VAX4ASF

NEW TECHNOLOGIES FOR AFRICANSWINE FEVER VACCINES

[D2.1] ASFV genes: Identification of ASFV genes modulating type I IFN production and signalling in stimulated BSRT7 /in HEK-293T cells

PROJECT FUNDED BY THE EUROPEAN COMMISSION		
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DELIVERABLE INFORMATION	
Deliverable Nº	D2.1
Deliverable Title	ASFV genes: Identification of ASFV genes modulating type I IFN production and signalling in stimulated BSRT7 /in HEK-293T cells
WP Nº	2
Lead beneficiary	CBMSO
Contributing partners	-
Type	R
Due Submission Date:	31/12/2024
Actual Submission Date:	19/12/2024

REVISION HISTORY	
DATE	**AUTHORS AND CONTRIBUTORS**
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This project has received funding from the European Union's Horizon Europe research and innovation programme under grant agreement No 101136439

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1. *In silico* identification and cloning of ASFV genes potentially involved in type I IFN control and virulence.

In order to identify ASFV genes involved in virulence and in type I IFN control, we previously performed an *in silico* analysis, comparing the genomes of several virulent and attenuated or partially attenuated strains of different genotypes. In particular, we have compared eight genomes belonging to either genotype I or genotype II: three from attenuated strains (NH/P68, OURT 88/3 and Estonia 2014), four from virulent strains (L60, E75, Georgia 2007/1, China 2018) and one Vero-adapted strain (Ba71V). A blastp was made using the protein sequences of all these genomes as the database and as the query, to compare all sequences to each other. We have used the Markov Cluster Algorithm (MCL) with this output, an algorithm for large-scale detection of protein families, using an inflation value of 4, resulting in 165 cluster of proteins comparing 8 strains. We have then identify those genes coding for proteins absent in attenuated strains (from genotype I, NH/P68 and OURT 88/3, 9 de 20 and/or from genotype II, Estonia 2014) and present in virulent strains analyzing each of these clusters. Thus, we identified 33 genes that were absent or modified in attenuated strains. In addition, we identified genes absent in an attenuated vaccine prototype, previously generated at the CBM (CSIC), where more than 40 genes were spontaneously deleted, most of them members of the so-called multigene families (MGFs) (Pérez-Núñez, et al., 2024, PMDI: 39460292). Finally, we chose those genes that were present in both groups, identifying up to 20 genes potentially involved in the control of type I IFN, specifically related to ASFV virulence.

The identified genes are the follows: MGF110-11L, MGF110-13L, MGF110-1L, MGF110-2L, MGF110-4L, MGF110-5L-6L, MGF110-9L, MGF360-10L, MGF360-11L, MGF360-6L, MGF360-1L, MGF360-2L, MGF360-3L, ASFV-G-ACD-00160, ASFV-G-ACD-00090, ASFV-G-ACD-0190, ASFV-G-ACD-00210, KP177R, L83L and L60L. In addition, we have added four other genes that were identified as potential type I IFN modulators in a previous EU ICRAD-funded proyect ICRAD: MGF100-1R, MGF100-3L, MGF360-4L, MGF360-15R, MGF505-1R and MGF505-3R.

Once these genes were identified, they were cloned into expression vectors, specifically, the pcDNA-myc vector (Thermofisher). This vector has a “myc” tag at the 5' end of the gene, so that it will later be possible to detect the expression of the protein of interest by Western Blot using specific antibodies against “myc”. For cloning, we use InFusion technology (Clontech). Briefly, specific PCRs are performed to (i) linearize the vector in the MCS, and (ii) to amplify the different genes, where the oligos will have specific terminations to hybridize with the vector, according to the indications of the InFusion technology. After purification of the PCR products, they are incubated with the InFusion solution, after which the resulting product is used to transform DH5α competent *Escherichia coli* bacteria. The cloned vectors were sequenced by Sanger (Macrogen) using specific oligos to check sequence accuracy.

Among the ASFV genes cloned are: MGF110-2L (used as expression control), MGF110-4L, MGF110-5L-6L, MGF360-6L, MGF360-10L, MGF360-11L, MGF110-11L or MGF110-13L. In addition, the cloning of some other genes, such as MGF100-1R, MGF100-3L, MGF360-4L, MGF360-15R, MGF505-1R and MGF505-3R, is still underway. Once the sequence fidelity of each vector was verified with regard to the ASFV-specific genes, these vectors were used for ectopic expression in different cellular systems, as detailed in the following section.

