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Supplementary Information for

Unexpected synergistic HIV neutralization by a triple microbicide produced in rice endosperm

Evangelia Vamvaka, Gemma Farré, Luis M Molinos-Albert, Abbey Evans, Anna Canela-Xandri, Richard M. Twyman, Jorge Carrillo, Raziel A. Ordóñez, R. Shattock, Barry R. O'Keefe, Bonaventura Clotet, Julian Blanco, Gurdev S Khush, P. Christou, T. Capell

Gurdev S Khush, P. Christou, T. Capell
Email: gurdev@khush.org, teresa.capell@pvcf.udl.cat; christou@pvcf.udl.es

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Supplementary Information Text

Supplementary Methods

Method S1 – HIV-neutralization assays. Serially diluted samples and controls were pre-incubated with 200 $TCID_{50}$ of pseudovirus stock for 1 h at 37ºC, in 96-well culture plates. We then added 10,000 TZM-bl luciferase-reporter target cells to each well. The plates were incubated at 37ºC in a 5% CO_2 atmosphere for 48 h. The TZM-bl cells were treated with dextran (Sigma-Aldrich, St Louis, MO, USA) to enhance infectivity. The luciferase substrate Britelite Plus (PerkinElmer Inc., Waltham, MA, USA) was used for the readout. Percent neutralization was determined by calculating the difference in average relative light units (RLUs) between virus control (cells + virus) and test wells (cells + sample + virus), dividing this result by the difference in average RLUs between the virus control and cell control wells and multiplying the result by 100. Neutralizing titers are expressed as the reciprocal dilution necessary to reduce the RLUs by 50% (IC_{50}). Alternatively, the actual inhibitor concentration was calculated for each dilution to determine the apparent inhibitory concentration 50 (IC_{50}). Failure to score at least 50% reduction of RLUs at any dilution was considered as a negative test. All data were fitted to an inhibitor vs. response (three parameters) model using GraphPad Prism v6 (GraphPad Software Inc., San Diego, CA, USA).

Method S2 – Preparation of expression constructs. The 2G12 transformation constructs were based on the binary vector pTRA, a derivative of pPAM (GenBank accession no. AY027531). The vector contains two tobacco RB7 scaffold attachment regions flanking the expression cassette (obtained from Dr. T. Rademacher, Fraunhofer IME, Aachen, Germany). The coding regions of the 2G12 heavy and light chains (obtained from Polymun, Vienna, Austria) included N-terminal signal sequences targeting the secretory pathway. The expression cassette comprised the endosperm-specific rice glutelin-1 promoter, the maize ubiquitin-1 first intron, the Tobacco etch virus 5′ leader which acts as a translational enhancer, the coding region, and the Cauliflower mosaic virus 35S terminator, resulting in final constructs pTRAgtiGH and pTRAgtiGL. The CV-N (N30Q/P51G) gene in vector pET30b was amplified by PCR in a 50-µL reaction comprising 1.25 units of GoTaq polymerase in the appropriate buffer (Promega Corp., Fitchburg, WI, USA), 1 µM each of forward primer 5′-GGG ATC CAT GCT TGG TAA ATT CTC CCA G-3′ and reverse primer 5′-CCG AAT TCT TAT TCG TAT TTC AGG GTA CCG-3′ (underlined nucleotides represent BamHI and EcoRI restriction sites), 0.2 mM of each dNTP and 250 ng template DNA. The reaction was heated to 94°C for 3 min followed by 35 cycles at 94°C for 45 s, 60°C for 45 s and 72°C for 3 min, and a final extension step at 72°C for 10 min. The products were transferred to the shuttle vector pGEM-T Easy (Promega) and introduced into competent *E. coli* cells, which were incubated overnight at 37°C under ampicillin selection. The integrity of the plasmid DNA was confirmed by sequencing (Universidad Autónoma de Barcelona, Barcelona, Spain) before digestion with BamHI and EcoRI to release the expression cassette, which was then inserted into vector pRP5 containing the endosperm-specific rice prolamin RP5 promoter and the rice α-amylase 3A signal peptide sequence (GenBank accession no. CAA39776). The GRFT gene in vector pET-28a(+) was prepared by PCR in the same manner as above using 1 µM each of forward primer 5′-TGC ATG CAT GGG CAG CAG CCA TCA T-3′ and reverse primer 5′-GGG GAG CTC TTA GTA CTG TTC ATA GTA G-3′ (the underlined nucleotides are SphI and SacI restriction sites introduced to facilitate cloning). The verified pGEM-T Easy shuttle

vector was digested with SphI and SacI to release the expression cassette, which was then inserted into vector pgZ63 containing the endosperm-specific maize γ-zein promoter which also functions in rice, the rice α-amylase 3A signal peptide sequence and a sequence encoding a N-terminal His_6 affinity tag.

