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CLONING GENES OF p49, p72, pE199L, pE248R AND CD2vINTO *PICHIA PASTORIS* GS115

Nguyen Trong Binh[1,✉], Thai Gia Hoa Pham[1], Trung Hieu Tran[2], Pham Thanh Phu Vu[2], Nguyen Ly Nguyen[2] and Phat Dinh Xuan[2]

[1]*Biotechnology Center of Ho Chi Minh City, 2374 Highway 1, Trung My Tay Ward, District 12, Ho Chi Minh City, Vietnam.*

[2]*Nong Lam University, Block 6, Linh Trung Ward, Thu Duc City, Ho Chi Minh City, Vietnam.*

✉To whom correspondence should be addressed. E-mail: ntbinh.snn@tphcm.gov.vn

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ABSTRACT

African swine fever (ASF), caused by the African swine fever virus (ASFV), poses a significant threat to pig populations worldwide. ASFV is a double-stranded DNA virus. In recent years, 54 structural proteins and more than 100 proteins have been involved in viral infection found in macrophages of diseased pigs. Among these, p49 and p72 are essential capsid proteins crucial for forming the viral capsid. Additionally, pE199L and pE248R, located in the inner viral membrane, are critical for membrane fusion, a necessary step for viral entry into host cells. Another important player is CD2v, a type I transmembrane protein involved in the infection process. In this research, to develop subunit vaccines against ASFV**,** we focused on cloning the genes encoding these five proteins—p49, p72, pE199L, pE248R, and CD2v—into the pPIC9K plasmid for expression in the *Pichia pastoris* GS115 yeast strain. The viral genomic DNA was extracted from blood samples of infected pigs, and the genes encoding the five proteins were successfully amplified using Phusion PCR. The PCR products of each gene were then digested with *Eco*RI and *Not*I restriction enzymes and ligated into the pPIC9K plasmid. After that, we transformed the recombinant plasmids into *Escherichia coli* DH5α for amplification and purification. The plasmids were subsequently linearized with *Sal*I and introduced into *P. pastoris* GS115 through electroporation. The selection of appropriate media and PCR analysis of the genomic DNA confirmed the successful generation of five recombinant *P. pastoris* GS115 strains. This work paves the way for the development of a recombinant protein vaccine against ASF by using the *Pichia pastoris* GS115 in the future.

Keywords: CD2v, p49, p72, pE199L, pE248R, *Pichia pastoris* GS115, pPIC9K plasmid, African swine fever virus

INTRODUCTION

African swine fever (ASF) has emerged as a significant issue in global livestock and public health. Caused by the African swine fever virus (ASFV), this disease is not transmissible to humans but has a mortality rate approaching 100% in pigs (Kalenzi Atuhaire et al., 2013). The impact of ASF on

the swine industry is immense, directly affecting the economy and livelihoods of millions of people worldwide. African swine fever is not only an animal health issue but also a significant threat to global food security. Pork remains a widely consumed source of protein in many parts of the world. The number of pigs lost to ASF can severely impact the global pork supply. Moreover, the spread of the disease affects market prices, leading to economic losses for farmers. African swine fever was first identified around 1909 in Africa. The disease gradually spread to European countries such as Portugal, Spain, and other countries in the 1960s (Cwynar et al., 2019). In 2018, ASFV was detected in China, spreading to surrounding countries, including Vietnam. On February 19, 2019, the first ASFV case was reported in Vietnam, resulting in damages exceeding 13 trillion Vietnamese dong and the culling of nearly 6 million infected pigs (Tran et al., 2020). ASFV belongs to the family Asfarviridae, genus Asfivirus (Dixon et al., 2013), and is characterized by its roughly 200 nm diameter icosahedral structures comprising nucleoid, core, inner membrane, capsid, and outer membrane components (Salas & Andrés, 2013). Depending on the strain, the genome length can range from 170 to 195 kb (de Villiers et al., 2010). Essential proteins of ASFV include p49 and p72, which are found on the capsid shell, and pE199L, pE248R located in the inner membrane, and CD2v on the outer membrane, all crucial for viral infectivity and host cell entry of ASFV (Salas & Andrés, 2013).

