# A screen of nodule-specific cysteine-rich (NCR) peptides for control of the HLB bacterium in citrus

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*Year 2 of 1 (100% Complete)*

## Objectives

1. Complete *in vivo* screen of NCR peptides
2. Deliver top candidate NCR peptides to CLas infected Asian citrus psyllids via artificial diet and assay impact on CLas transmission by treated psyllids

## Problem and Significance

The gram-negative bacterium '*Candidatus* Liberibacter asiaticus' (CLas) is the causal agent of citrus huanglongbing (HLB), a destructive disease of *Citrus* species worldwide. The CLas bacterium is transmitted between citrus trees in the U.S. by the invasive Asian citrus psyllid, *Diaphorina citri*, which spread to the United States in the 1990s, devastating the Florida citrus industry and now threatening citrus growers in California and other citrus growing U.S. states. While a variety of therapeutic strategies are being explored to remedy HLB disease, antimicrobial peptides are especially promising since they are naturally occurring and small, making them amenable to synthesis for laboratory testing, and can be delivered (genetically or via injection) directly into citrus and other crops. In particular, the nodule-specific cysteine-rich (NCR) peptides are a family of legume derived antimicrobial peptides with wide-spectrum bactericidal activity, including soil-dwelling bacteria closely related to CLas. However, the utility of NCR peptides as therapeutics to stop HLB disease in citrus trees is unknown. Coupled to novel citrus delivery strategies under development, NCR peptides may provide CA citrus growers a new, effective tool to help combat HLB disease spread.

## Benefit to Industry

Citrus growers in California and worldwide will have the option to use products based on naturally occurring plant-derived antimicrobial NCR peptides for the control and management of HLB in commercial citrus groves, nurseries and residential areas. NCR peptide products will provide growers with an effective new tool for HLB management. Due to their unique chemistry, NCR peptides are structurally stable antimicrobials that exhibit diverse modes of action. Hence, a set of NCR peptides can be combined with other existing methods as well as approaches being developed by other research projects to create an effective resistance management program for HLB control.

## Progress Summary

For Objective 1, we have finished an *in vivo* screen of 14 (out of 15) of our top NCR peptide candidates (out of a total of 182 previously tested *in vitro*) for their antimicrobial activity against the CLas bacterium in detached leaf assays. The *in vivo* assay revealed that 7/14 (50%) of the NCR peptides tested resulted in a significant reduction in the CLas 16S transcript-to-gene ratio over a seven day incubation period, suggesting robust inhibition of CLas *in vivo* by the NCR peptides (Figure 1). While we did note some decline in CLas activity in potassium phosphate buffer only controls (0.1 mM, pH 5.8) over the seven-day incubation, antimicrobial peptide treatments tended to demonstrate greater declines in detectable CLas activity (e.g., see positive control PMB, Figure 1).

To address Objective 2, we tested three of our top performing NCR peptides (803364, 803543, and 803570, Figure 1) in excised leaf acquisition assays.