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2. Ectopic expression of cloned ASFV genes in different expression systems.

Having identified and cloned a number of ASFV genes potentially involved in ASFV virulence, we will now identify those involved in the control of type I IFN through different screenings. For this purpose, ectopic expression of these genes in different cell systems, in particular HEK-293T cells and BSRT7 cells, is necessary. The pcDNA-MGF110-2L-myc vector, previously cloned and expressed at the CBM (CSIC), was used as expression control. Genes expressed in HEK-293T cells could be tested for their type I IFN control by both luciferase and qPCR assays. Those genes that are only expressed in BSRT7 by co-infection with VV-T7 could only be tested by qPCR.

First, we transfected the different vectors into HEK-293T cells, using Metafecten transfectant (Biontex) according to the manufacturer's instructions, at a ratio of 1µg DNA/10^6 cells. At 24h post transfection (hpt), transfected cells were collected and lysed using RIPA buffer (50 mM Tris-HCl pH 7.5, 1% NP40, 0.25% Na-deoxycolate, 150 mM NaCl, 1 mM EDTA) supplemented with protease and phosphatase inhibitors (Roche). Protein extracts were quantified using the Pierce BCA Protein Assay kit, based on the spectrophotometric acid spectrophotometric method (Thermo Scientific). Cell lysates (30 µg total protein) were fractionated by SDS-PAGE and electrophoretically transferred to an Immobilon extra membrane (Amersham) and the separated proteins were reacted with anti-myc primary antibody (Cell signaling, ref: 9B11) at 1/1000 dilution. The membranes were exposed to peroxidase-conjugated secondary anti-myc anti-mouse antibody (1/2000 dilution) followed by chemiluminescence (ECL, Amersham Biosciences) by autoradiography.

As shown in Figure 1A, expression of the ASFV genes MGF110-4L, MGF110-5L-6L is visible by Western blot, in addition to that of the MGF110-2L gene, which was used as a positive control. However, no expression of MGF110-11L, MGF110-13L, MGF360-6L, MGF360-10L or MGF360-11L genes was obtained in HEK-293T cells.

