PNAS
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Supplementary Information for

A stable antimicrobial peptide with dual functions of treating and preventing citrus Huanglongbing

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Supplementary Text

Materials and Methods

Materials and Methods

Nb and tomato Material, *C*Lso Inoculation

Nb and tomato (*Solanum lycopersicum, S*l, 'money maker') plants were grown in a plant growth room at 23±1 °C with 12-h light/12-h dark photoperiod. The 10-day-old *Nb* seedlings (with 2 true leaves emerged) were exposed to 10 *C*Lso-positive potato psyllid nymphs for five days. To detect *C*Lso titer in *Nb* plants, 6 new leaves were collected from each plant and the plant DNA was purified following the CTAB protocol from the midvein of collected leaves (*1*). *C*Lso was detected by qPCR in the plant using the primers and TaqMan probe designed against *CLso* 16S rDNA (SI Appendix, Table S1). The primers and probe of the positive internal control set was designed to amplify the ubiquitin gene from *Nb* (*NbUbi*). Dilutions of plasmid DNA pLso (containing the target DNA sequence from *C*Lso in pGEM-T easy vector, Promega) were set as a standard curve for evaluating the titer of *C*Lso.

MASAMP treatment on *Nb* plants with genes knockdown by VIGS

VIGS was performed by infiltration with *A. tumefaciens* strain containing the bipartite TRV, pTRV1, and pTRV2 vectors (Liu et al., 2002). Equal volumes of *A. tumefaciens* (OD_{600}= 0.5) suspensions carrying pTRV1 and pTRV2-*siSGT1 (2), pTRV2-siBAK1/SERK3 (3)* or pTRV2-siRB construct (*4*), then co-inoculated into the third and fourth true leaves of the *Nb* plant by infiltration with 1ml syringe. The pTRV2-*siNPR1* construct that can silence *NbNPR1* was designed by the SGN VIGS Tool (*5*) and the primer sets used for amplifying the fragment are shown in SI Appendix, Table S1. The leaves of inoculated plants were collected after three weeks for RNA extraction.

SAMP immunoprecipitation from plant tissue

Two grams of citrus leaf tissue were frozen with liquid nitrogen and ground into a fine powder. 10ml of extraction buffer (20 mM Tris-HCl [pH7.5], 150 mM NaCl, 5 mM $MgCl_2$, 5 mM dithiothreitol [DTT], 1% Nonidet P40**,** 30 ***μ***l of plant protease inhibitor [sigma p9599]) was added to the powder. After being agitated at 4 ^{0}C for 30 minutes, the protein lysate was filtered through a 45 ***μ***m nylon mesh and total protein amount was evaluated using BradFord reagent (BioRad). The total protein lysate was pre-incubated with 10 µl of protein A beads (Roche) for one hour at 4^{0}C to clean the non-specific binding of the beads. Next, 5***μ***g of anti-SAMP antibodies were added to the lysate for 2 hours at 4^{0}C. Finally, 20 µl of protein A beads were added to the lysate for one more hour. After incubation, the protein A beads were collected by spinning and washed three times (5 min each) with 5 ml IP washing buffer (20 mM Tris-HCl [pH7.5], 150 mM NaCl, 5 mM $MgCl_2$, 5 mM dithiothreitol [DTT], 0.5% Triton X-100, 5 ***μ***l of plant protease inhibitor [sigma p9599]). The washed beads were resuspended in 1XSDS-loading buffer and resolved in an 18% SDS-PAGE gel. The SAMP was detected by western blot and quantified using ImageJ (*7*).

SAMP Peptide Production

The SAMP used in this study was produced by *E. coli* or by chemical synthesis. The long and short SAMP genes were cloned into a pET28a protein expression vector using the primer list in Table S1. The N-terminal His-tag fusion SAMPs were expressed in *E. coli* strain C41(DE3) with 0.5 mM IPTG at 28 °C for 2 hours. The native protein purification

was performed using Ni-NTA Agarose (Qiagen) following the manufacture's protocol. The chemical synthetic MaSAMP, double helix hairpin (SAMP**Δ**N**Δ**C, HIFESTFESTE-GVAEYVSHPSHVEYANLFLANLEKVLVIDYK), helix1 (MaSAMP-helix1, THIFE-STFESTEGVAEYVSHP), and helix2 only (MaSAMP-helix 2, SHVEYANLFLANLE-KVLVIDYK) is >75% purity in net weight produced by GenScript or Biomatik.

