manthey, J.A., Baldwin, E.A., Ferrarezi, R.S., Gloria, M.B.A. (2018) Effect of huanglongbing or greening disease on orange juice quality, a review. Front. Plant Sci., 9: 1976.

Da Graça, J.V., Douhan, G.W., Halbert, S.E., Keremane, M.L., Lee, R.F., Vidalakis, G., Zhao, H. (2016) Huanglongbing: An overview of a complex pathosystem ravaging the world's citrus. J. Integr. Plant Biol., 2016. 58: 373-87.

Hu, J., Jiang, J., Wang, N. (2018) Control of citrus huanglongbing via trunk injection of plant defense activators and antibiotics. Phytopathol., 108: 186-195.

Huan, Y., Kong, Q., Mou, H., Yi, H. (2020) Antimicrobial peptides: Classification, design, application and research progress in multiple fields. Front. Microbiol., 11: 582779.

Huang, C.Y., Araujo, K., Sánchez, J.N., Kund, G., Trumble, J., Roper, C., Godfrey, K.E., Jin, H. (2021) A stable antimicrobial peptide with dual functions of treating and preventing citrus huanglongbing. Proc. Natl. Acad. Sci. U.S.A.,. 118: e2019628118.

Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S.A.A., Ballard, A.J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., Back, T., Petersen, S., Reiman, D., Clancy, E., Zielinski, M., Steinegger, M., Pacholska, M., Berghammer, T., Bodenstein, S., Silver, D., Vinyals, O., Senior, A.W., Kavukcuoglu, K., Kohli, P., Hassabis, D. (2021) Highly accurate protein structure prediction with AlphaFold. Nature, 596: 583-589.

Ma, W., Pang, Z., Huang, X., Xu, J., Pandey, S.S., Li, J., Achor, D.S., Vasconcelos, F.N.C., Hendrich, C., Huang, Y., Wang, W., Lee, D., Stanton, D., Wang, N. (2022) Citrus huanglongbing is a pathogen-triggered immune disease that can be mitigated with antioxidants and gibberellin. Nat. Commun., 13: 529.

Salse, J. (2016) Ancestors of Modern Plant Crops. Curr. Opin. Plant Biol., 30: 134-42.

Xiaoke, F., Xiaojiao, H., Tao, C., Jing, Z., Yan, W., Qing, C., Qingliang, L., Haoru, T., Xiaorong, W. (2017) A new citrus rootstock 'Pujiang Xiangcheng' (*Citrus junos*). J. Fruit Sci.,. 34: 917-920.

Xu, Q., Chen, L.L., Ruan, X., Chen, D., Zhu, A., Chen, C., Bertrand, D., Jiao, W.B., Hao, B.H., Lyon, M.P., Chen, J., Gao, S., Xing, F., Lan, H., Chang, J.W., Ge, X., Lei, Y., Hu, Q., Miao, Y., Wang, L., Xiao, S., Biswas, M.K., Zeng, W., Guo, F., Cao, H., Yang, X., Xu, X.W., Cheng, Y.J., Xu, J., Liu, J.H., Luo, O.J., Tang, Z., Guo, W.W., Kuang, H., Zhang, H.Y., Roose, M.L., Nagarajan, N., Deng, X.X., Ruan, Y. (2013). The draft genome of sweet orange (*Citrus sinensis*). Nat. Genet., 45: 59-66.