



# Characterization of three African swine fever viruses from different clinical settings revealed a potential attenuation mechanism

Jiajia Zhang<sup>1,2,3,4</sup>, Yuening Wang<sup>1,2,3,4</sup>, Kaili Zhang<sup>1,2,3,4</sup>, Dafu Deng<sup>1,2,3,4</sup>, Kai Peng<sup>1,2,3,4</sup>, Wanglong Zheng<sup>1,2,3,4</sup>, Nanhua Chen<sup>1,2,3,4</sup>, Penggang Liu<sup>1,2,3,4</sup>, Shaobin Shang<sup>1,2,3,4</sup>, Ruichao Li<sup>1,2,3,4\*</sup> and Jianzhong Zhu<sup>1,2,3,4\*</sup>

## Abstract

African swine fever (ASF) is an acute and fatal hemorrhagic disease in domestic pigs and wild boars caused by African swine fever virus (ASFV) that currently threatens the pig industry worldwide. Since the 2018 ASF outbreak in China, ASFV has evolved and caused diverse clinical manifestations, such as chronic and asymptomatic infections. Therefore, it is important to understand the molecular mechanisms underlying ASFV attenuation in the field. Here, we isolated three ASFVs from one diseased and two asymptomatic pigs by using primary porcine alveolar macrophages (PAMs) from both domestic pigs and Bama minipigs. The three ASFVs exhibited similar phenotypes in cell culture, including cytopathic effects (CPEs), hemadsorptions (HADs), viral protein expressions and growth curves. Genome sequencing revealed that all three ASFVs were genotype II strains. Genomic comparisons suggested that the disruption of the viral genes MGF360 and MGF110, rather than EP402R and EP153R, is likely involved in the potential attenuation of ASFV via the upregulation of innate immune responses.

**Keywords** African swine fever virus, Virus isolation, Genome sequence, Genotype II, Attenuation

## Introduction

African swine fever (ASF), a highly lethal hemorrhagic disease of domestic pigs and wild boars, causes enormous economic losses worldwide (Dixon et al. 2019; Li et al. 2022). African swine fever virus (ASFV), the causative

agent of ASF, is the only member of the family Asfarviridae and the only known DNA arbovirus to date (Karger et al. 2019). The ASFV genome is 170–193 kb in size and encodes more than 150 open reading frames (ORFs), with the central regions conserved and both sides highly variable (Karger et al. 2019). It is highly contagious and associated with high mortality; however, there are no effective and safe vaccines or antiviral drugs available to fight ASFV infection (Arabyan et al. 2019; Urbano and Ferreira 2022).

ASFV was first discovered and isolated in Kenya in 1921, after which it spread to other countries outside Africa (Sanchez-Vizcaino et al. 2012). The disease was first identified in Georgia in 2007 and subsequently spread to Eastern Europe and Asia in 2018 (Guinat et al. 2016; Zhou et al. 2018). On August 3, 2018, China reported the first case of ASF (Zhou et al. 2018). Since the outbreak, ASF has spread rapidly in China, and a

Handling editor: Wentao Li

\*Correspondence:

Ruichao Li

006820@yzu.edu.cn

Jianzhong Zhu

jzzhu@yzu.edu.cn

<sup>1</sup> College Veterinary Medicine, Yangzhou University, Yangzhou, China

<sup>2</sup> Joint International Research Laboratory of Agriculture and Agri-Product Safety, Yangzhou University, Yangzhou, China

<sup>3</sup> Comparative Medicine Research Institute, Yangzhou University, Yangzhou, China

<sup>4</sup> Jiangsu Coinnovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University, Yangzhou 225009, China



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number of variant ASFV strains have appeared in recent years, posing a serious threat to domestic pigs (Sun et al. 2021a; Sun et al. 2021b; Zhang et al. 2021; Zhao et al. 2023). In the field of endemic regions, infected domestic pigs exhibit different clinical symptoms, ranging from highly lethal to chronic to asymptomatic infections (Sun et al. 2021a; Sun et al. 2021b; Zhang et al. 2021). Therefore, it is necessary and important to isolate and characterize ASFV strains from different clinical settings to provide an understanding of viral evolution, which is useful for the prevention and control of ASF in China.

Here, we isolated three ASFVs from one diseased pig and two asymptomatic infected pigs by using primary porcine alveolar macrophages (PAMs) from both domestic pigs and Bama minipigs. Monocytes/macrophages are the main target cells of ASFV and the first line of defense against pathogens (Carrascosa et al. 2011a). Among the various types of monocytes/macrophages assessed, PAMs were suggested to be more susceptible to ASFV infection than were bone marrow-derived macrophages or blood monocytes (Oh et al. 2021). The three ASFVs exhibited similar culture features in PAMs. Additionally, the genomes of three ASFVs were sequenced, and all belonged to genotype II strains. Comparisons of the three genomes indicated that disruptions of ASFV MGF genes other than EP402R and EP153R may be involved in the attenuation of ASFV by upregulating innate immune

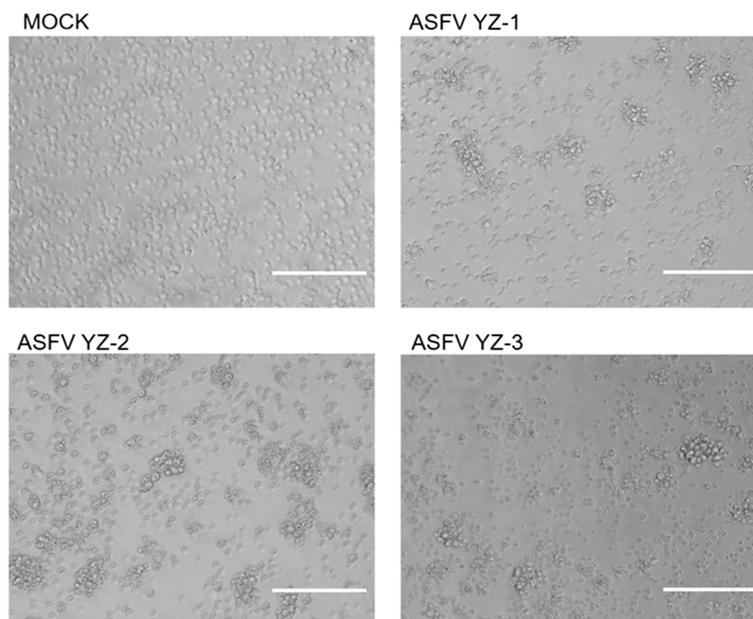
responses. Thus, our work significantly enriches the understanding of ASFV evolution and pathogenesis.