Separation of Outer and Inner Membrane Fractions

Lcr cell fractionation was carried out by using two subsequent sets of sucrose density gradients as described in Marani *et al (6)*. 3L of *Lcr* cells with OD_{600} around 0.5 were collected and resuspended in 3 mL of 200nM SAMP in 1xPBS pH7.3 and incubated at room temperature for 20 minutes. This MaSAMP-bacteria suspension was added to 3 mL of buffer K (1xPBS, 500 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol [DTT], one complete ULTRA tablet[Roche]/50ml, lysozyme [Sigma] 2mg/ml, at pH 7.3), incubated on ice for 15 minutes and lysed by sonication in position 5, 6 sec ON/10 sec OFF pulses repeating for 10 times. The unbroken cells were clarified from the lysate by 20-min centrifugation at 8,000g. The supernatant was transferred to the top of two layers of sucrose gradient: 1 mL 55% (w/w) on the bottom and 5.5 mL 9% (w/w) on the top. All sucrose gradients were prepared in buffer M: 50 mM Tris-HCl, 1 mM EDTA, and 1 mM DTT (pH7.5). The gradients were spun at 200,000g for 3 h with a Beckman SW 32.Ti rotor. The membrane fraction which contained the entire membranes was collected from the top of the 55% sucrose layer. This fraction was diluted 1:1 with buffer M and transferred on top of a six-layer sucrose gradient to separate the inner and outer membrane fractions. The second gradient was as follows (from bottom to top): 1 mL at 55%; 2.0 mL at 50%, 45%, 40%, and 35%; 0.8 mL at 30% (all w/w) and 3.3 mL of the sample faction. The gradients were spun for 18h at 200,000g in a Beckman SW 32.Ti rotor. The inner and outer

membrane fractions were collected from the top of the 40% and 50% sucrose steps, respectively.

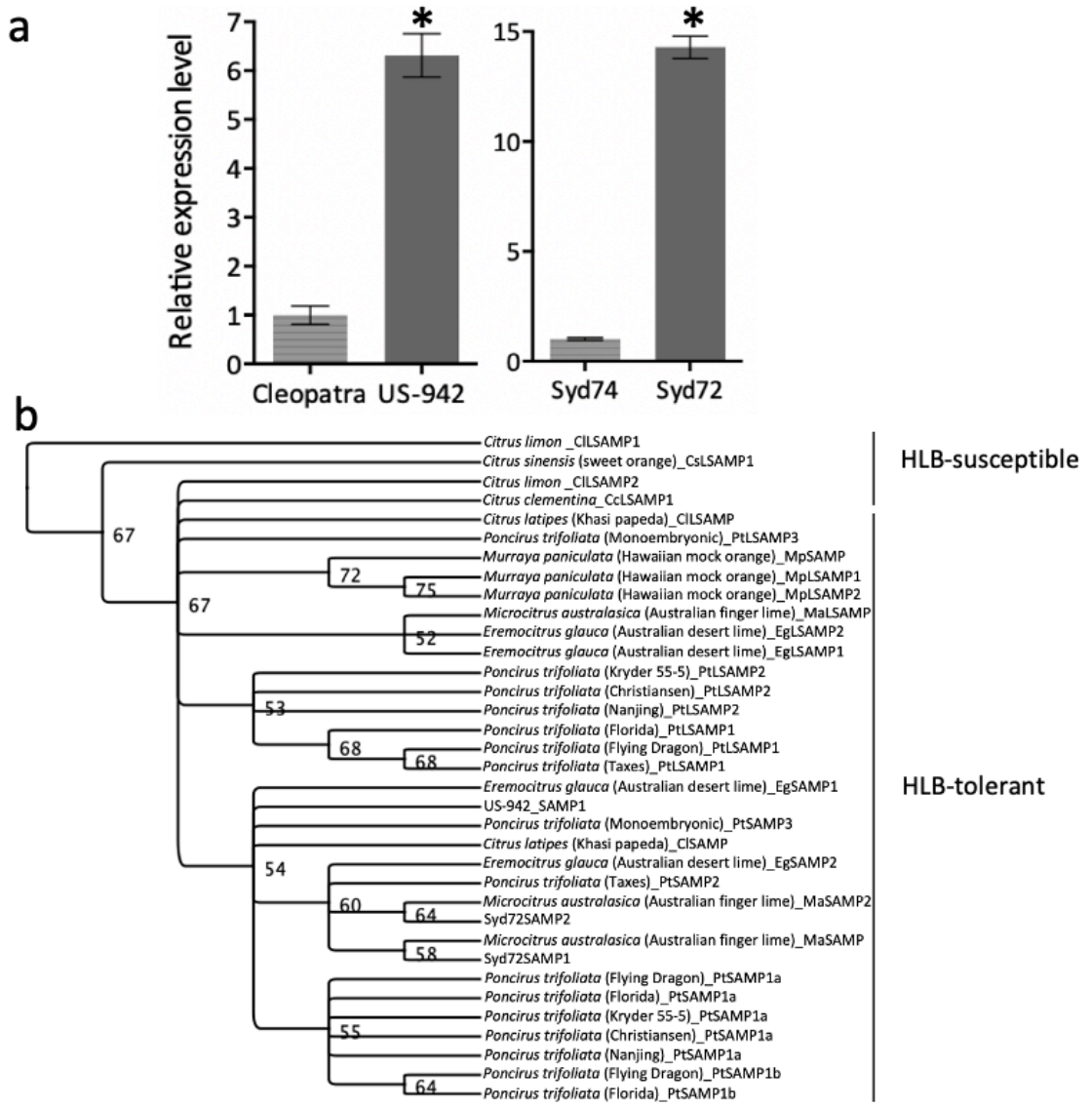

Figure S1. Identification of SAMPs from HLB-tolerant citrus relatives.