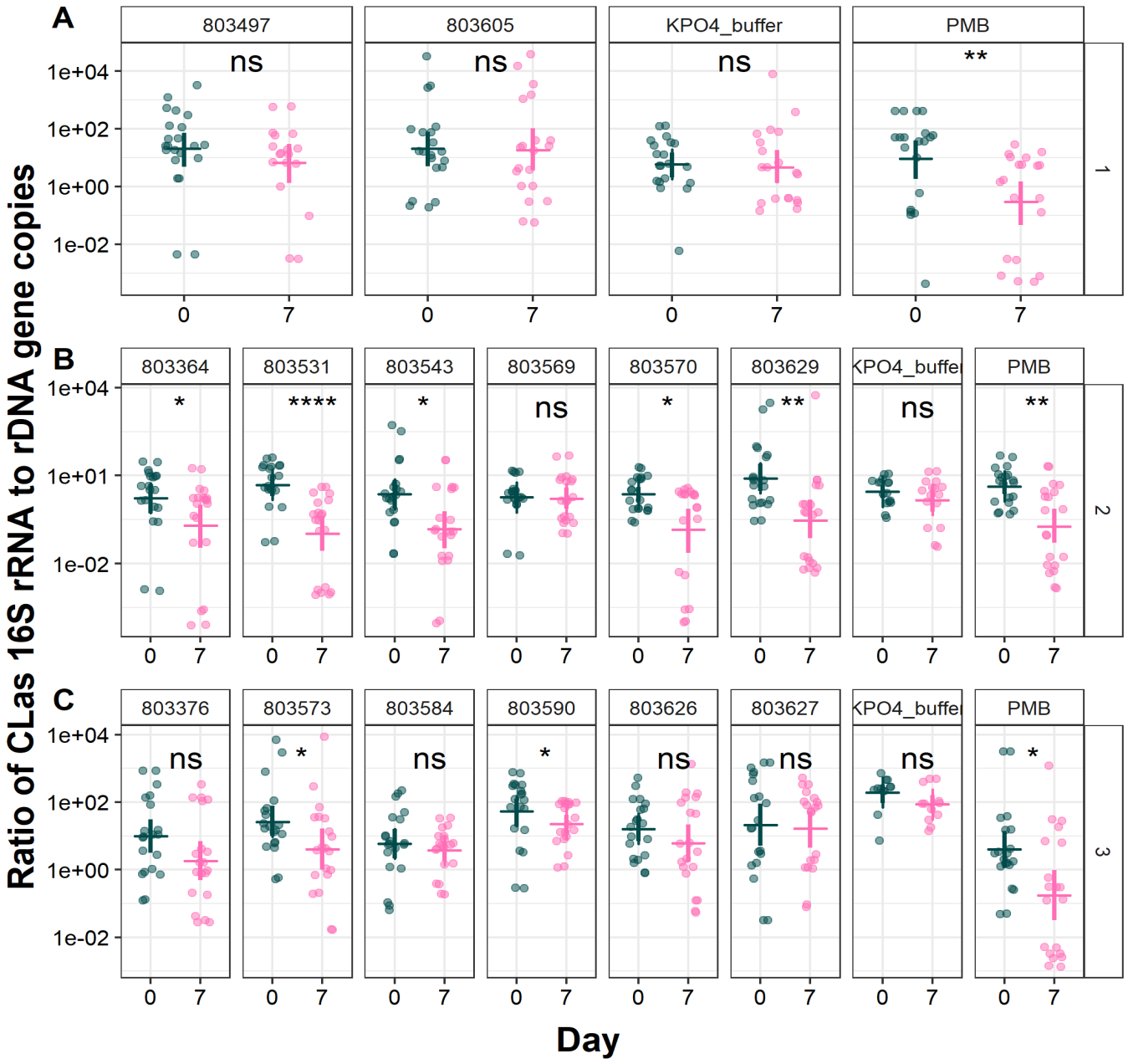


*Figure 1. CLas activtiy (the ratio of CLas 16S rRNA to rDNA gene copies) measured in detached Citrus medica (citron) leaves at 0 and 7 days following exposure to 0.1 mM potassium phosphate buffer (pH 5.8; label KPO4_buffer), NCR peptides have label formats of 803XXX, or the antimicrobial peptide polymyxin B sulfate (label PMB in figure). Each point represents a technical duplicate qPCR result from the DNA and RNA extracted from leaves used in each treatment. The The "ns" in figure panels indicate wilcoxon rank sum test not significant at α = 0.05. Asterisks indicate significance of Wilcoxon rank sum tests at p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), and p ≤ 0.0001 (****). Cross bar and vertical lines in each panel represent a nonparametric bootstrapped population mean and 95% confidence intervals (1,000 bootstraps; "mean_cl_boot" option in R stat_summary() function).*

Briefly, CLas-infected *Citrus medica* (citron) leaves (n = 10 per treatment) were provided with potassium phosphate buffered solutions containing either 1 mg $ml^{-1}$ NCR peptides, 0.5 mg $ml^{-1}$ of the antimicrobial peptide polymyxin B sulfate (PMB), or buffer only. After insertion of the detached citrus leaf into buffered solution, we added between five and eight uninfected 4th or 5th instar stage *D. citri* nymphs to each leaf, which were incubated for 20 days in 50 ml falcon tubes with a sealed mesh opening at 75°F with a 14 h:10 h day:night light cycle. While two out of three NCR peptides (803364 and 803543) appeared to demonstrate reduced acquisition of CLas by *D. citri* nymphs, only the polymyxin B sulfate treatment demonstrated significantly lower CLas titers compared to the buffer only control (Figure 2; Dunn test Z = 3.37, p =0.008).