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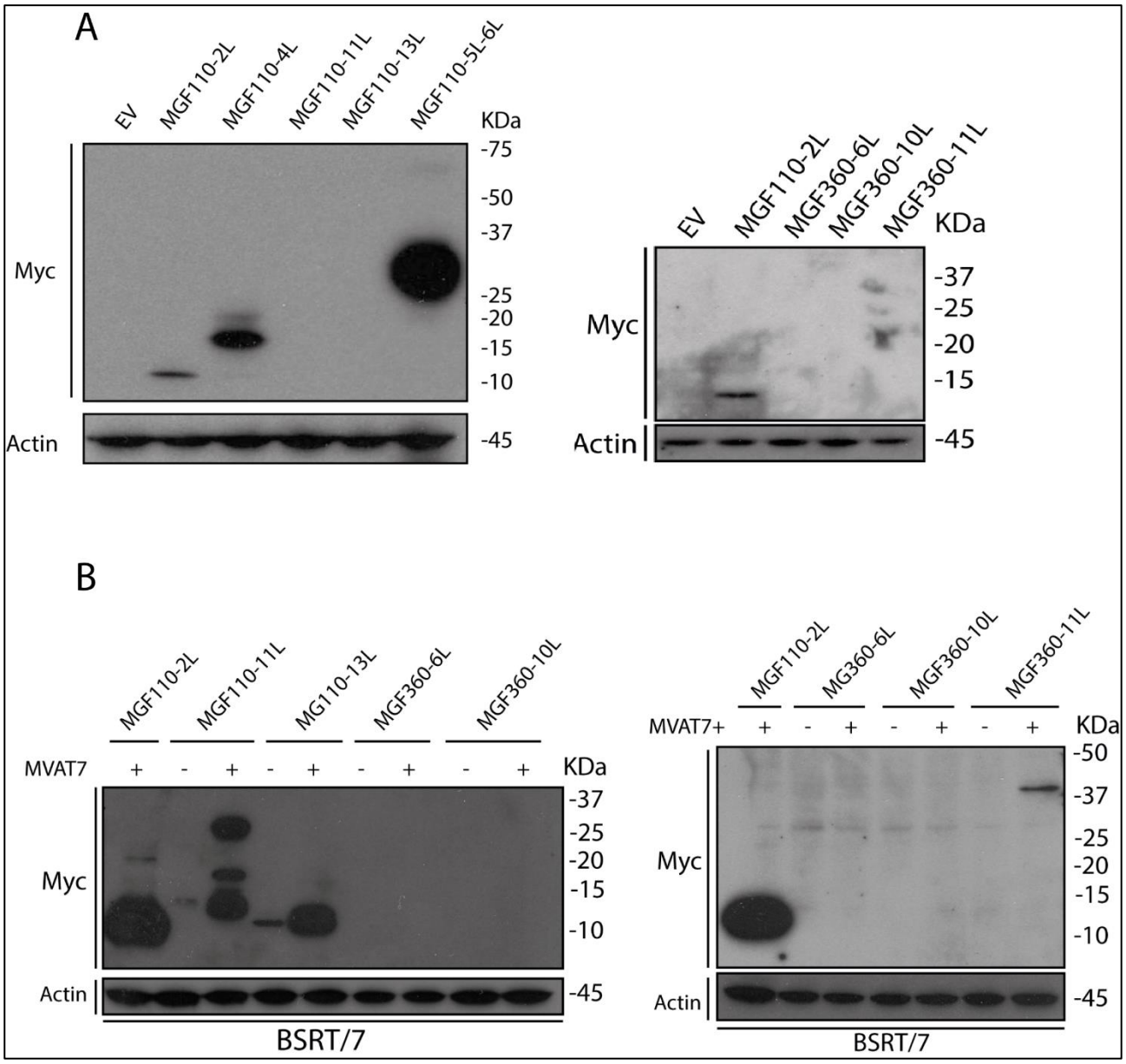

Figure 1. Ectopic expression of ASFV genes in HEK-293T cells (A) or BSRT7 cells (B). A: HEK-293T cells were transfected with the expression vectors specific for each viral gene, and at 16h were collected for Western blotting. B: BSRT7 cells were transfected with the specific expression vectors for each viral gene, and at 6h were infected with VV-T7 (MOI=0.5) for 16h. All samples were lysed and treated for Western blot, analyzed by (A) anti-myc (1/1000) and anti-actin (1/6000).

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These genes that were not expressed in HEK-293T cells were tested for expression in BSRT7 cells co-infected with VV-T7. Co-infection with VV-T7 has been widely used for ectopic expression of ASFV genes (Pérez-Núñez et al., 2023; PMID: 37768082). For this, cells were similarly transfected with Metafecten (Biontext) following the manufacturer's instructions, followed, at 6hpt, by infection with VV-T7 at MOI=0.5 for 16h. Samples were then collected as indicated above to determine the expression of each gene by Western blot using anti-myc antibody. Thus, as can be seen in Figure 1B, the MGF110-11L, MGF110-13L and MGF360-11L genes could be expressed, although only during co-infection with VV-T7. In contrast, expression of the MGF360-6L and MGF360-10L genes was not possible under any of these conditions, so that alternative expression systems to pcDNA-myc would be necessary.

In conclusion, we have identified a number of ASFV genes potentially involved in type I IFN control, which have been cloned into expression vectors. Of these, in addition to the positive control MGF110-2L, we have achieved successful expression in HEK-293T of the MGF110-4L and MGF110-5L-6L genes. In addition, we have alternatively achieved expression in BSRT7 co-infected with VV-T7 of the MGF110-11L, MGF110-13L and MGF360-11L genes.

3. *In vitro* screening for the identification of ASFV genes involved in type I IFN control.