a. The relative expression level of small RNA 942si1007 and *SAMP* in US-942 compared to the HLB-susceptible parental line Cleopatra, and Syd72 compared to the other HLB-susceptible hybrids Syd74. The expression level was analyzed by qRT-PCR and normalized to *Actin.* The significant difference is indicated by *($P<0.05$ analyzed by t-test).

b. The phylogenetic analysis of SAMPs cloned from different citrus and citrus-relatives collected from the Citrus Variety Collection at the University of California, Riverside.

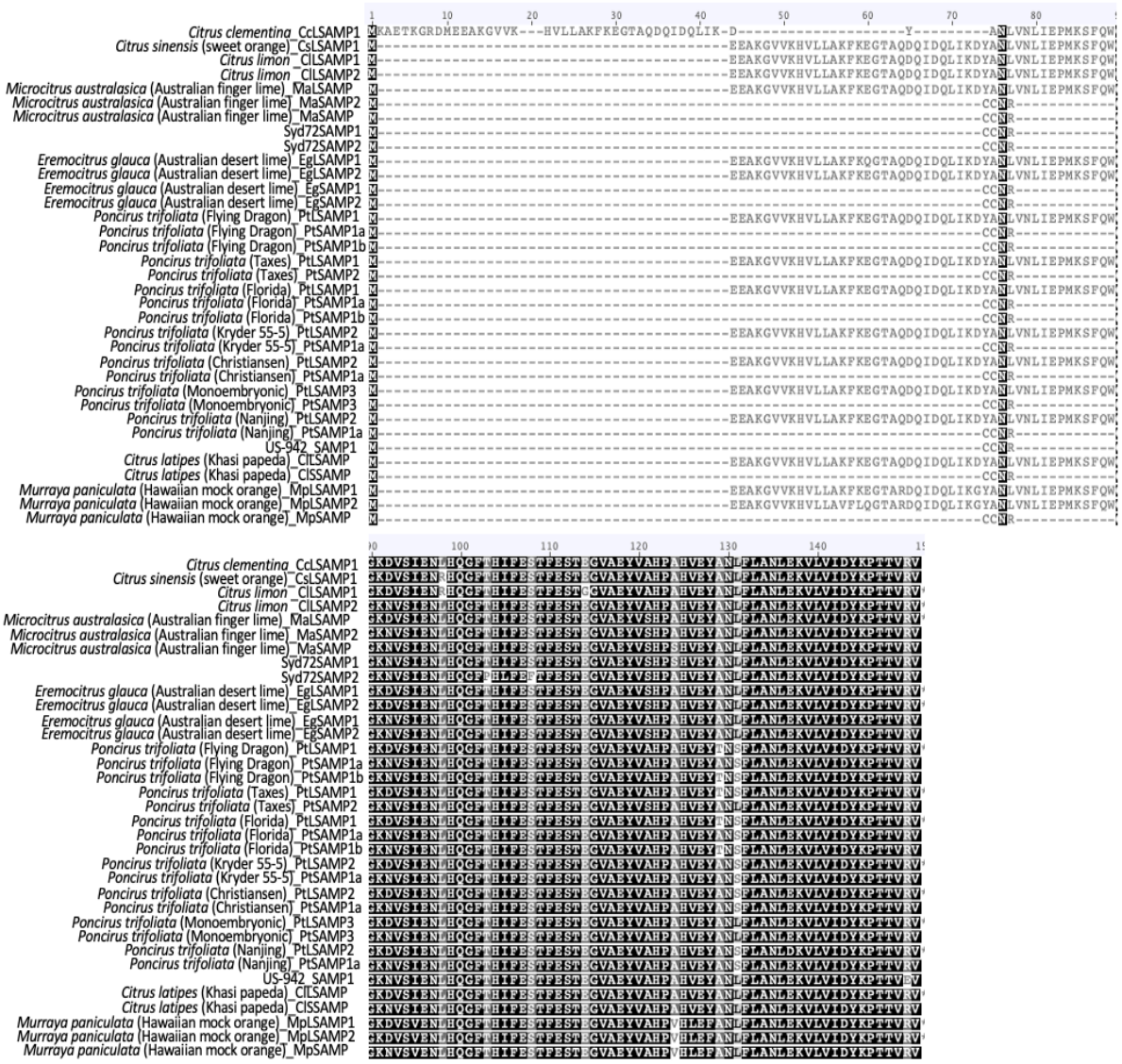

Figure S2. The sequence alignment of identified SAMP genes in citrus and citrus-relatives.