## Results

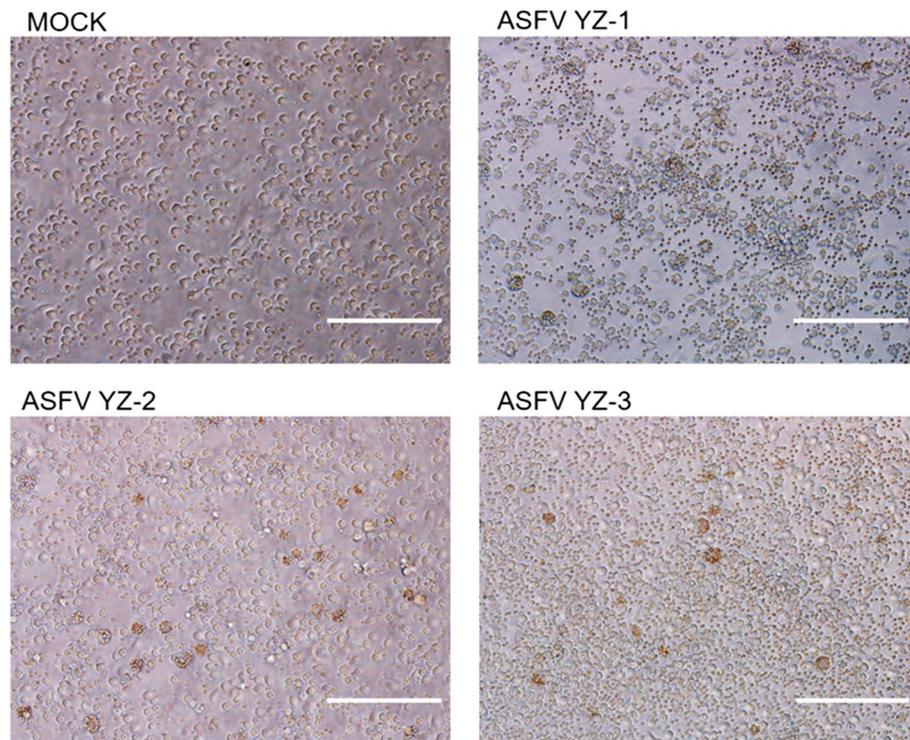
### The isolation and characterization of three ASFVs

Using primary porcine alveolar macrophages (PAMs) from both domestic pigs and Bama minipigs, we isolated ASFVs from the spleen, lung and lymph node tissues of diseased and dead pigs. Furthermore, we also isolated ASFVs from the spleen tissues of two asymptomatic pigs. The three ASFVs from three pigs were named pig/China/Yangzhou/202201, pig/China/Yangzhou/202202 and pig/China/Yangzhou/202203, respectively, with the abbreviations YZ-1, YZ-2 and YZ-3, respectively. As shown in Fig. 1, the three ASFV-infected PAMs exhibited clear cytopathic effects (CPEs) of cell shrinkage, exfoliation and aggregation within 72–96 h post infection. The hemadsorption of the isolated ASFVs was tested, and all three ASFVs exhibited similar hemadsorption by porcine red blood cells within 48 h post infection (Fig. 2).

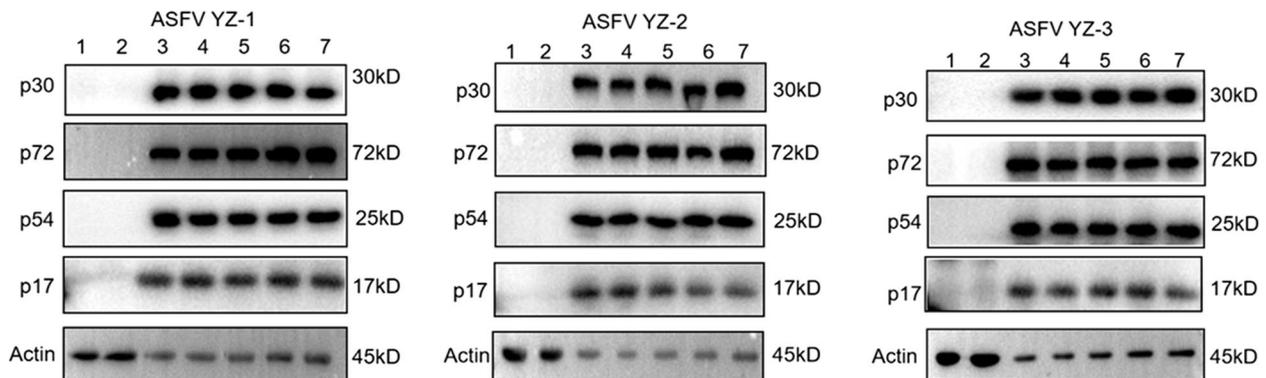
The three isolated ASFVs (first passage, P1) were passaged in PAMs of domestic pigs for five generations (P1 to P5). The viral structural protein expression levels of p30, p54, p72 and p17 were examined in each generation. Western blotting revealed similar expression levels of four viral structural proteins in each generation of the three ASFV-infected PAMs (Fig. 3). Similarly, p30, p72 and p17 expression was also detected in three



**Fig. 1** The cytopathic effects (CPEs) caused by three ASFV isolates. The PAMs of Bama minipigs were infected with the spleen tissue homogenates of three pigs (ASFV YZ-1, YZ-2 and YZ-3). At 72–96 h post infections, ASFV CPEs were observed under microscopy. The PAMs of domestic pigs had identical CPEs with infections of three ASFV isolates. The scale bar is 200  $\mu$ M



