Supplementary Data, Table and figures

Table S1: qRT-PCR of lung cells of IFNAR$^{-/-}$ mice vaccinated with plant produced chimaeric AHSV-1/5 VLPs or AHSV-5 VP2, 1X PBS buffer as negative control and OBP binary ethyleneimine (BEI) inactivated AHSV-5 monovalent vaccine as positive control. Group 0 were non-vaccinated and non-challenged, only serum samples taken at specific time points) were not detected (ND) whilst mice in group 3 (1X PBS plus adjuvant) all the mice died after the challenge and the lung samples not harvested at the time. Higher Cycle threshold (Ct) values are indicative of virus suppression.

Group 1	Animal IDs	Ct	Ct	AVG	Overall AVG
1 VLPs	1.1	ND	ND		
	1.2	38.68	38.59	38.64 ± 0.032	
	1.3	38.46	ND	38.46	
	1.4	38.52	38.93	38.73 ± 0.145	
	1.5	ND	37.52	37.52	
	1.6	ND	ND		38.34 ± 0.554
2 VP2	2.1	34.87	34.19	34.53 ± 0.24	
	2.2	ND	ND		
	2.3	ND	38.78	38.78	
	2.4	ND	ND		
	2.5	33.17	33.61	33.39 ± 0.156	
	2.6	31.64	31.85	31.75 ± 0.074	34.61 ± 3.005
4 OBP BEI	4.1	28.36	28.41	28.39 ± 0.018	
	4.3	38.52	ND	38.52	33.45 ± 7.167

Ct = Cycle threshold

Soluble AHSV-5 VP2, sample 1: 77% coverage, 206 unique peptides

MASEFGVLLTDK**VEGDALEKTNCEVILTR**SGRVRRREVDGVR**GYEWEFTDHRLGLCEVEHTMSM**
ADFFYNQIKCEGAYPIFPHYITDVLKYGKMVDR**NDHQVRVDRDVK**ELSK**ILIQPYFGEAYFSPEFYT**
STFLKRQAIQMNVEMLRAFVPK**RVAFYEDDMRRGSTINGNWIGALQAWKK**KADLQMSREGNSQ
TNCVDHNADVIYQHMKKLR**FGLLYPHYYMLNSEYTVEEKSKGGLIANWLVK**EKTAGR**AEDSPM**
YSGVGPLNTLRERIEKDELDEKVIQEIIAYGSKFSTYAGTRAGDLTLNELVKYCESLTTFVHKKKK
EGEDETAREFFKSKWVQGMPKMNFENEMIMSRKSWANTK**FFWSIDMFKRNNGVDIDPNGKNW**
KDYKK**KVQEQLDEAQKKNNNEPYKVMVDGVNIMTNKKYGSVENWVDWVVNYIMLSHVK**RLVK
DYKFK**RLKPDNLMSGMNKLVGALR**CYAYCLILALYDYFGEDIEGFK**KGTNAASIVETVSQMFPQF**
RKEVSETFGITLNTKDVKYELFVARDMSAGEAQFGEVGYRFQYGWR**KTDQR**VMSDYADILSEKV**
EALYQALLSGRKWSDIADDTEEYFIDDLYVNKPDRVFERAGLNPERHIK**VKGVMNELTTYFSKRFI**
SYWYKITKVEAR**DLLTLTDIGGDAKKYTQFDPDDFKPMAVAELGAHASTYVYQNLILGR**NRGEK**I**
DDAKEIVWYDLSLTNFECSRSLDSCWVGSVAR**SELNLR**FHLVSAIFER**YQHDAR**RSSFYEIIFDLPS**
RKER**IFPSYKHYYVALLQNIFNDTQRLEVMDYCERLMNPETRMSALLSLQGFRNCVESEFVAPTL**
KMNALLWVLADMENIDINYSNKRMPLLLSTEKGLRVISIDMFNGMLGVSYSGWIPYLERICSEVN
LQRRLRADELKLK**KWFISYYATYEVERR**AEPRMSFK**MEGISTWIGSNCGGVQDYVLHLIPSR**KPKP
GLLFLIYADDGDVGWVANMLSDVIGSEGSLGFIFINDR**TFVNKSQLK**VRTLKIYNRGMLDR**LILISGG**
NYTFGNKFLLSKLLAK**TEK**