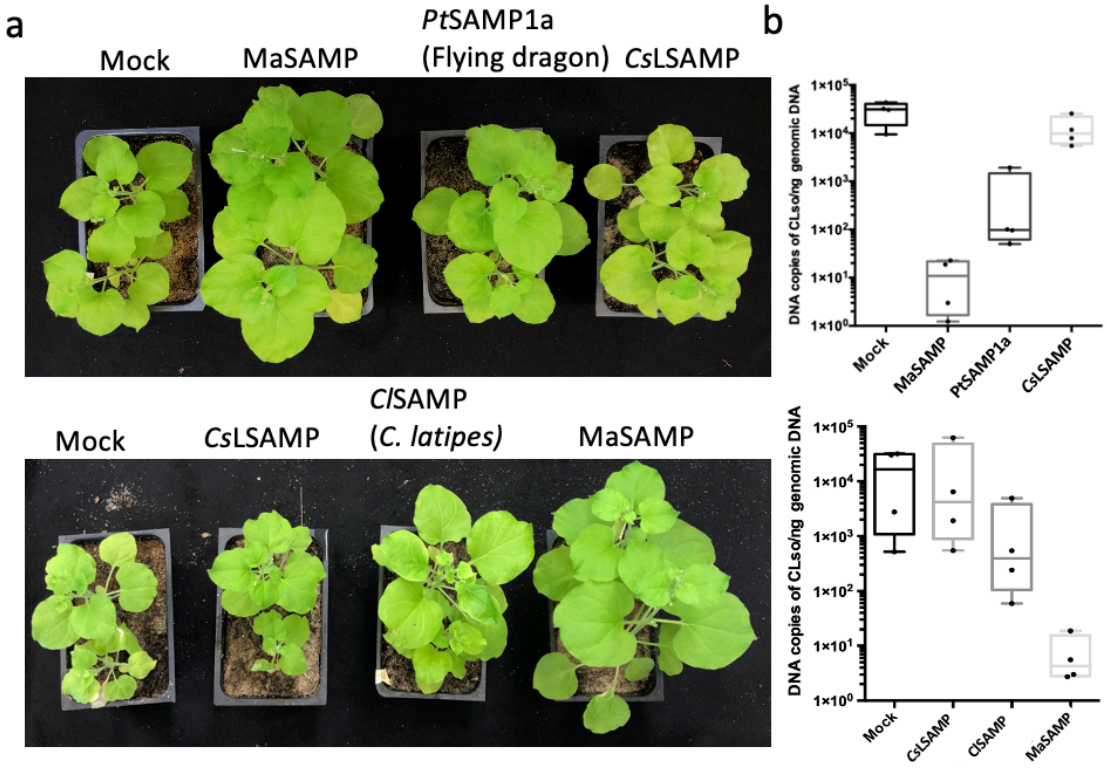

Figure S3. Screening for the most effective SAMP using the *Nb*/CLso growth assay.

a. The *C*Lso infected *Nb* plants injected with different SAMPs after 4 weeks. b. The *C*lso titer in the *Nb* plants showed in (a). Six leaves were collected from each plant for DNA extraction. Four plants were evaluated for each treatment. The *C*Lso titer was analyzed by qPCR.