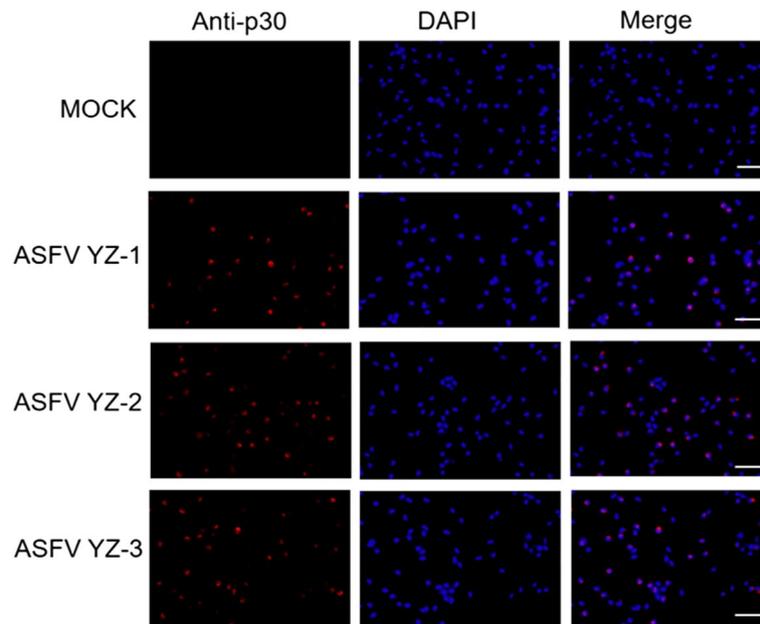
**Fig. 2** The hemadsorption (HAD) caused by three ASFV isolates. The PAMs of Bama minipigs were infected with the three ASFVs (YZ-1, YZ-2 and YZ-3) at a MOI (multiplicity of infection) of 0.1 for 24 h. The porcine erythrocytes were added for another 24 h, and the blood red cell rosette formations were observed under microscopy. The PAMs of domestic pigs had identical HAD with infections of three ASFV isolates. The scale bar is 200  $\mu$ M



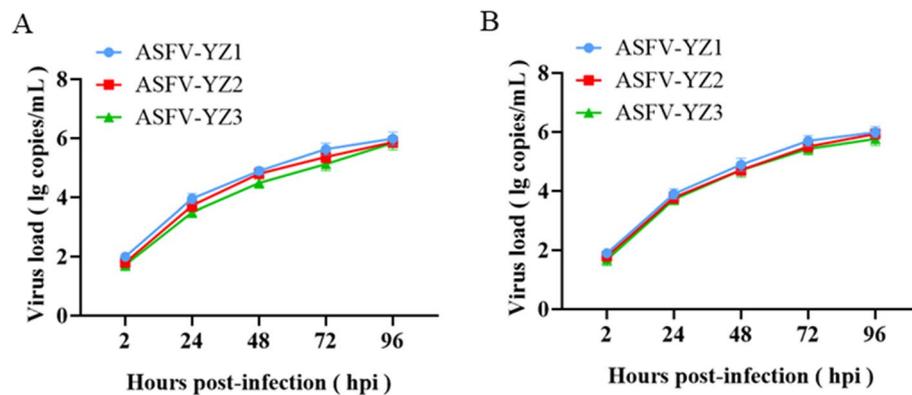
**Fig. 3** The expressions of viral structural proteins by ASFVs of different passages. The PAMs of domestic pigs were infected with ASFV YZ-1, ASFV YZ-2 and ASFV YZ-3 of different passages (P1, P2, P3, P4 and P5) at a MOI (multiplicity of infection) of 0.1 for 72–96 h. The infected cells were harvested and subjected for detection of the viral p30, p54, p72 and p17 protein expressions by Western blotting. Lane 1, mock infection controls; lane 2, infection with normal tissues; lanes 3–7, infections with ASFV P1, P2, P3, P4 and P5, respectively

ASFV-infected PAMs by immunofluorescence, with p30 and p17 mainly localized in the cytoplasm of infected cells (Fig. 4 and Fig S1). The growth kinetics of the three ASFVs in PAMs were measured by qPCR detection of viral p72 gene (B646L) copy numbers at 2 h, 24 h, 48 h, 72 h and 96 h post infection, and the results showed that

the three ASFVs had similar growth curves in PAMs of domestic pigs and Bama minipigs (Fig. 5). Together, the above results suggested that the three ASFVs from different clinical settings exhibited similar characteristics in cell culture.



**Fig. 4** The detection of ASFV p30 expressions by immunofluorescence assay. The PAMs of domestic pigs grown in 24-well plates were infected with three ASFVs (P5) at a MOI (multiplicity of infection) of 0.1. At 48 h post infections, the cells were fixed, permeabilized, sequentially probed with anti-p30 mAb and goat anti-mouse IgG H&L Alexa Fluor 594, and counterstained with nucleus marker DAPI. The stained cells were examined for fluorescence signals by fluorescence microscope. The scale bar is 100  $\mu$ M