Once the ASFV infects pigs, it targets macrophages in the blood and bone marrow. Infected pigs initially exhibit a high fever and loss of appetite, which can progress to more severe symptoms like cyanosis, erythema, hemorrhages, and nosebleeds (Li et al., 2022; Yoon et al., 2020). Within a few days of infection, pigs can die with a mortality rate approaching 100%.

For these reasons, many current studies are focused on researching and developing vaccines to prevent ASFV. However, recent research results have not yielded promising outcomes due to various challenges. Currently, there is no specific effective vaccine against ASFV; inactivated vaccine technologies have not proven to be protective even in the presence of adjuvants (de Villiers et al., 2010). Attenuated vaccines offer better protection but are strain-specific and less effective against heterologous virulent strains (Gómez-Puertas et al., 1996). Additionally, attenuated vaccines often lead to adverse effects such as skin ulcers, joint swelling, or even abortion (King et al., 2011). Subunit vaccines are promising solutions for the future to reduce these risks (Arias et al., 2017). Subunit vaccines contain only specific antigens of the virus, making them safer as they do not include the whole virus and cannot cause disease. However, the effectiveness of these vaccines still requires extensive research and further exploration. In the past, subunit vaccines using p30, p54, and p72 were studied and tested in 1996, but the results did not protect animals against ASFV (Gómez-Puertas et al., 1996). Another trial in 2004 by Neilan and colleagues tested a vaccine on pigs using a mixture of baculovirus expressing p30, p54, p72, and p22 (Neilan et al., 2004). However, this vaccine failed to protect the test pigs from the Pr4 strain of ASFV. Recent studies on subunit vaccines include research by Zhang G and colleagues in 2022, which developed a subunit vaccine using p30 and

p54, and research by Lopera-Madrid and colleagues in 2021, which focused on a subunit vaccine using p30 with poxviruses as delivery vectors (Lopera-Madrid et al., 2021; Zhang et al., 2022). Despite these efforts, further investigation is needed to evaluate the protective efficacy of these vaccines. In summary, subunit vaccines represent a promising and safer approach to the development of vaccines against ASFV. Recombinant protein technologies have shown promise with recombinant proteins identified in prokaryotic cells like *E. coli*, but not yet in eukaryotic yeast cells like *P. pastoris* (Ferrer-Miralles & Villaverde, 2013). Therefore, our study aims to develop gene constructs encoding inner and outer membrane structural proteins of ASFV in *P. pastoris* GS115 yeast cells, encouraging the development of recombinant protein vaccines in the future. *Pichia pastoris* is chosen for its numerous advantages over bacterial strains and other expression systems. The *P. pastoris* GS115 yeast cells are easy to cultivate and can express proteins rapidly, allowing for the production of large quantities in a short timeframe. As an eukaryotic protein expression system, *P. pastoris* benefits from its glycosylation capabilities, which enable the produced proteins to resemble those found in higher eukaryotic cells. This contrasts with *E. coli*, which operates as a prokaryotic system and often results in structural differences in the expressed proteins. Consequently, utilizing yeast expression systems can yield more accurate results. Additionally, compared to other cellular expression systems, yeast offers advantages such as lower costs, faster expression times, and easier purification of recombinant proteins (Yao et al., 2022). These features contribute to the widespread use of *Pichia pastoris* cells in large-scale production.

MATERIALS AND METHODS

The African swine fever virus (ASFV) was isolated from blood samples of infected pigs in Đồng Nai province in 2020. The viral DNA was extracted and verified using real-time PCR, ensuring accurate identification of ASFV DNA (Tran et al., 2023). The bacterial strain *E. coli* DH5α, the yeast strain *Pichia pastoris* GS115, as well as the plasmids pJET1.2/blunt and pPIC9K, were supplied by Thermo Scientific, USA. The growth media for culturing *E. coli* and for *P. pastoris* was provided by Biobasic, Canada.