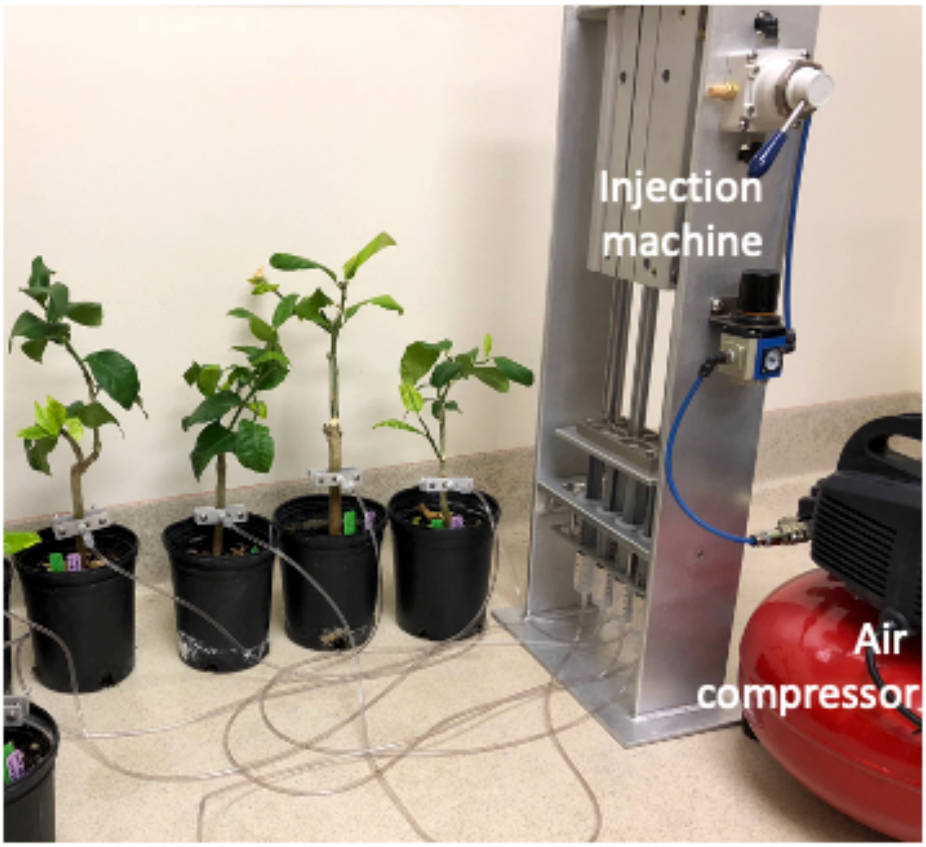

Figure S4. Pneumatic trunk injection system used to deliver solution into the vasculature of the citrus trees.

The system allows for an accurate amount of solution to be injected into trees in a period of time under control pressure. The air compressor is connected to the injection machine which pushes syringes connected to trees with tubes.

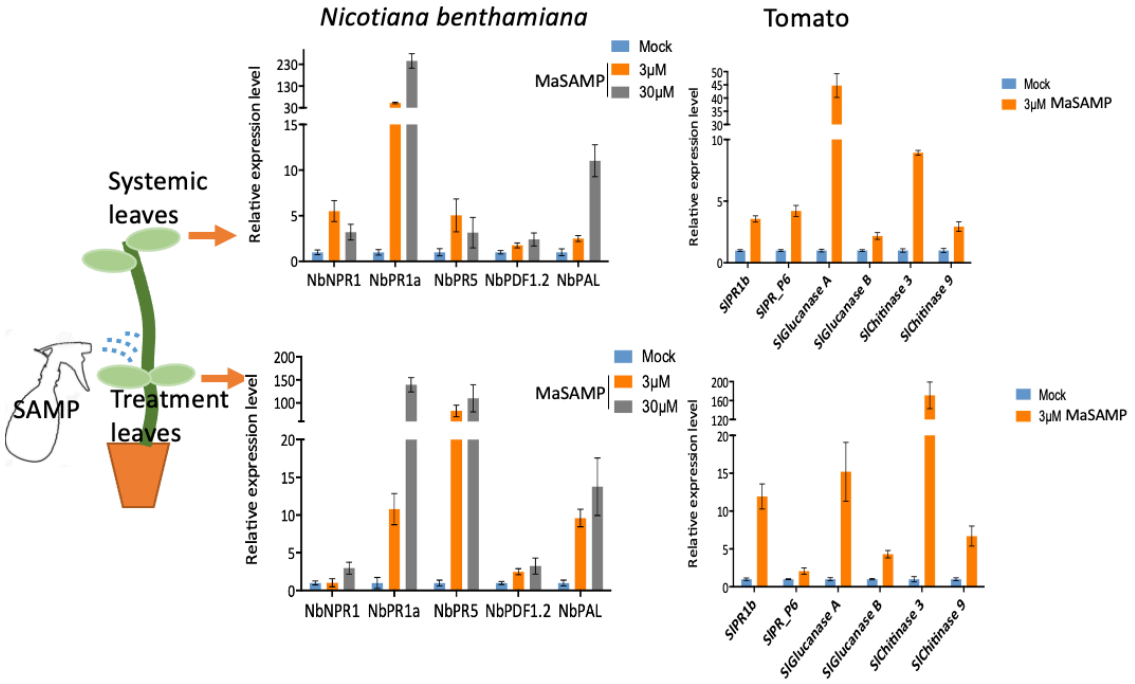

Figure S5. The defense marker genes were induced in *Nb* and tomato post MaSAMP treatment.

The expression levels of defense marker genes in the MaSAMP treated leaves (lower panel) and the systemic leaves (upper panel) of *Nb* and tomato. The relative expression level was analyzed by qRT-PCR and normalized to *Ubiquitin*.