From genes that were successfully expressed in HEK-293T cells, we performed luciferase assays to determine whether these ASFV genes are able to downregulate type I IFN expression. For this, we used a system whereby HEK-293T cells are transfected with: luciferase reporter (consisting of luciferase gene cloned under IFN-β promoter) 100ng/10^6 cells; Renilla vector (used to normalize the real amount transfected from each sample) 50ng/10^6 cells. In addition, we also transfected 100ng/10^6 cells from the TBK-1 protein expression vector, which we used to stimulate the IFN-β promoter. Finally, we transfect the ASFV gene we want to analyze (2µg/10^6 cells) or, alternatively, the empty vector (pcDNA-myc) under the same conditions. Cells are transfected with FuGEN (Promega) 24h and then lysed using the Luc-Pair™ Duo-Luciferase HS Assay Kit, LF004 (GeneCopoeia) following the manufacturer's instructions, and then the luminescence of each sample, indicative of IFN-β promoter signal, is analyzed using FLUOstar OPTIMA reader (BMG LabTech). All analyses of each sample are performed in triplicate.

As seen in Figure 2A, the expression of TBK-1, a protein involved in the signaling of several pathways leading to IFN-β production, is able to significantly increase the activity of the IFN-β promoter compared to the sample not transfected with TBK-1. Furthermore, the expression of MGF110-4L and MGF110-5L-6L genes is able to downregulate the expression of the IFN-β promoter that has been previously stimulated by TBK-1 transfection compared to the sample stimulated and transfected with empty vector (EV). This indicates that both genes would be potential modulators of IFN-β expression mediated by TBK-1 activation.

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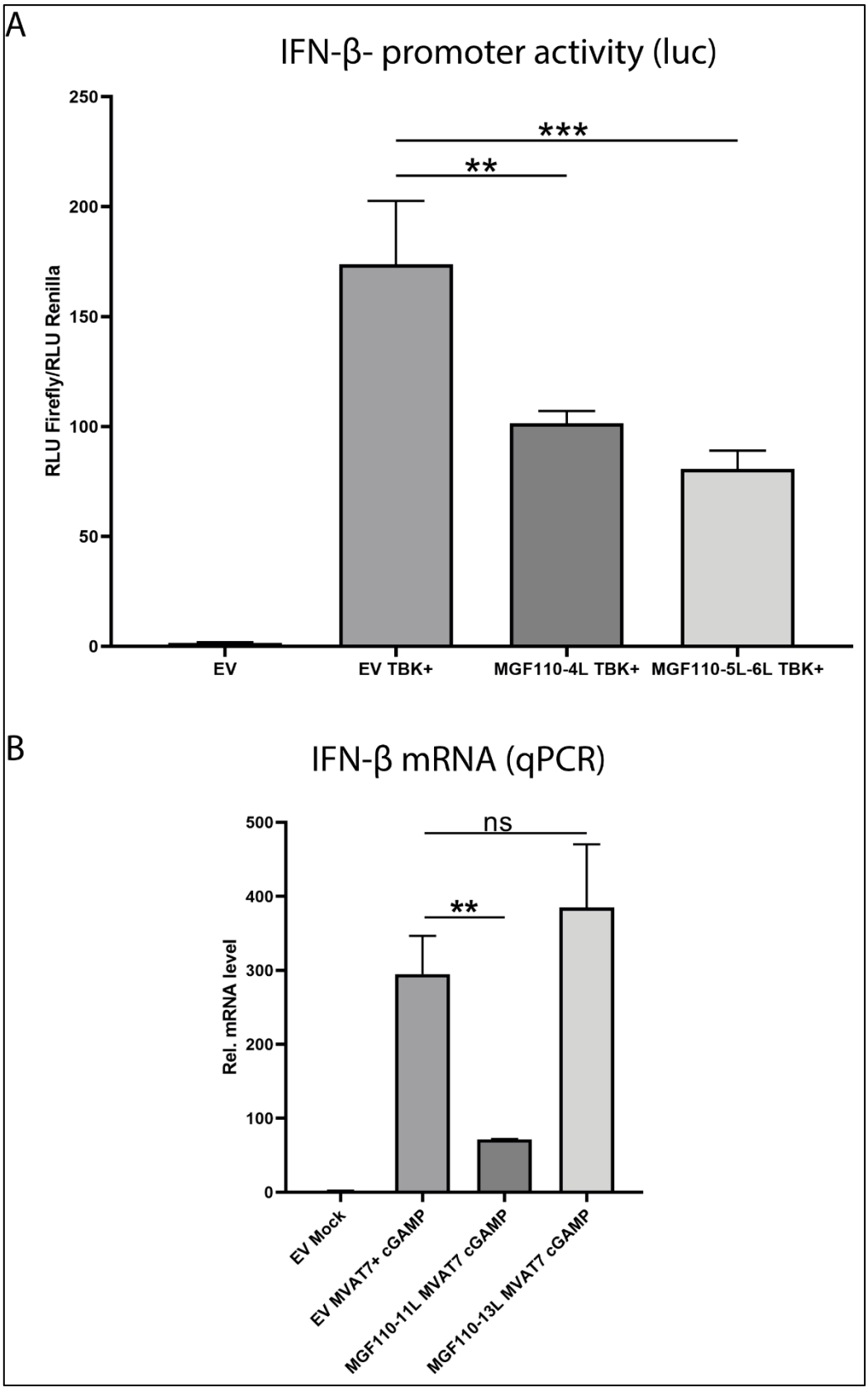