Table1: The sequences of the primers were designed.

Primer	Primer sequences	Expected PCR product sizes (in bp)	Restriction enzyme
p49 F	5'-GAATTCATGTATCATGATTATGCTTCAAAG-3'	1314	*EcoRI*
p49 R	5'-GCGGCCGCCAATGATGGAGATATAGATGAG-3'		*Not* I
p72 F	5'-GAATTC ATGGCATCAGGAGGAGCTTTTTG-3'	1938	*EcoRI*
P72 R	5'-GCGGCCGC GGTACTGTAACGCAGCACAGC-3'		*Not* I
pE199L F	5'-GAATTC ATGTCTTGCATGCCAGTTTC-3'	597	*EcoRI*

pE199L R	5'-GCGGCCGC AAAATTGTTTAGGTTTGAAAAAATAAG-3'		*Not* I
pE248R F	5'-GAATTC ATGGGAGGCTCTACAAGCAAAAATTC-3'	744	*EcoRI*
pE248R R	5'-GCGGCCGC CGAAACGGCAGCATTTTTTAATACC-3'		*Not* I
CD2v F	5'-GAATTC ATGATAATACTTATTTTTTTAATATTTTC-3'	1274	*EcoRI*
CD2v R	5'-GCGGCCGC AATAATTCTATCTACGTGAATAAG-3'		*Not* I

Table 2: Reference primer sequences from Thermo Scientific

Primer	**Primer sequences**	**Expected PCR product sizes (in bp)**	**Primer binding sites**
AOX1 F	5'-GACTGGTTCCAATTGACAAG C -3'	732 (without gene of interest)	855-875 (Pairing on the promoter of the plasmid pPIC9K.)
AOX1 R	5'-GCAAATGGCATTCTGACATC C -3'		1253-1586 (Pairing at the transcription termination site of the plasmid pPIC9K.)

To amplify the target gene, we based our primer design on the sequence from GenBank: MK795938.1 from NCBI for the gene encoding pE248R, with the forward primer spanning positions 1-26 and the reverse primer from positions 720-744. For the gene encoding p49, we used GenBank: MK128995.1, with the forward primer at positions 97521-97544 and the reverse at 96231-96252. Similarly, for p72, the forward primer is from positions 105525-105547 and the reverse from 103610-103630; for pE199L, the forward primer spans 166451-166470 and the reverse 165874-165900; and for CD2v, the forward primer is from 73383-73411 and the reverse from 74439-74462. All primer sequences were designed using the AnnHyb 4.946 software. The primer sequences are detailed in Table 1.

Table 2 includes the AOX1 F and AOX1 R primer sequences referenced from Thermo Scientific. The expected size of the PCR product is 732 bp in the absence of the target gene. Additionally, the AOX1 F primer binds at positions 855-875 on the promoter of the plasmid pPIC9K, while the AOX1 R primer binds at positions 1253-1586 in the transcription terminator region of the same plasmid.

Specific primers for the target genes encoding p49, p72, pE199L, pE248R, and CD2v were created to include *Eco*RI recognition sites at the forward primer and *Not*I at the reverse primer positions. PCR amplification of the target genes was performed using the following reaction mixture: 1X Phusion HF Buffer, 200 µM dNTPs, 3% DMSO, 0.5 µM of each primer,

0.02 U/µL Phusion DNA Polymerase, and 50 ng of gene template DNA. After an initial denaturation step at 98°C for 30 seconds, 30 amplification cycles were performed with the following requirements per cycle: denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 90 seconds. A final extension step at 72°C for 10 minutes concluded the reaction, followed by a hold at 10°C (Nguyễn et al., 2023).