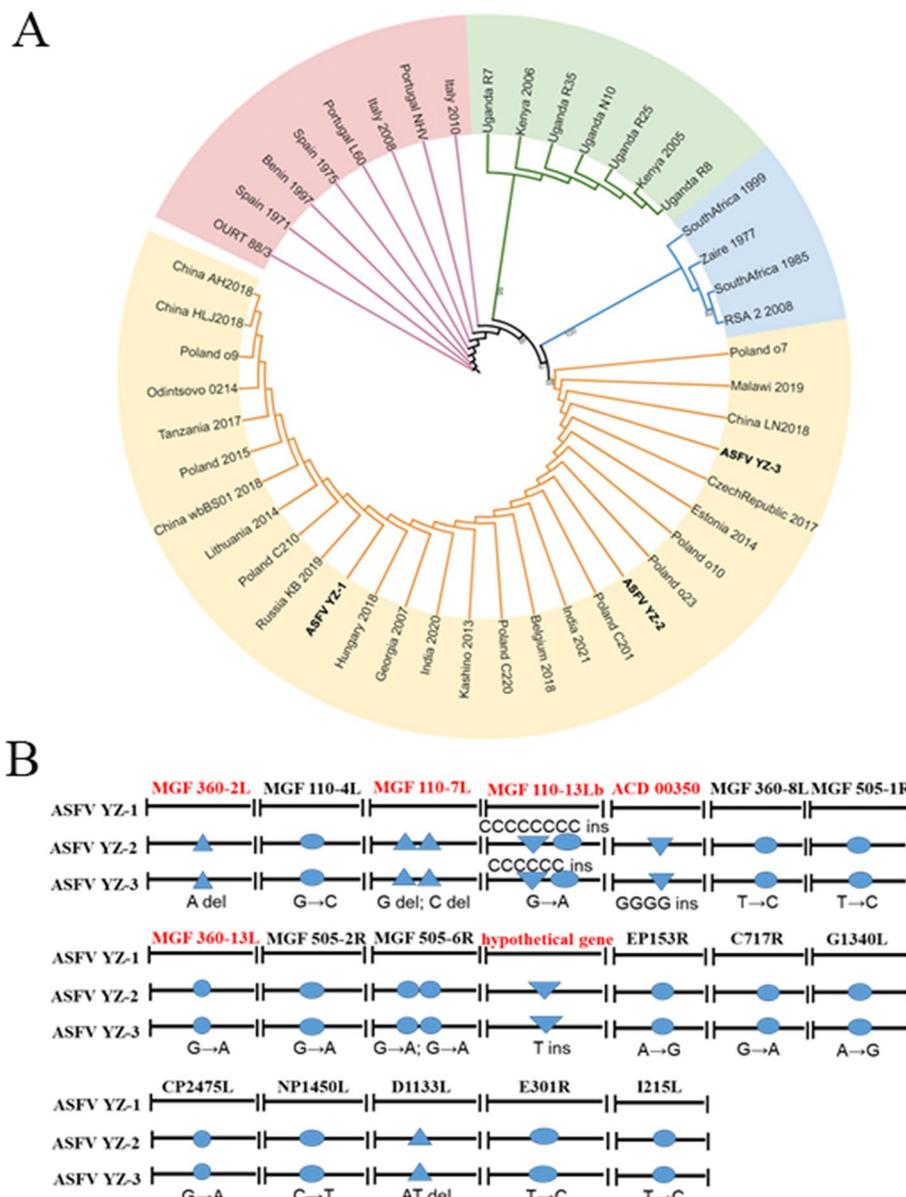


**Fig. 5** The growth kinetics of three ASFVs in two types of PAMs. The PAMs of Bama minipigs (A) and domestic pigs (B) were infected with three ASFVs (P5, multiplicity of infection = 0.1) for the indicated time periods. The cell supernatants were harvested and virus yields were titrated by qPCR targeting the viral genomic *B646L* gene. The growth curve of ASFV was determined and shown as Lg genomic copies/mL. Each time point represents the mean of three independent experiments with different batches of PAMs

### Genomic sequencing and genome comparison of three ASFVs

The genomic DNA of ASFV YZ-1, ASFV YZ-2, and ASFV YZ-3 was sequenced by using a second-generation sequencing approach. The assembled genomes of ASFV YZ-1, YZ-2 and YZ-3 were 187,951 bp, 187,836 bp and 187,835 bp in length, respectively, and were deposited in GenBank with the accession numbers ON456300,

OP823268 and OP823269, respectively. A phylogenetic tree based on the p72 sequences from 44 other ASFV strains in GenBank (Table S1) revealed that all three ASFVs were Genotype II strains (Fig. 6A). BLAST results revealed that the three ASFV genomes had the highest similarity (99.98%) to several China Genotype II ASFV isolates, including Pig/HLJ/2018 (GenBank: MK333180.1) (Wen et al. 2019), China/2018/



**Fig. 6** Phylogenetic analysis of three ASFVs and the encoding gene comparison between ASFV YZ-2/3 and ASFV YZ-1. **A** The rootless phylogenetic tree was constructed based on the p72 protein sequences from three ASFVs and 44 ASFV strains in GenBank (Table S1). The ASFV YZ-1, YZ-2 and YZ-3 in this study are marked in bold. Genotype I strains are shadowed with pink and Genotype II strains are shadowed with yellow. Green shadow includes Genotypes IX and X, whereas blue shadow contains Genotypes IV, VII, XX and XXII. **B** The differences of encoding genes in ASFV YZ-2/3 and ASFV YZ-1 genomes. The nucleotide deletion (del), insertion (ins) and mutation (→) are signed by ▲, ▼ and ●, respectively. The common differences between ASFV YZ-2 and 3 to ASFV YZ-1 are written below the encoding genes, whereas the specific differences between ASFV YZ-2 and 3 to ASFV YZ-1 are given above the encoding gene MGF110-13Lb. The significant changed encoding genes of ASFV YZ-2/3 relative to ASFV YZ-1 are marked in red color. The more detailed comparison between ASFV YZ-2/3 and YZ-1 is shown in Table 1

AnhuiXCGQ (GenBank: MK128995.1) (Bao et al. 2019), and Wuhan 2019-1 (GenBank: MN393476.1).

Compared with the international Genotype II ASFV strain Georgia 2007/1 (GenBank: FR682468.2) (Chapman et al. 2011), ASFV YZ-1 has seven nucleotide deletions, eight nucleotide insertions and 12 nucleotide

mutations (Table S2). The YZ-2 and YZ-3 genomes are almost identical except for a 2-nucleotide difference in MGF110-13Lb, and both have eight nucleotide deletions, six nucleotide insertions and 17 nucleotide mutations relative to Georgia 2007/1 (Table S2). Among the differences, five nucleotide deletions, three nucleotide

insertions and five nucleotide mutations commonly existed in three ASFVs relative to ASFV Georgia 2007/1 (Table S2). Among the three common nucleotide insertions, the 10-nucleotide “TATATAGGAA” tandem repeat sequence (TRS), located between I73R and I329L, was first found in wild boar ASFVs in Lithuania and Poland in 2014 (Goller et al. 2015) and was also detected in Chinese ASFV isolates (Wen et al. 2019).

ASFVs YZ-2 and YZ-3 were both from asymptomatic pigs and are very likely to be naturally attenuated. We compared the genomes of YZ-2 and YZ-3 with the genome of virulent YZ-1 from a diseased pig (Fig. 6B and Table 1). There were 33 nucleotide differences in both the YZ-2 and YZ-3 genomes, including eight deletions, five insertions and 20 mutations (Table 1). Among the 33 nucleotide differences, three deletions, three insertions and one mutation caused significant alterations in the encoding genes, including *MGF110-7L*, *MGF110-13Lb*, *MGF360-2L*, *MGF360-13L*, *ACD00350* and a hypothetical gene (Fig. 6B and Table 1). However, no alterations in

the *EP402R* or *EP153R* genes, which are closely related to naturally attenuated ASFV, were detected (Table 1).

### MGF genes disrupted in ASFV YZ-2 and YZ-3 affect innate immune responses

The *MGF* genes of ASFV play important roles in ASFV immune evasion and pathogenicity (Huang et al. 2022; Zhu, et al. 2021). Since several *MGF* genes, including *MGF110-7L*, *MGF110-13L*, *MGF360-2L* and *MGF360-13L*, are disrupted in ASFVs YZ-2 and YZ-3 (Fig. 6B and Table 1), we sought to examine whether these *MGF* genes present in YZ-1 disturb cellular innate immune responses. The four *MGF* genes were cloned and inserted into expression plasmids, and the effects of these *MGF* genes on innate immune responses were examined in cells transfected with porcine cGAS-STING, which is mainly responsible for sensing and controlling ASFV infection (García-Belmonte et al. 2019). In the promoter assay, *MGF110-7L*, *MGF110-13L* and *MGF360-13L* significantly suppressed the ISRE promoter and NF- $\kappa$ B promoter activities (Fig. 7A). Consistently, in the RT-qPCR