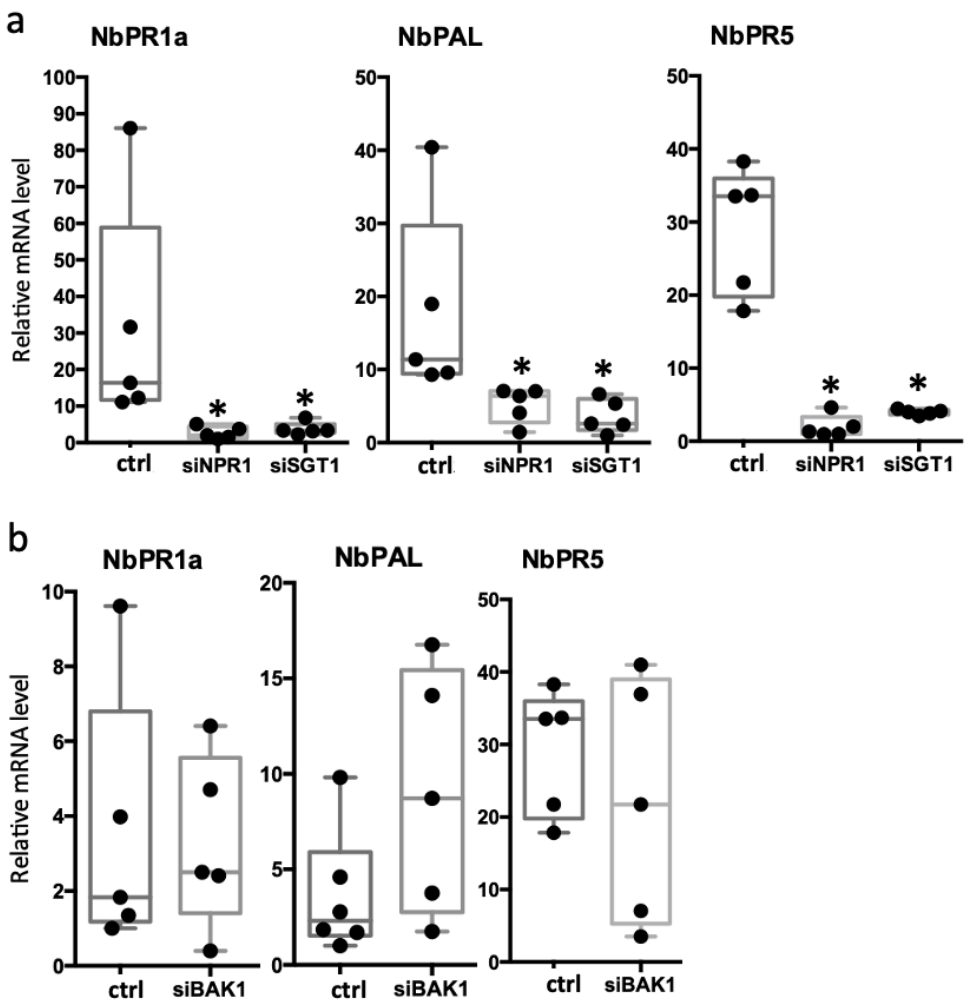

Figure S6. The immune responses activated by MaSAMP is dependent on *NPR1* and *SGT1*, but not BAK1.

a and b. Expression level of defense response genes *PR1a, PAL* and *PR5* in *Nb* plants at 20 hours post MaSAMP foliar spray treatment were analyzed by qRT-PCR and normalized to *Ubiqutin* gene (*NbUbi)*. Two leaflets per plants were collected and five plants per treatment were used. Data are shown as means ± SE. Significant difference was determined by

ANOVA Dunnett's multiple comparisons test, compared to siBR (*$P < 0.05$), the non-target vector control (ctrl).

a

	MaSAMP detected in midribs of 20 leaves (μg)	Vascular fluid collected (μl)	MaSAMP concentration (μM)
Sample1	1.30	10	22
Sample2	1.09	15	12
Sample3	1.06	18	9.8

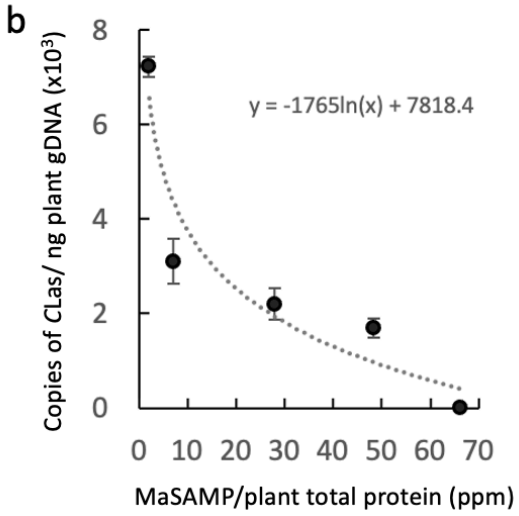

Figure S7. The MaSAMP level in leaf midribs is negatively correlated with the *C*Las titer.

a. MaSAMP content in leaf midribs of 'Madam Vinous' sweet orange (*Citrus sinensis*) trees. The lower leaves were wiped with 135μg of MaSAMP at 10 μM and the upper systemic leaves were collected for vascular fluid collection. MaSAMP levels were probed by western blot and quantified using ImageJ. b. The amount of MaSAMP was negatively correlated with *C*Las titer in the leaves of 'Madam Vinous' sweet orange trees after 6 weeks post foliar spray of MaSAMP. MaSAMP was detected by western blot after

immunoprecipitation with anti-SAMP antibodies and quantified using ImageJ. The *C*Las titer was detected by qPCR.