To clone each target gene into the expression plasmid, the purified PCR products of the target genes and the pPIC9K expression plasmid were digested with *Eco*RI and *Not*I restriction enzymes. The digested products were separated on a 1% agarose gel to isolate the target gene and pPIC9K plasmid bands. Recombinant plasmids containing the target genes were created using T4 DNA ligase. The ligation products were transformed into competent *E. coli* DH5α cells using electroporation. Only *E. coli* DH5α cells carrying the recombinant plasmids with the kanamycin resistance gene were capable of forming colonies on LB agar plates containing kanamycin (20 µg/ml). Candidate colonies containing the pPIC9K plasmid with or without the target genes were screened using bacterial colony PCR with AOX1 primers on the plasmid. The expression plasmids containing the target genes were purified and sequenced using AOX1 primers to confirm the product expressed the correct protein of interest. These primers attach to the pPIC9K plasmid and can detect the inserted gene sequence within the Multiple Cloning Site (MCS) region. The sequencing results were compared with gene and amino acid sequence data from NCBI GenBank using Clustal X software.

The pPIC9K plasmids containing the target genes were linearized using the *Sal*I enzyme (restriction sites pre-existing on the pPIC9K plasmid) and transformed into *Pichia pastoris* GS115 cells using electroporation at 25 µF, 186 Ω, and 1.5 kV (Wu & Letchworth, 2004). Recombinant yeast strains were selected on the Minimal Dextrose (MD) medium, where only *Pichia pastoris* GS115 strains capable of histidine self-synthesis could grow. Subsequently, selected yeast strains were cultured on Yeast Extract Peptone Dextrose (YPD) medium containing Geneticin antibiotic. Colonies grown on the Geneticin-resistant medium were extracted for the entire gene using 10% SDS, and the presence of the pPIC9K segment containing the inserted target gene in the *Pichia pastoris* GS115 host genome was verified using PCR.

RESULTS

Clone genes encoding proteins p49, p72, pE199L, pE248R, and CD2

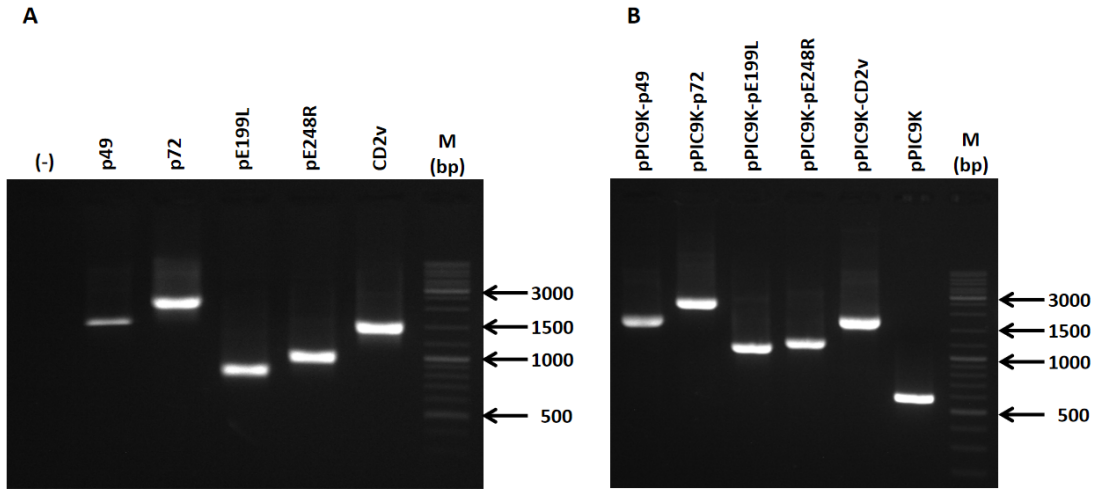

Figure 1. (A) Electrophoresis of PCR products amplifying specific genes p49, p72, pE199L, pE248R, and CD2v from the ASFV genome using corresponding primer pairs: p49F and p49R; p72F and p72R; pE199LF and pE199LR; pE248R F and pE248R R; CD2v F and CD2v R. (B) *E. coli* DH5α bacterial colonies after transformation with pPIC9K plasmid containing each target gene pPIC9K-p49, pPIC9K-p72, pPIC9K-pE199L, pPIC9K-pE248R, and pPIC9K-CD2v, with AOX1 primer pair. M represents the DNA size marker.