Soluble AHSV-5 VP2, sample 2: 86.8% coverage, 235 unique peptides

MASEFGVLLTDK**VEGDALEKTNCEVILTR**SGRVRR**REVDGVRGYEWEFTDHRLGLCEVEHTMSM**
ADFFYNQIKCEGAYPIFPHYITDVLKYGKMVDR**NDHQVRVDR**DVKELSK**ILIQPYFGEAYFSPEFYT**
STFLKRQAIQMNVEMLRAFVPK**RVAFYEDDMRRGSTINGNWIGALQAWKK**KADLQMSR**EGNSQ**
TNCVDHNADVIYQHMKKLRFGLLYPHYYMLNSEYTVEEKSKGGLIANWLVKEKTAGRAEDSPM

YSGVGPLNTLRERIEKDELDEKVIQEIIAYGSKFSTYAGTRAGDLTLNELVKYCESLTTFVHKKKK
EGEDETAREFFKSKWVQGMPKMNFENEMIMSRKSWANTKFFWSIDMFKRNNGVDIDPNGKNW
KDYKKKVQEQLDEAQKKNNNEPYKVMVDGVNIMTNKKYGSVENWVDWVVNYIMLSHVKRLV
KDYKFKRLKPDNLMSGMNKLVGALRCYAYCLILALYDYFGEDIEGFKKGTNAASIVETVSQMFP
QFRKEVSETFGITLNTKDVKYELFVARDMSAGEAQFGEVGYRFQYGWRKTDQRVMSDYADILSE
KVEALYQALLSGRKWSDIADDTEEYFIDDLYVNKPDRVFERAGLNPERHIKVKGVMNELTTYFSK
RFISYWYKITKVEARDLLTLTDIGGDAKKYTQFDPDDFKPMAVAELGAHASTYVYQNLILGRNRG
EKIDDAKEIVWYDLSLTNFECSRSLDSCWVGSVARSELNLRFHLVSAIFERYQHDARRSSFYEIIFDL
PSRKERIFPSYKHYYVALLQNIFNDTQRLEVMDYCERLMNPETRMSALLSLQGFRNCVESEFVAP
TLKMNALLWVLADMENIDINYSNKRMPLLLSTEKGLRVISIDMFNGMLGVSYSGWIPYLERICSE
VNLQRRLRADELKLKKWFISYYATYEVERRAEPRMSFKMEGISTWIGSNCGGVQDYVLHLIPSRK
PKPGLLFLIYADDGDVGWVANMLSDVIGSEGSLGFIFINDRTFVNKSQLKVRTLKIYNRGMLDRLI
LISGGNYTFGNKFLLSKLLAKTEK

AHSV-5 VP2 assembled in a chimaeric AHSV-1/5 VLP, sample 3: 73% coverage, 144 unique peptides of AHSV-5 VP2

MASEFGVLLTDKVEGDALEKTNCEVILTRSGRVRRREVDGVRGYEWEFTDHRLGLCEVEHTMSM
ADFFYNQIKCEGAYPIFPHYITDVLKYGKMVDRNDHQVRVDRDVKELSKILIQPYFGEAYFSPEFY
TSTFLKRQAIQMNVEMLRAFVPKRVAFYEDDMRRGSTINGNWIGALQAWKKKADLQMSREGNS
QTNCVDHNADVIYQHMKKLRFGLLYPHYYMLNSEYTVEEKSKGGLIANWLVKEKTAGRAEDSP
MYSGVGPLNTLRERIEKDELDEKVIQEIIAYGSKFSTYAGTRAGDLTLNELVKYCESLTTFVHKKK
KEGEDETAREFFKSKWVQGMPKMNFENEMIMSRKSWANTKFFWSIDMFKRNNGVDIDPNGKNW
KDYKKKVQEQLDEAQKKNNNEPYKVMVDGVNIMTNKKYGSVENWVDWVVNYIMLSHVKRLVK
DYKFKRLKPDNLMSGMNKLVGALRCYAYCLILALYDYFGEDIEGFKKGTNAASIVETVSQMFPQFR
KEVSETFGITLNTKDVKYELFVARDMSAGEAQFGEVGYRFQYGWRKTDQRVMSDYADILSEKVE
ALYQALLSGRKWSDIADDTEEYFIDDLYVNKPDRVFERAGLNPERHIKVKGVMNELTTYFSKRFIS
YWYKITKVEARDLLTLTDIGGDAKKYTQFDPDDFKPMAVAELGAHASTYVYQNLILGRNRGEKID
DAKEIVWYDLSLTNFECSRSLDSCWVGSVARSELNLRFHLVSAIFERYQHDARRSSFYEIIFDLPSR
KERIFPSYKHYYVALLQNIFNDTQRLEVMDYCERLMNPETRMSALLSLQGFRNCVESEFVAPTLK
MNALLWVLADMENIDINYSNKRMPLLLSTEKGLRVISIDMFNGMLGVSYSGWIPYLERICSEVNL
QRRLRADELKLKKWFISYYATYEVERRAEPRMSFKMEGISTWIGSNCGGVQDYVLHLIPSRKPKPG
LLFLIYADDGDVGWVANMLSDVIGSEGSLGFIFINDRTFVNKSQLKVRTLKIYNRGMLDRLILISGGNY
TFGNKFLLSKLLAKTEK