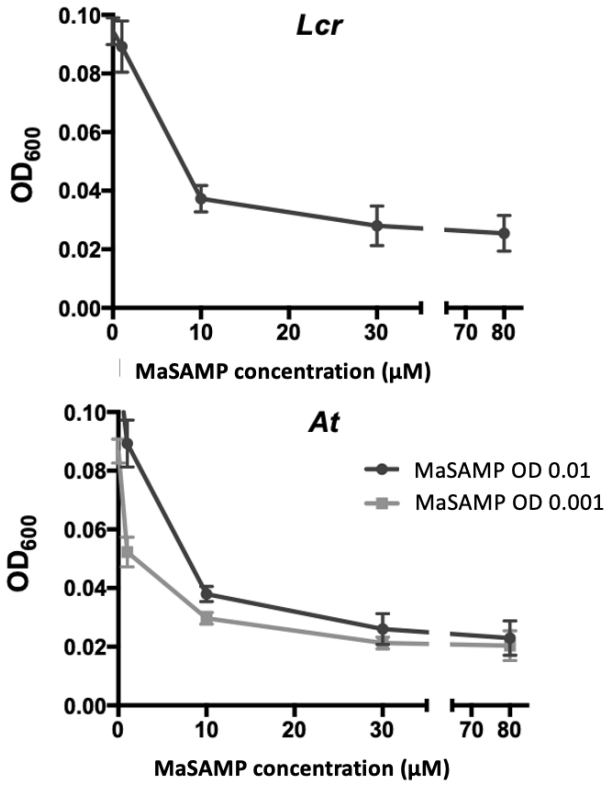

Figure S8. The minimum inhibitory concentration of MaSAMP is between 10 μM to 30 μM.

Bacteria growth assay of different concentrations of MaSAMP with *Lcr* and *At.* The initial culture of *Lcr* was started at OD_{600} 0.01 and evaluated after 4 days of growth at 25°C. The initial culture of *At* was started at OD_{600} 0.01 or 0.001 in Agrobacterium minimal medium and evaluated after 4 days of growth at 28°C.

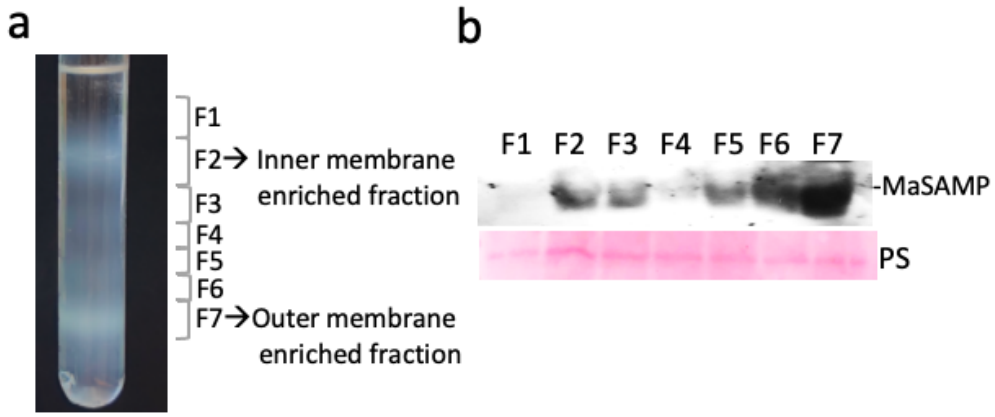

Figure S9. MaSAMP is mainly associated with the membrane fraction from *Lcr*.

a. The fraction separation of membrane from *Lcr* after ultracentrifugation. The fraction was collected according to the labeling F1 to F7.

b. MaSAMP was detected by immunblot with anti-SAMP antibody in different fractions indicated in (a). Ponceas staining (PS) was used as loading control.