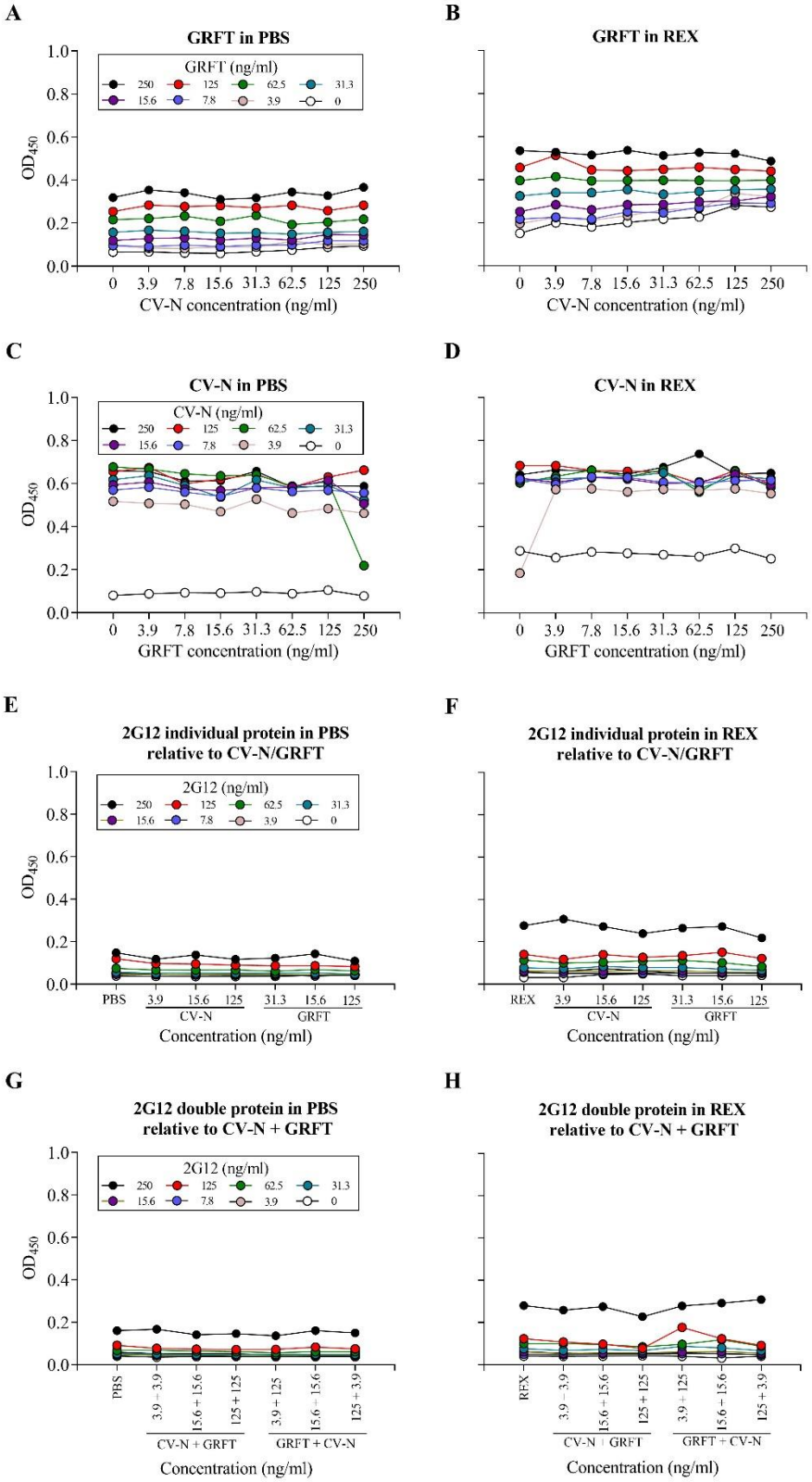
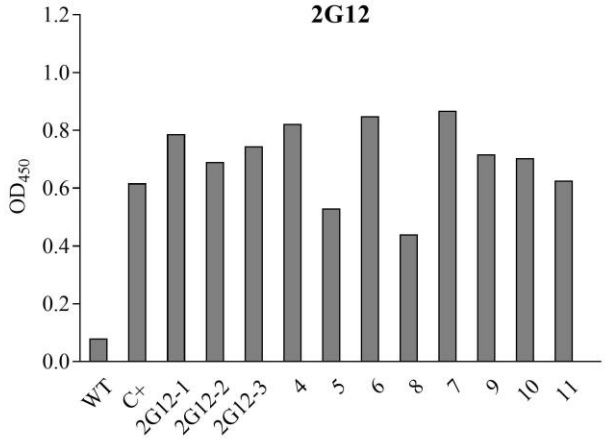
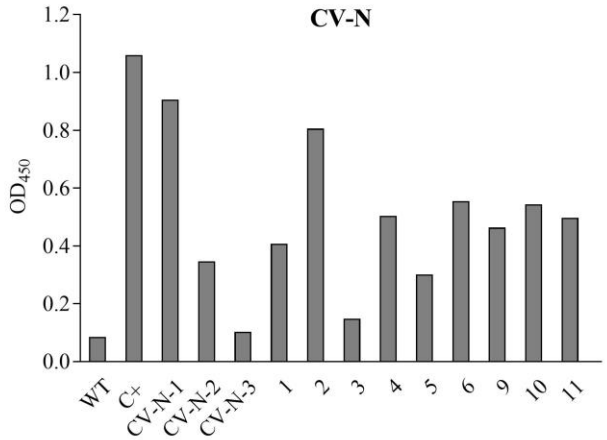