The PCR products of the target genes encoding p49, p72, pE199L, pE248R, and CD2v have sizes of approximately 1314 bp, 1938 bp, 597 bp, 744 bp, and 1274 bp, respectively, suitable with the designed and predicted sizes (Figure 1A). These target genes were cloned into the expression plasmid pPIC9K at the *Eco*RI and *Not*I restriction sites to create pPIC9K-p49, pPIC9K-p72, pPIC9K-pE199L, pPIC9K-pE248R, and pPIC9K-CD2v. The pPIC9K expression plasmids contain only one of the target genes. To confirm the presence of the target genes in pPIC9K, plasmids containing these target genes were subjected to PCR using AOX1 primers specific to the plasmid. The results showed p49, p72, pE199L, pE248R, CD2v, and AOX1 of pPIC9K with approximate sizes of 1806 bp, 2430 bp, 1089 bp, 1236 bp, 1766 bp, and 492 bp, respectively (Figure 1B). These are the size of the target genes plus the AOX1 primer segment.

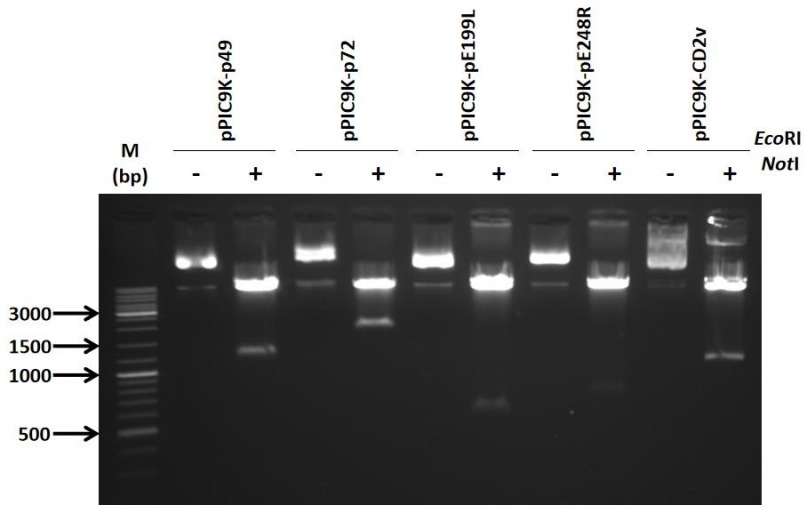

Figure 2. The electrophoretic profiles of plasmids pPIC9K-p49, pPIC9K-p72, pPIC9K-pE199L, pPIC9K-pE248R, and pPIC9K-CD2v with the absence (-) or presence (+) of *Eco*RI and *Not*I enzymes. M represents the DNA size ladder.

The bacterial colonies containing the target genes were subjected to plasmid extraction and purification, followed by digestion with the restriction enzymes *Eco*RI and *Not*I. The electrophoresis results of the digested products of the genes p49, p72, pE199L, pE248R, and CD2v at the (+) showed bands with sizes approximately 1314 bp, 1938 bp, 597 bp, 744 bp, and 1274 bp, respectively (Figure 2). The size of these bands corresponds to the size of the target genes, indicating that the target gene segments were inserted correctly into the pPIC9K plasmid at the designated positions. The (+) wells also show the presence of a brighter band that corresponds to the size of the pPIC9K vector. In the remaining (-) wells, the absence of the *Eco*RI and *Not*I restriction enzymes results in bands corresponding to pPIC9K-p49, pPIC9K-p72, pPIC9K-pE199L, pPIC9K-pE248R, and pPIC9K-CD2v, respectively.