AHSV-5 VP2 assembled in a chimaeric AHSV-1/5 VLP, sample 4: AHSV-5 VLPs VP2 77.4% coverage, 133 unique peptides

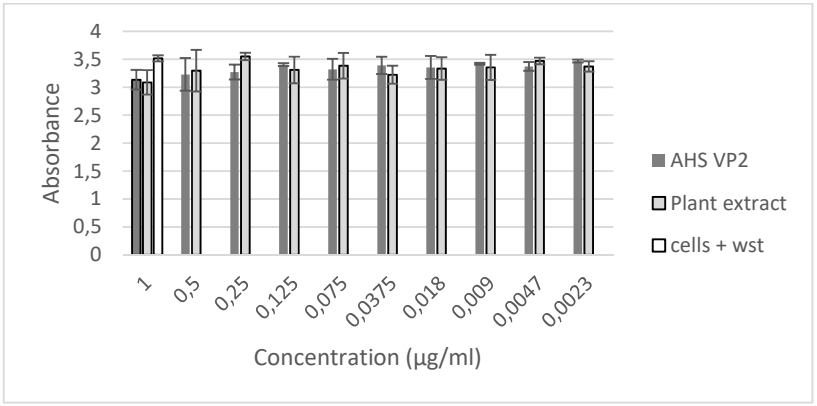
MASEFGVLLTDKVEGDALEKTNCEVILTRSGRVRRREVDGVRGYEWEFTDHRLGLCEVEHTMSM
ADFFYNQIKCEGAYPIFPHYITDVLKYGKMVDRNDHQVRVDRDVKELSKILIQPYFGEAYFSPEFYT
STFLKRQAIQMNVEMLRAFVPKRVAFYEDDMRRGSTINGNWIGALQAWKKKADLQMSREGNSQT
NCVDHNADVIYQHMKKLRFGLLYPHYYMLNSEYTVEEKSKGGLIANWLVKEKTAGRAEDSPMYS
GVGPLNTLRERIEKDELDEKVIQEIIAYGSKFSTYAGTRAGDLTLNELVKYCESLTTFVHKKKKE
GEDETAREFFKSKWVQGMPKMNFENEMIMSRKSWANTKFFWSIDMFKRNNGVDIDPNGKNWK
DYKKKVQEQLDEAQKKNNNEPYKVMVDGVNIMTNKKYGSVENWVDWVVNYIMLSHVKRLVKD
YKFKRLKPDNLMSGMNKLVGALRCYAYCLILALYDYFGEDIEGFKKGTNAASIVETVSQMFPQFRK
EVSETFGITLNTKDVKYELFVARDMSAGEAQFGEVGYRFQYGWRKTDQRVMSDYADILSEKVEA
LYQALLSGRKWSDIADDTEEYFIDDLYVNKPDRVFERAGLNPERHIKVKGVMNELTTYFSKRFISY
WYKITKVEARDLLTLTDIGGDAKKYTQFDPDDFKPMAVAELGAHASTYVYQNLILGRNRGEKID
DAKEIVWYDLSLTNFECSRSLDSCWVGSVARSELNLRFHLVSAIFERYQHDARRSSFYEIIFDLPSR
KERIFPSYKHYYVALLQNIFNDTQRLEVMDYCERLMNPETRMSALLSLQGFRNCVESEFVAPTLK
MNALLWVLADMENIDINYSNKRMPLLLSTEKGLRVISIDMFNGMLGVSYSGWIPYLERICSEVNL
QRRLRADELKLKKWFISYYATYEVERRAEPRMSFKMEGISTWIGSNCGGVQDYVLHLIPSRKPKP
GLLFLIYADDGDVGWVANMLSDVIGSEGSLGFIFINDRTFVNKSQLKVRTLKIYNRGMLDRLILIS
GGNYTFGNKFLLSKLLAKTEK

Figure S1: Protein confirmation using LC-MS/MS based peptide sequencing of AHSV-5 VP2 as assembled in a VLP and soluble VP2 proteins. The images above show the capsid protein peptides and sequence coverage for AHSV-5 VP2. LC-MS/MS based peptide sequence analysis for excised bands resulted in the percentage sequence