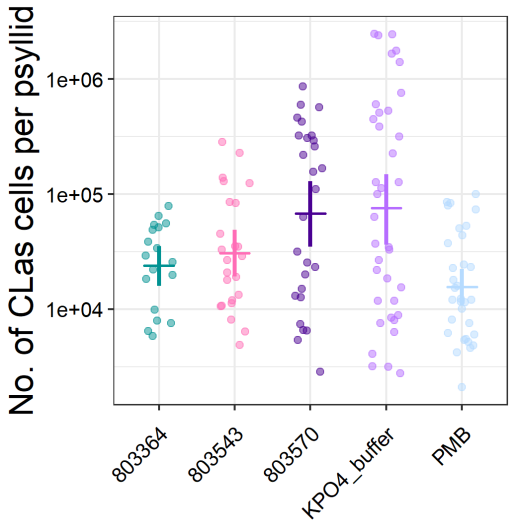


*Figure 2. CLas 16S rRNA gene abundance in adult Diaphorina citri after a 20 day incubation period from 4th instar nymphal stage on CLas-infected citrus leaves treated with 0.1 mM potassium phosphate buffer (pH 5.8; label KPO4_buffer), NCR peptides (label 803364, 803543, 803570), or the antimicrobial peptide polymyxin B sulfate (label PMB in figure). CLas 16S rRNA gene copies in the above figure are shown only for D. citri individuals with a CLas Cq ≤ 40. Each point represents a technical duplicate qPCR result from the DNA extracted from D. citri individuals used in each treatment. Cross bar and vertical lines in each panel represent a nonparametric bootstrapped population mean and 95% confidence intervals (1,000 bootstraps; "mean_cl_boot" option in R stat_summary() function).*

The number of individual *D. citri* with CLas Cq values in a quantifiable range (Cq ≤ 35) was two times greater than two NCR peptides and the PMB treatment (Table 1). While promising, the assay should be repeated since detectable CLas acquisition by individual *D. citri* in this assay was low overall. Additional NCR peptides can be used to further support a reduction in CLas acquisition by *D. citri* nymphs compared to controls when reared on citrus leaves exposed to these substrates. Unexpectedly, there were high levels of mortality in the excised leaf assays with the NCR peptides. This effect was not quantified in the first experiment because it was unexpected; however, we will repeat these experiments to test if the NCR peptides are insecticidal against *D. citri*.

| Treatment | Cq ≤ 30 | Cq ≤ 35 | Cq ≤ 40 |
|---|---|---|---|
| 803364 | 0 | 5 | 38 |
| 803543 | 0 | 6 | 37 |
| 803570 | 0 | 9 | 44 |
| KPO4_buffer | 1 | 12 | 44 |
| PMB | 0 | 6 | 56 |

*Table 1. Number of individual* D. citri *adults with CLas Cq values below a Cq threshold cutoff of 30, 35, and 40.*

Finally, Drs. Heck, Shatters and Trimmer are cooperating on a new USDA Emergency Citrus Disease Research and Extension grant which will encompass large-scale tree delivery and field trials of the promising NCR and other antimicrobial compounds discovered in this CRB-funded research.

## Conclusions

We have identified a total of seven NCR peptides that demonstrate a significant reduction in the activity (as measured by the 16S rRNA/rDNA ratio) of CLas in *in vivo* detached leaf assays compared to buffer only controls. We have also observed a reduction in the total quantity of CLas bacterium acquired by *D. citri* nymphs reared on citrus leaves in phosphate buffered solutions containing NCR peptides compared to buffer only controls. These top-performing peptides (and their combinations) can also be tested in psyllid artificial diet assays to assess CLas transmission by psyllids reared on

uninfected citrus leaves in the presence or absence of NCR peptides. The team is developing a plan for technology transfer, including filing a use patent to protect the intellectual property and further research on the *in planta* delivery strategy.

**CRB Project # 5300-202**

## Publications and Presentations

Heck et al. 2020. Scientists screen for plant-based antibacterials. *Citrograph* 11(1): 62-63.

Igwe et al. 2021. An excised leaf assay to measure acquisition of 'Candidatus Liberibacter asiaticus' by psyllids associated with citrus huanglongbing disease. *Phytopathology* 0: 0ja. https://doi.org/10.1094/PHYTO-03-21-0124-SC

M. Heck. 2021. Eureka! Taking a discovery through the tech transfer process in the USDA ARS. All Hands Meeting Presentation, USDA ARS, Ithaca, NY.