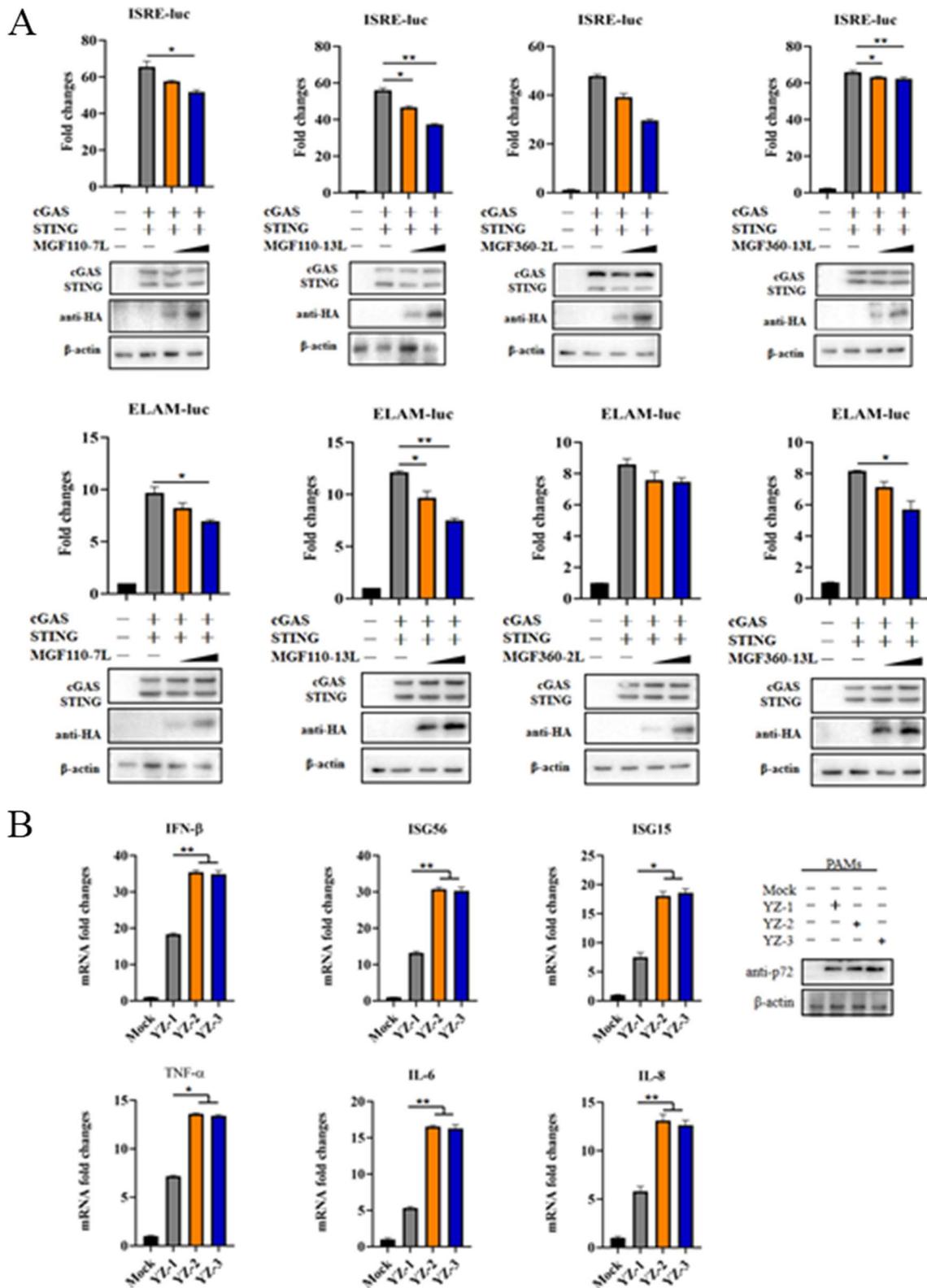
**Table 1** The genome comparison of ASFVs YZ-2 and YZ-3 with ASFV YZ-1

locations	YZ-2	YZ-3	CDS-locations	Interpretations
429	A deletion	same as YZ-2	repeat gene-MGF 360-2La	non-encoding region
2021	A deletion	same as YZ-2	MGF 360-2L	truncated gene
3902	C→T	same as YZ-2	L83L-L60L	non-encoding region
7657	G→C	same as YZ-2	MGF 110-4L	His→Asp change at #108aa
7930	A→C	same as YZ-2	ASFV G ACD 00120-MGF 110-5L	non-encoding region
7959	T→C	same as YZ-2	ASFV G ACD 00120-MGF 110-5L	non-encoding region
7969-7970	TC→CT	same as YZ-2	ASFV G ACD 00120-MGF 110-5L	non-encoding region
9295	G deletion	same as YZ-2	MGF 110-7L	YZ-2 and 3 have same genes as Georgia 2007/1, YZ-1 has a truncated gene
9369	C deletion	same as YZ-2	MGF 110-7L	truncated gene
14339	CCCCCCC insertion	CCCCC insertion	MGF 110-13Lb	YZ-2 has 3 Gly addition, and YZ-3 has 9 aa addition
14535	G→A	same as YZ-2	MGF 110-13Lb	synonymous mutation
16291	GG deletion	same as YZ-2	MGF 360-4L-ASFV G ACD 00300	non-encoding region
16507	G insertion	same as YZ-2	MGF 360-4L-ASFV G ACD 00300	non-encoding region
18461	GG insertion	same as YZ-2	ASFV G ACD 00320-ASFV G ACD 00330	non-encoding region
18661	GGGG insertion	same as YZ-2	ASFV G ACD 00350	YZ-2 and 3 have same genes as Georgia 2007/1, YZ-1 has a truncated gene
23592	T→C	same as YZ-2	MGF 360-8L	YZ-2, 3 have same gene as Georgia 2007/1, YZ-1 has Val→Met change at #17aa
28222	T→C	same as YZ-2	MGF 505-1R	synonymous mutation
30996	G→A	same as YZ-2	MGF 360-13L	truncated gene
33983	G→A	same as YZ-2	MGF 505-2R	synonymous mutation
38820	G→A	same as YZ-2	MGF 505-6R	Met→Ile change at #46aa
39209	G→A	same as YZ-2	MGF 505-6R	Arg→Lys change at #176aa
49939	T insertion	same as YZ-2	hypothetical gene	truncated gene due to generation of stop codon
54108-54109	CC→TT	same as YZ-2	A179L-A137R	non-encoding region
72662	A→G	same as YZ-2	EP153R	YZ-2, 3 have same gene as Georgia 2007/1, YZ-1 has Cys→Tyr change at #63aa
82912	G→A	same as YZ-2	C717R	Glu→Lys change at #392aa
101997	G deletion	same as YZ-2	B602L-B385R	non-encoding region
110603	A→G	same as YZ-2	G1340L	Leu→Pro change at #728aa
117778	G→A	same as YZ-2	CP2475L	synonymous mutation
131875	C→T	same as YZ-2	NP1450L	synonymous mutation
143403-143404	AT deletion	same as YZ-2	D1133L	synonymous mutation at the start condon ATG
163924	T→C	same as YZ-2	E301R	synonymous mutation
172052-172062	TATATAGGAA deletion	same as YZ-2	I73R-I329L	non-encoding region
173665	T→C	same as YZ-2	I215L	YZ-2, 3 have same genes as Georgia 2007/1, YZ-1 has Arg→Lys change at #153aa