A
GRFT in PBS
GRFT (ng/ml)
250
125
62.5
31.3
15.6
7.8
3.9
0
OD450
CV-N concentration (ng/ml)
B
GRFT in REX
CV-N concentration (ng/ml)
C
CV-N in PBS
CV-N (ng/ml)
GRFT concentration (ng/ml)
D
CV-N in REX
GRFT concentration (ng/ml)
E
2G12 individual protein in PBS relative to CV-N/GRFT
2G12 (ng/ml)
PBS
CV-N
GRFT
Concentration (ng/ml)
F
2G12 individual protein in REX relative to CV-N/GRFT
REX
G
2G12 double protein in PBS relative to CV-N + GRFT
CV-N + GRFT
GRFT + CV-N
H
2G12 double protein in REX relative to CV-N + GRFT

Fig. S1. Competition ELISA experiments. GRFT/CV-N competition ELISA in PBS (A, C) or rice endosperm extract (REX) (B, D) probed with antibodies against GRFT or CV-N. (A, B) GRFT binding at increasing concentrations of CV-N (colored lines show different concentrations of GRFT). (C, D) CV-N binding at increasing concentrations of GRFT (colored lines show different concentrations of CV-N). 2G12/individual-lectin competition ELISA in PBS (E) or REX (F) probed with antibodies against 2G12. (G, H) 2G12/double-lectin competition ELISA in PBS (G) or REX (H) probed with antibodies against 2G12. 2G12 binding with no lectin (lane 1), at increasing concentrations of CV-N (lanes 2–4) and at increasing concentrations of GRFT (lanes 5–7) (colored lines show different concentrations of 2G12).

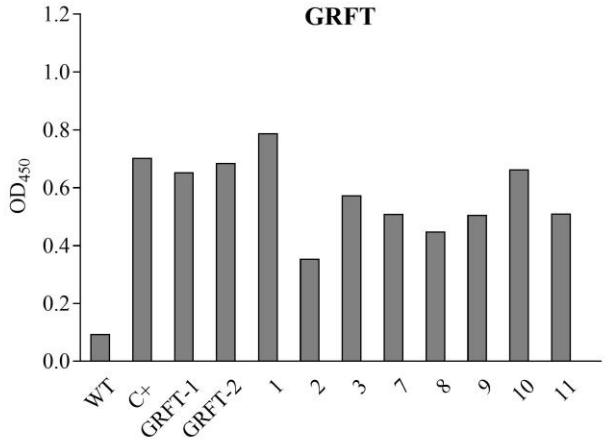
A



B



C



Independent events

Fig. S2. Binding activity of crude extracts from all transgenic lines containing ^{OS}CV-N, ^{OS}GRFT and ^{OS}2G12 alone or in combination with gp120. (A) Binding activity of 2G12. (B) Binding activity of CV-N. (C) Binding activity of GRFT. Numbers on the *x*-axis refer to the transgenic lines listed in Table 2. C+ = ^{EC}CV-N, ^{EC}GRFT or ^{OS}2G12 positive controls (starting concentration 250 ng/mL) where EC = *Escherichia coli* and OS = rice (*Oryza sativa*). WT = wild-type endosperm extracts as a negative control. OD_{450} = optical density at 450 nm. Representative lines are shown in Figure 2.