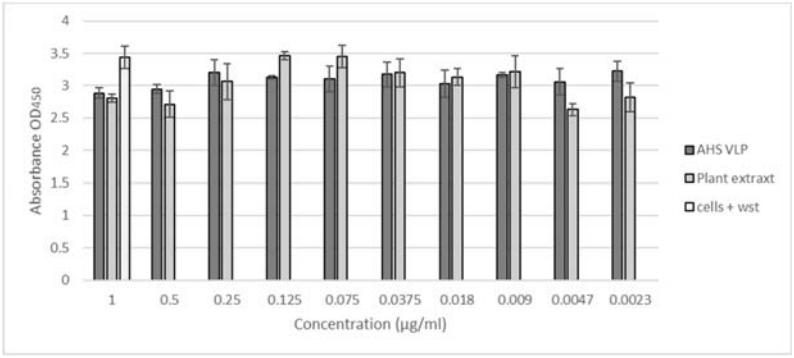
coverage as indicated with a certain number of unique peptides identified with >95% confidence. Peptides with >95% confidence are highlighted in green, those 50-95% confidence in yellow and <50% confidence in red. No peptides were identified for the non-highlighted regions of the sequence (grey).

Cytotoxicity study

Vero (African green monkey kidney, ATCC® CCL-81™) cells were cultured in tissue culture flasks in complete Dulbecco's Modified Eagle Medium (DMEM) (Gibco) (10% foetal calf serum (FCS) (Gibco), 1% of each penicillin and streptomycin) at 37°C and 5% CO_2. Cells (50 000 cells/well) were seeded in microplates (tissue-culture grade, 96 wells, flat bottom) in a final volume of 100 µl/well culture medium (complete DMEM) and incubated in a humidified atmosphere, 37°C and 5% CO_2 for 24 hours. After 24 hours the media was removed and the various compounds and partially purified plant extracts (1 µg to 2.3 ng) in different concentrations were added to a final volume of 100µl per well. The plates were again incubated as described before for 24 hours. After this incubation period, 10µl of the cell proliferation reagent WST-1 (CELLPRO-RO Cell Proliferation Reagent WST-1, Sigma) was added to each well. The plates were incubated at 37°C and 5% CO_2 for 3 hours after which the absorbance of the formazan product was measured at 440nm in an ELISA plate reader. Both media alone or cells with WST-1 served as negative controls. The assay is based on the cleavage of the slightly red tetrazolium salt WST-1 to form a dark red formazan dye by metabolically active cells and thus only occurs in viable cells. As the formazan dye formed is soluble in aqueous solutions, it is directly quantified using a scanning multi-well spectrophotometer (ELISA reader).



Concentration (µg/ml); p < 0.05 = significant										
T-test	**1**	**0.5**	**0.25**	**0.125**	**0.075**	**0.0375**	**0.018**	**0.009**	**0.0047**	**0.0023**
AHS VP2 vs plant extract	0.7893	0.8352	0.0762	0.6212	0.7338	0.3345	0.9238	0.7277	0.2942	0.2961
AHS vs cells + wst	0.0646	0.2805	0.0968	0.1211	0.2624	0.3880	0.3888	0.1368	0.1626	0.3630



Concentration (µg/ml); p < 0.05 = significant										
T-test	**1**	**0.5**	**0.25**	**0.125**	**0.075**	**0.0375**	**0.018**	**0.009**	**0.0047**	**0.0023**
AHS VLP vs plant	0.2843	0.1259	0.5346	0.0035	0.1344	0.8999	0.5178	0.7862	0.0724	0.0866
AHS vs cells + wst	0.0073	0.0102	0.1845	0.0372	0.0872	0.1501	0.0594	0.1239	0.0682	0.1765

Figure S2: Cytotoxicity studies in Vero cells validating both plant produced AHSV-5 VLPs and soluble VP2.

A cytotoxicity study in Vero cells was conducted to confirm the safety of the plant produced and partially purified VLPs and VP2 of concentrations ranging from 1 µg to 2.3 ng serial dilutions per well, prior to the pre-clinical trials. This was achieved by means of a colorimetric cell proliferation assay based on tetrazolium salt (WST-1), which is reduced to water-soluble orange formazan in viable cells by cellular mitochondrial dehydrogenase. The method was done as per the manufacturer's instructions. The results indicated that with all the concentrations tested for both the VLP and VP2 antigens, that there were less than 20% decrease in cell viability as compared to the standard control.