The significant gene alterations are marked in red color. The sign # denotes amino acid position

(See figure on next page.)

**Fig. 7** The effects of ASFV *MGF* genes on cell innate immune responses. **A** 293 T cells in 96-well plate were transfected with pcGAS-HA (10 ng/well), pSTING-HA (10 ng/well), ISRE-Fluc or NF- $\kappa$ B-Fluc (10 ng/well) and Rluc (0.2 ng/well), together with *MGF110-7L-2HA*, *Myc-MGF110-13L*, *Myc-MGF360-2L* and *Myc-MGF360-13L* (10 ng and 20 ng each *MGF* plasmid) for 24 h. The promoter activity was measured as described in the Methods and the expressions of different proteins were examined by WB with relevant antibodies including anti-HA, anti-Myc and anti- $\beta$ -Actin mAbs. **B** PAMs in 12-well plates were infected with three ASFVs (MOI=0.1) for 96 h. The cells were examined for the mRNA expressions of IFN- $\beta$ , ISG56, ISG15, TNF- $\alpha$ , IL-6 and IL-8 by RT-qPCR assay. The three ASFV replications were monitored by using the WB detection of viral p72 expression



**Fig. 7** (See legend on previous page.)

assay, they significantly inhibited IFN- $\beta$ , IFN-stimulated gene (ISG) and proinflammatory cytokine expression triggered by the STING agonist cGAMP (Fig S2). Accordingly, the ASFV YZ-2- and YZ-3-infected PAMs produced significantly higher levels of IFN- $\beta$ , ISG and proinflammatory cytokine gene expression (Fig. 7B). These several lines of evidence suggested that the disruption of MGF genes may be responsible for the attenuation of YZ-2 and YZ-3.

## Discussion

In this study, three ASFVs were successfully isolated from the spleen tissues of a diseased pig and two asymptomatic infected pigs. According to the sequence analysis of the ASFV B646L gene encoding the viral major capsid p72 protein (Qu et al. 2022), the three ASFVs all belong to genotype II. The three ASFVs exhibited very similar characteristics in cell culture, including expression of CPE, HAD, the viral structural proteins p30, p54, p72 and p17, and growth kinetics. Since ASF was first reported in Kenya in 1921, it has spread to most parts of the world and has shown a trend of evolution from a virulent strain to a naturally attenuated strain in endemic regions by adaptation (Ito et al. 2022). Considering that ASF has been endemic for more than 5 years in China, it is not surprising for naturally occurring low-virulence ASFV to appear in the field (Sun et al. 2021a; Sun et al. 2021b; Zhang et al. 2021). Therefore, the two ASFVs from two asymptomatic infected pigs (YZ-2 and YZ-3) are likely to be naturally attenuated strains despite their similar characteristics in cell culture to the virulent ASFV from a diseased pig (YZ-1). The emergence of such naturally attenuated ASFVs with asymptomatic infection in pigs may pose a particular challenge for the control of ASF by promoting ASFV spread and recombination in the field, which deserves more attention.

Our sample size is limited to only three isolates, and expanding the number of ASFV isolates from diverse clinical settings is necessary to provide a more generalizable conclusion and enhance the robustness of our findings. Furthermore, the availability of detailed clinical data, such as the total number of animals affected and disease severity and transmission rates, will increase the practical relevance, impact and applicability of our study. Nevertheless, our current study and findings stimulate interest in further investigations in the future.

Recent analysis of the genetic variation and evolution of 5 attenuated ASFVs and the relevant virulent ASFVs revealed that MGF family genes account for a large proportion of differential genes, especially in field attenuated strains prior to 2014, with MGF360 most prone to deletion, followed by MGF110 (Zhenzhong et al. 2022). EP402R and EP153R are the most important virulence

genes in ASFV (Zhenzhong et al. 2022) and together mediate viral HAD activity, and deletions of both HAD genes are present in four of the five field attenuated strains, including two Chinese strains, HuB20 (GenBank: MW521382.1) (Zhang et al. 2021) and Pig/Heilongjiang/HRB1/2020 (GenBank: MW656282.1) (Sun et al. 2021b). EP402R was shown to exhibit genetic variability among 67 field ASFV isolates collected from infected wild boars and pigs, indicating ASFV evolution (Fraczyk et al. 2016). Nevertheless, the two ASFVs we isolated from asymptomatic infected pigs (YZ-2 and YZ-3) were intact EP402R and EP153R and exhibited HAD similar to that of the virulent ASFV strain (YZ-1). These results suggest that genes other than EP402R and EP153R, such as MGF360-2L, MGF360-13L, MGF110-7L and MGF110-13L, are likely involved in the potential attenuation of the ASFV strains YZ-2 and YZ-3 in the field. Our experiments showed that MGF360-13L, MGF110-7L and MGF110-1 L evade cellular innate immune responses by inhibiting IFN- $\beta$ , ISG and proinflammatory cytokine expression. Although the upregulated innate immune responses in YZ-2- and YZ-3-infected PAMs did not dampen the two types of ASFV replication relative to YZ-1, such changes in innate immune responses in vivo were likely to attenuate YZ-2 and YZ-3 relative to YZ-1. In addition, several studies have shown that the deletion of different MGF genes attenuates ASFVs in vivo through the upregulation of cytokine production, despite similar in vitro replication in cell culture between ASFV-deleted and parental ASFV strains (Zhao et al. 2023; Bourry et al. 2022; Kitamura et al. 2023; Li et al. 2021a; Li et al. 2021b; O'Donnell et al. 2015). While our study focused on MGF genes, we did not exclude other genes involved in the attenuation of ASFV, and extensive validation of additional genes implicated in immune evasion and attenuation would provide a more complete picture of the viral mechanisms at play.