To confirm the product correctly expressed the proteins of interest, we sequenced the plasmid samples containing the target genes. The AOX1 primer pair was used to determine the sequence of the gene segment inserted into the Multiple Cloning Site (MCS) of the pPIC9K plasmid. The sequencing results were processed and compared with NCBI gene data using Clustal X software (SFI). The results showed a 100% nucleotide sequence match compared to the original sequence with no modifications to the composition or amino acid sequence. These findings indicate that we have successfully cloned the target gene segments into the pPIC9K expression plasmid. Also, the PCR amplification using Phusion DNA Polymerase did not introduce any differences to the original target genes.

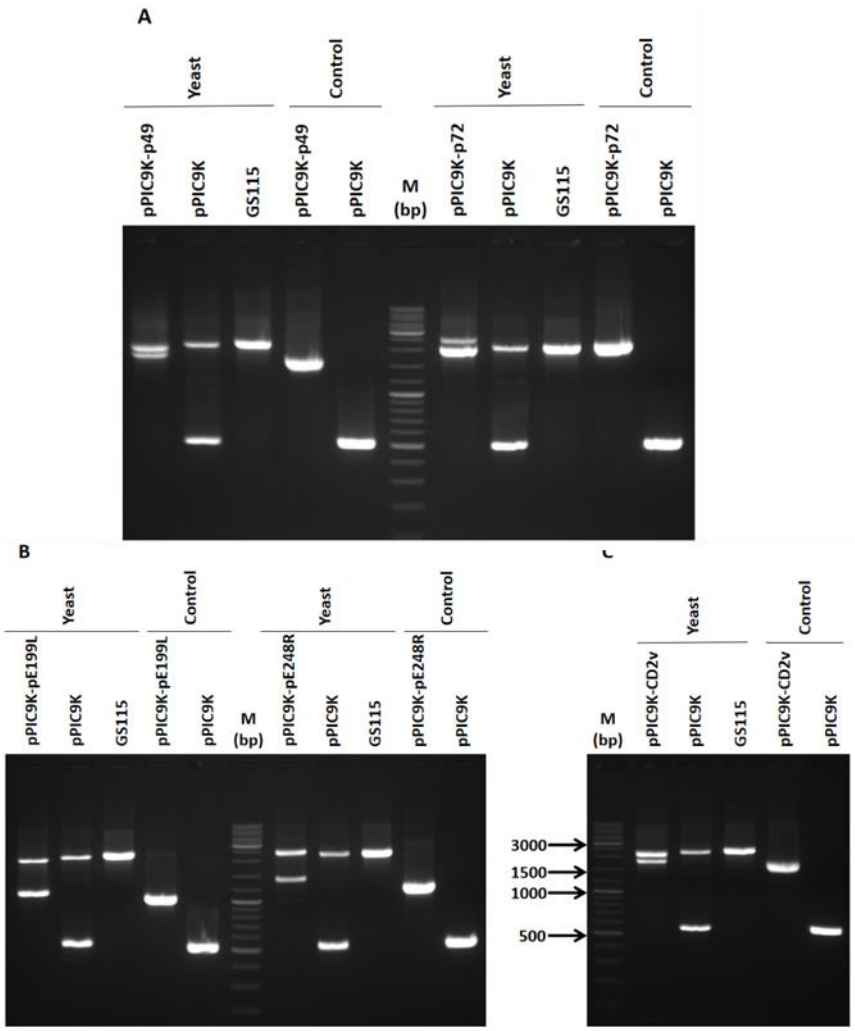

Figure 3. Electrophoresis of PCR products from *Pichia pastoris* GS115 yeast strains: *P. pastoris* GS115, *P. pastoris* GS115 containing empty pPIC9K, and *P. pastoris* GS115 containing pPIC9K with target genes: pPIC9K-p49 and pPIC9K-p72 (A), pPIC9K-pE199L and pPIC9K-pE248R (B), pPIC9K-CD2v (C). The control group includes strains with only empty pPIC9K or pPIC9K with target genes detected using the AOX1 primer pair. M represents the DNA size marker.