Figure 2. Modulation of IFN-I production by ASFV genes. A: Selected genes were used in a Luciferase (luc) assay. HEK-293-T cells were transfected with the indicated ASFV genes and co-transfected or not with TBK-1, in order to stimulate IFN-I production. Graphs represent the means of the Firefly luciferase RLU (Relative Luminiscence Units)

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values divided by its Renilla luciferase RLU values from biological triplicates. EV: Empty vector. B: Selected genes were used in a qPCR assay. BSRT7 cells were transfected with the indicated ASFV genes and co-infected with MVA-T7 (MOI= 0.5) and cGAMP was added in order to stimulate the cGAS/STING pathway. 24hpt, cells were lysed, RNA extracted, retrotranscribed to cDNA and qPCR was performed. Expression levels of genes of interest were normalized against 18s ribosomal RNA (rRNA) expression, and these values were relativized against the mean of the values obtained in the mock.

On the other hand, genes that could not be expressed in HEK-293T, but could be expressed in BSRT7+VVV-T7, were assayed by qPCR to measure their potential ability to counteract IFN-β production. In this case, IFN-β stimulation is achieved by adding to the medium the metabolite 2'-3'-cGAMP, which is the stimulator of the cGAS/STING pathway and leads to IFN-β production. In this case, we transfected with 2 µg/10^6 cells (FuGEN) with both the vectors coding for ASFV genes and the empty vector, pcDNA-myc (EV), performing the assay in triplicate. At 16hpt they are co-infected with MVA-T7 (MOI= 0.5) o/n, and the next day 10 µg/µl of 2'3'-cGAMP (Invivogen) is added per condition, and incubated for 2h. Samples are then collected using RNA extraction kit (Quiagen) according to the manufacturer's instructions. Prior to qPCR analysis, a quality test of the extracted RNAs is performed using the Bioanalyzer (Agilent). Those RNA samples with an RNA Integrity Number (RIN) below 5.9 were discarded. Equivalent amounts of RNA were then retrotranscribed using the NZY first-strand cDNA kit (NZYTech), and RT-PCR was performed in technical triplicate with a cDNA amount of 12 ng/sample on ABI PRISM 7900HT SDS (Applied Biosystems,Waltham, MS, USA) thermal cycler using SYBR Green (NZYTech). Expression levels of genes of interest were normalized against 18s ribosomal RNA (rRNA) expression, and these values were relativized against the mean of the values obtained in the mock.

As can be seen in Figure 2B, addition of the 2'-3'-cGAMP metabolite induced IFN-β mRNA synthesis in the sample transfected with the EV empty vector compared to the unstimulated MOCK sample. Expression of the ASFV gene MGF110-11L was able to significantly reduce 2'-3'-cGAMP-stimulated IFN-β expression, indicating that it is able to counteract the activation of the cGAS/STING pathway. On the other hand, however, expression of the ASFV gene MGF110-13L was not able to counteract such activation, suggesting that it is not involved in modulating IFN-β expression, or at least not through activation of the cGAS/STING pathway. This indicates that MGF110-11L would be a potential candidate for controlling IFN-β expression, while ruling out under these conditions a role for MGF110-13L.
In conclusion, we have already identified up to three genes that are potentially virulence genes for their role in modulating IFN-β expression, as follows: **MGF110-4L, MGF110-5L-6L and MGF110-11L**. These genes are potential candidates for deletion, both in combination and individually, from the vaccine prototype(s), should there be a need to improve their safety.

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