Bama minipigs are a unique experimental animal resource in China with a high degree of inbreeding and stable genetics (Zhang et al. 2014). In this study, we isolated three ASFVs from PAMs of not only domestic pigs but also Bama minipigs. Both types of PAMs exhibit similar morphologies and susceptibilities to ASFV infection. In addition to SPF pigs, PAMs isolated from domestic farm pigs are often infected with other pathogens, especially PRRSV, which interferes with ASFV isolation and growth. In our previous study, we found that PAMs of Bama minipigs are not susceptible to PRRSV (not shown) and that the use of these PAMs is more conducive to the isolation of PAMs. Therefore, Bama minipigs are a better experimental model than domestic pigs for ASFV research. Similarly, a recent study revealed that Bama minipigs infected with ASFV exhibited similar

characteristic organ lesions to those of domestic pigs and wild boars (Lv et al. 2022).

The tropism of ASFV isolates derived from pigs is restricted to primary monocytes/macrophages (Oh et al. 2021). Despite previous reports showing the productive replication of field ASFVs in different continuous cell lines (Weingartl et al. 2002; Sánchez et al. 2017; Rai et al. 2020; Masujin et al. 2021; Portugal et al. 2020), our results demonstrated that none of the three isolated ASFVs could replicate significantly and be passaged in a variety of cell lines we tested, including 3D4/21, MA-104, Huh-7.5.1, Cos-1, Vero and Vero cells with different gene knockouts (not shown). These results are reflected by a recent investigation of four cell lines, 3D4/21, PK-15, MA-104 and Marc-145, which exhibited distinct defects during virus early transcription-translation, genome replication and late protein synthesis (Gao et al. 2022).

In summary, we isolated genotype II ASFVs from different clinical settings in the field, and analysis of the virus culture and viral genomes deepened our understanding of ASFV evolution and pathogenesis.

## Conclusion

The three genotype II ASFV strains isolated from different clinical settings exhibited similar phenotypes in cell culture. Genomic comparisons between the three ASFVs suggested that the disruption of the viral genes MGF360 and MGF110, rather than EP402R and EP153R, is likely involved in the potential attenuation of ASFV by upregulating innate immune responses. This work helps elucidate the molecular mechanisms underlying ASFV attenuation in the field.

## Methods

### Facility statement

All ASFV infection experiments were performed at the animal biosafety level 3 (ABSL-3) of Yangzhou University and were approved by the Ministry of Agriculture and Rural Affairs (07140020201109–1).

### Specimen description

The first batch of specimens was obtained from a diseased and dead farm pig in the Yangzhou region, Jiangsu, China, in which lesions were observed in the liver, spleen, lung, kidney and lymph nodes. The Ct values of the liver, spleen, lung and lymph node tissues were 20–22 according to the ASFV qPCR assay performed in our laboratory. The second and third batches of specimens were obtained from two 180-day-old farm pigs in the Nantong region of Jiangsu, China. The euthanized pigs did not exhibit any visual lesions; however, the Ct values of the lymph node, spleen and lung tissues were 25–38 according to

the commercial qPCR assay (Animal Detection U<sup>+</sup> Probe Master Mix, Cat # QV110, Vazyme, Nanjing China).

### Cell culture and ASFV isolation

Primary porcine alveolar macrophages (PAMs) were obtained from both 1-month-old domestic pigs and Bama minipigs by bronchoalveolar lavage as previously described. PAMs were cultured in RPMI 1640 medium (HyClone Laboratories, Logan, Utah, USA) supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin and 10% FBS (Gibco, Grand Island, NY, USA). The cells were grown at 37°C in a 5% CO<sub>2</sub> humidified incubator. The liver, spleen, lung and lymph node samples of the first specimens and the spleen tissue samples of the second and third specimens were processed by homogenization, followed by inoculation into PAMs with the filtrated homogenates. The cell supernatants from 3–5 d post inoculation (first passage, P1) were collected for qPCR detection, and ASFV P1 was inoculated into PAMs for viral passage. The inoculations were also tested by qPCR to confirm the absence of other porcine viruses, such as swine fever virus (CSFV), porcine circovirus 1 (PCV), pseudorabies virus (PRV), and porcine respiratory and reproductive syndrome virus (PRRSV).

### Quantitative PCR (qPCR)

For the detection of ASFV and other DNA viruses, genomic DNA was extracted from tissue homogenates or cultured cells using a DNA mini kit (Cat No: D3121-02, Magen, Guangzhou, China), and then, TaqMan qPCR was performed using 2×Taq master mix (Vazyme, Nanjing, China) on StepOne Plus equipment (Applied Biosystems) with a qPCR program of 95°C for 3 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For the detection of the RNA viruses CSFV and PRRSV, RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Shanghai, China) and reverse transcribed into cDNA with a HiScript<sup>®</sup> 1st Strand cDNA Synthesis Kit (Vazyme). Then, the cDNA was used for TaqMan qPCR. For detection of cellular gene expression, cell RNA was extracted and reverse transcribed, and cDNA was used for SYBR Green qPCR. SYBR qPCR was performed at 95°C for 30 s followed by 40 cycles of 95°C for 10 s and 60°C for 30 s by using ChamQ Universal SYBR qPCR Master Mix (Vazyme). The relative gene levels were normalized to β-actin and calculated using the 2<sup>-ΔΔCT</sup> method. The qPCR primers and probes used in this study are listed in Table S3 and were designed, validated and used in our laboratory.

### Hemadsorption (HAD) assay

Porcine red blood cells (1%) were prepared in PBS (pH 7.4) from anticoagulated blood taken from a healthy pig.

The HAD assay was performed according to a previous study with minor adjustments. Specifically, PAMs cultured in 96-well plates were infected with a 10-fold series of diluted ASFV solutions, with 8 replicates for each concentration. After 24 h of infection, 1% porcine erythrocytes were added (8  $\mu$ L/well) for another 24 h. Upon completion, the cell plates were gently shaken to avoid false positive reactions, and the characteristic red cell rosettes were observed via microscopy. The 50% HAD doses (HAD50) were calculated using the Reed-Muench method.

#### Western blotting (WB) analysis

The PAMs in 24-well plates were infected with ASFV at different passages at a multiplicity of infection (MOI) of 0.1 for 72–96 h. The cell protein samples were separated on 6–10% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked using 5% nonfat dry milk in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST). Then, the membrane was incubated with primary antibodies (1:2000 each) at 4°C overnight. The antibodies used included ASFV p30, p54, p72 and p17 mouse monoclonal antibodies (mAbs) developed in our laboratory, as well as mouse anti-HA, anti-Myc, and anti- $\beta$ -Actin mAbs (TransGen, Beijing, China). Next, the membranes were incubated with HRP-conjugated goat anti-mouse IgG (BBI, Shanghai, China) for 1 h. Protein signals were visualized and captured by a Western blot imaging system (Tanon, Shanghai, China).