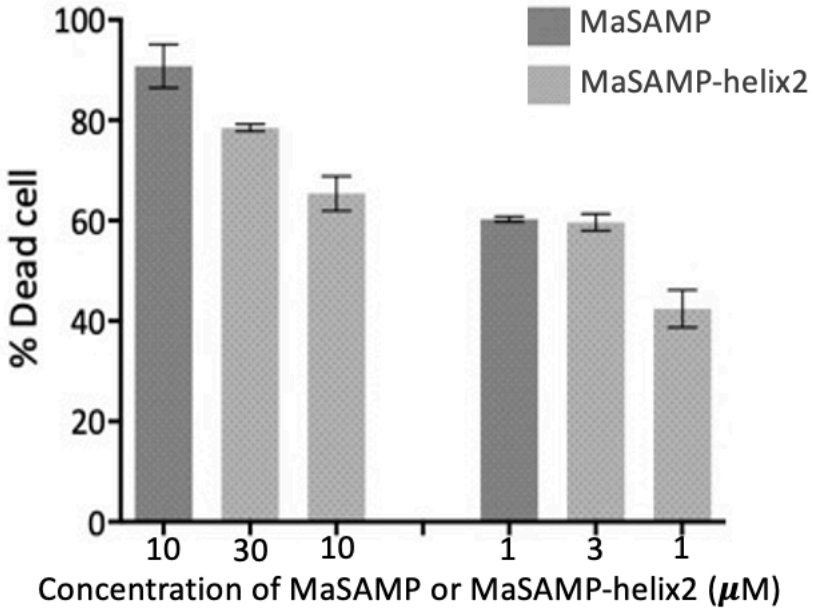

Figure S10. Tripling the amount of MaSAMP-helix2 can reach 90% of the antibacterial activity of MaSAMP. Antibacterial activity testing of different concentrations of MaSAMP and MaSAMP-helix2 using *Lcr* viability/cytotoxic assays.

Table S1. Primer list

Primer	Sequence 5'->3'	
For citrus genes relative expression level detection		
CsPR1qRTF	GACCGATGAGATGGGACAAC	
CsPR1qRTR	GTAAGGCCGTTTACCAGCA	
CsPR2qRTF	TCCACTGCCATCGAAACTG	
CsPR2qRTR	GTAATCTTGTTTAAATGAGCCTCTTG	
CsPAL1rtF	CTCGGCCCTCAGATCGAA	
CsPAL1rtR	CCGAGTTGATCTCCCGTTC	
SAMPrtF	AACAGGGGCAAGAATGTGAGCAT	
SAMPrtR	ACACGTACTGTTGTCGGTTTGTAGTCA	
CsUbiF	ACCATCGACAATGTCAAGGC	
CsUbiR	CCTTTTGGATGTTGTATTCGGC	
For *Sl* genes relative expression level		
SlGluA_rtF	CTTTTACTTGTTGGGCTTCT	
SlGluA_rtR	ACTTCCTTTGAGGGCATT	
SlGluB_rtF	GGACAGATTTCACTTCCGTAT	
SlGluB_rtR	CCAAAAGCACCAGCAGA	
SlChi9_rtF	GGAAGAGCCATTGGAGTG	
SlChi9_rtR	GCTTTGGGGATTGAGGA	
SlChi3_rtF	ACGCCATCCCCTAAAGA	
SlChi3_rtR	TGGACCCATCCCACATT	
SlPR1b1_rtF	CATCCCGAGCACAAAAC	
SlPR1b1_rtR	TGAAGTCACCACCACCCT	
SlPR-P6_rtF	GTACTGCATCTTCTTGTTTCCA	
SlPR-P6_rtR	TAGATAAGTGCTTGATGTGCC	
SlEF_rtF	GATTGGTGGTATTGGAACTGTC	
SlEF_rtR	AGCTTCGTGGTGCATCTC	
For *Nb* genes relative expression level		
NbPR1a_rtF	CCTCGTACATTCTCATGGTC	
NbPR1a_rtR	ATTGTTACACTGAACCCTAGC	
NbPR5_rtF	CCGAGGTAATTGTGAGACTGGAG	
NbPR5_rtR	CCTGATTGGGTTGATTAAGTGC	
NbPDF1.2_rtF	GGAAATGGCAAACTCCATGCG	
NbPDF1.2_rtR	TCCTTCGGTCAGACAAACG	
NbNPR1_rtF	CATCAGCGGAAGCAGTAG	
NbNPR1_rtR	GTCGGCGAAGTAGTCAAAC	
NbPAL_rtF	TTATGCTCTTAGAACGTCGCCC	
NbPAL_rtR	CCGTGTAATGCCTTGTTTCTTG	
For SAMP genes cloning		
SAMP_S_CACC	CACCATGTGCTGCAACAGGGGCAAGA	pEntry cloning
SAMP_L_CACC	CACCATGGAAGAAGCTAAAGGAGTGGTGAAG	pEntry cloning
SAMP_R	CTACTAGTACAACTCAGACACG	pEntry cloning
SAMP_S_SalF	TACGCGTCGACTGTGCTGCAACAGGGGCAAGAATG	pET28a cloning
SAMP_L_SalF	TACGCGTCGACTGGAAGAAGCTAAAGGAGTGGTGAAG	pET28a cloning
SAMP_R_XhoI	ATCCGCTCGAGTCAGACACGTACTGTTGTCGG	pET28a cloning
For NbNPR1 VIGS		
NbsiNPR1_attB1	AAAAAGCAGGCTTC TACTGGATATTCTTGACAAAATTGC	
NbsiNPR1_attB2	AGAAAGCTGGGTGTTA GCAACATCTGCAGTAATTCAACAT	

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