#### Immunofluorescence assay

The PAMs were seeded in 24-well plates and infected with ASFV at a multiplicity of infection (MOI) of 0.1 for 72 h. The cells were fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.5% Triton X-100, and then blocked with 5% BSA. The treated cells were incubated with primary p30, p72 and p17 mAbs (1:500 each) overnight and then incubated with goat anti-mouse IgG H&L Alexa Fluor 594 (Abcam, Shanghai, China) (1:500) for 1 h. Then, the cells were stained with DAPI (Beyotime, Shanghai, China) for 15 min and observed under a microscope.

#### ASFV genome next-generation sequencing

Viral genomic DNA was extracted from the ASFV-infected PAMs. The quality and purity of the extracted DNA were evaluated using a Qubit 4 Fluorometer and NanoDrop (Thermo Scientific). Then, a total of 200  $\mu$ g of DNA was randomly fragmented and used for construction of a short-read paired-end library with the MGIEasy Universal DNA Library Prep Set Kit (Cat No. 1000006986, MGI Tech Co., Ltd., Shenzhen, China) following the manufacturer's protocol. The qualified

libraries were sequenced on the MGI SEQ-2000 platform via the PE150 strategy. After the raw data were acquired, the adaptor and low-quality sequences were trimmed using fastp with default parameters (Chen et al. 2018). The trimmed sequences were taxonomically classified using Kraken2 with the K2 standard database, and sequences belonging to ASFV were extracted using the 'extract\_kraken\_reads.py' tool in the KrakenTools package (Wood et al. 2019). Finally, the extracted ASFV genomic sequences were assembled using SPAdes v3.15.0 (with default parameters) (Bankevich et al. 2012).

#### Molecular cloning

The *MGF110-7L* gene was PCR amplified from the ASFV YZ-1 genomic DNA, and the purified PCR product was cloned and inserted into the *EcoRI* and *EcoRV* sites of the pCAGGS-2HA vector by using MultiF Seamless Assembly Mix (AB clone, Wuhan China). The resulting recombinant plasmid was named pCAGGS-MGF110-7L-2HA. MGF110-13L, MGF360-2L and MGF360-13L were PCR amplified from the ASFV YZ-1 genomic DNA, and the purified PCR products were subsequently cloned and inserted into the *SalI* and *KpnI* sites of pCMV-Myc by using MultiF Seamless Assembly Mix. The resulting recombinant plasmids were named pCMV-Myc-MGF110-13L, pCMV-Myc-MGF360-2L and pCMV-Myc-MGF360-13L, respectively. The cloning PCR primers are shown in Table S4.

#### Promoter-driven luciferase reporter gene assay

293 T cells in 96-well plates were cotransfected with Lipofectamine 2000 (Thermo Fisher Scientific, Shanghai, China) and the ISRE or ELAM (NF- $\kappa$ B) firefly luciferase (Fluc) reporter (10 ng/well) or the  $\beta$ -actin Renilla luciferase (Rluc) reporter (0.2 ng/well) together with the indicated plasmids or vector control (5–40 ng/well). The total DNA per well was normalized to 50 ng by adding the corresponding empty vectors. Twenty-four hours post-transfection, the cells were lysed, and the Fluc and Rluc activities were measured by a double-luciferase reporter assay kit (Vazyme). The results are expressed as the fold change in the expression of ISRE or ELAM (NF- $\kappa$ B)-Fluc relative to that of the vector controls after Fluc normalization to the corresponding Rluc.

#### Statistical analysis

All the results are representative of two or three similar experiments. The data are shown as the mean  $\pm$  standard deviation (SD) and were analyzed using GraphPad Prism 8.0. Student's *t* test was used for statistical analysis, and  $p < 0.05$  was considered to indicate statistical significance, as follows: \*  $p < 0.05$  and \*\*  $p < 0.01$ .

## Abbreviations

ASF	African swine fever
ASFV	African swine fever virus
PAM	Porcine alveolar macrophage
CPE	Cytopathic effect
HAD	Hemadsorption
ORF	Open reading frame
TRS	Tandem repeat sequence
MGF	Multiple gene family

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s44149-024-00130-1>.

Supplementary Material 1: Table S1. Information on the ASFV strains used in this study for the construction of the phylogenetic tree.

Supplementary Material 2: Table S2. Genomic comparison of three ASFVs with ASFV Georgia 2007/1.

Supplementary Material 3: Table S3. The qPCR primers and probes used in this study. Table S4. The cloning PCR primers used in this study.

Supplementary Material 4: Fig S1. ASFV p72 and p17 expression was detected by immunofluorescence. The PAMs of domestic pigs grown in 24-well plates were infected with three ASFVs (P5) at an MOI of 0.1. At 48 h post infection, the cells were fixed, permeabilized, sequentially probed with anti-p72 mAb (A), anti-p17 mAb (B) and goat anti-mouse IgG H&L Alexa Fluor 594, and counterstained with the nuclear marker DAPI. The stained cells were examined for fluorescence signals by fluorescence microscopy. The scale bar is 100  $\mu$ M.

Supplementary Material 5: Fig S2. The effects of the ASFV MGF gene on STING-activated innate immune responses in porcine macrophages. The 3D4/21 porcine macrophages in 12-well plates were transfected with MGF110-7L (A), MGF110-13L (B), MGF360-2L (C) or MGF 360-13L (D) (0.5  $\mu$ g and 1  $\mu$ g of each plasmid) for 12 h, followed by stimulation with the STING agonist 2'3'-cGAMP (2  $\mu$ g/mL) for another 12 h. The cells were examined for the mRNA expression of IFN- $\beta$ , ISG56, ISG15, TNF- $\alpha$ , IL-6 and IL-8 by RT-qPCR.

Supplementary Material 6.

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## Authors' contributions

JJ.Z. conceived and designed the experiments; JJ.Z., Y.W., K.Z., D.D., K.P., and W.Z. performed the experiments; N.C., P.L. and S.S. provided the resources; R.L. analyzed the sequencing data; JJ.Z. and JZ.Z. wrote the paper. All authors contributed to the article and approved the submitted version.

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## Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

## Declarations

## Ethics approval and consent to participate

N/A.

## Competing interests

No potential competing interests were reported by the authors.

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