The plasmid pPIC9K containing the target genes was linearized using the *Sal*I enzyme, transformed into the *P. pastoris* GS115 yeast strain, and spread on MD plates lacking histidine. The colonies grown on these plates were transferred to YPD plates containing the Geneticin antibiotic. The colonies resistant to Geneticin were subjected to PCR using the AOX1 primers.

The results of the electrophoresis of the PCR products for the target genes are shown in Figure 3: pPIC9K-p49 and pPIC9K-p72 (Figure 3A), pPIC9K-pE199L and pPIC9K-pE248R (Figure 3B), and pPIC9K-CD2v (Figure 3C). In all cases, a band of approximately 2.2 kb appeared for all target genes in the yeast expression system, corresponding to the size of the AOX1

segment in the GS115 yeast strain. Additionally, the wells for pPIC9K-p49, pPIC9K-p72, pPIC9K-pE199L, pPIC9K-pE248R, and pPIC9K-CD2v displayed bands of approximately 1806 bp, 2430 bp, 1089 bp, 1236 bp, and 1766 bp, respectively, which correspond to the sizes of AOX1 containing the genes p49, p72, pE199L, pE248R, and CD2v. The sizes of the products matched the predicted result, as shown in Figure 1B. The pPIC9K wells showed a band at 492 bp, corresponding to the size of the AOX1 segment in the pPIC9K vector.

DISCUSSION

For this study, we successfully generated strains of *Pichia pastoris* GS115 containing plasmids pPIC9K-p49, pPIC9K-p72, pPIC9K-pE199L, pPIC9K-pE248R, and pPIC9K-CD2v. The pPIC9K plasmids have been shown to carry one of the target genes: p49, p72, pE199L, pE248R, and CD2v, as demonstrated by PCR methods using the AOX1 primer pair (Figure 1B) and by electrophoresis of the digested products (Figure 2). Throughout the experiment, the sizes of the target genes were recorded as 1314 bp for p49, 1938 bp for p72, 597 bp for pE199L, 744 bp for pE248R, and 1274 bp for CD2v (Figures 1A and 2). Using PCR with the AOX1 primer pair for the plasmid, the plasmids containing the target genes—specifically pPIC9K-p49, pPIC9K-p72, pPIC9K-pE199L, pPIC9K-pE248R, and pPIC9K-CD2v—showed sizes of 1806 bp, 2430 bp, 1089 bp, 1236 bp, and 1766 bp, respectively (Figures 1B and 3). In conclusion, the plasmids pPIC9K-p49, pPIC9K-p72, pPIC9K-pE199L, pPIC9K-pE248R, and pPIC9K-CD2v have been successfully constructed in the *Pichia pastoris* GS115 yeast strain. Moreover, we screened *P. pastoris* GS115 strains on MD media to select those capable of Histidine biosynthesis, which helps create the His+ phenotype. This helps increase the efficiency of obtaining colonies containing multiple inserted segments. Subsequently, we further selected His+ strains on the YPD medium supplemented with Geneticin antibiotic to identify strains resistant to high concentrations of Geneticin. The purpose of these strategies was to identify *P. pastoris* strains capable of optimal protein expression upon methanol induction, thereby supporting future research.

Currently, there are no reports worldwide regarding the cloning and expression of African Swine Fever Virus proteins in *P. pastoris*. Furthermore, protein expression in this yeast strain holds promise over previous systems like *E. coli* due to its potential for enhanced structural biology functions and lower production costs compared to expensive systems such as HEK-293 cells. *Pichia pastoris* yeast strain not only enables easy cultivation and protein expression but also can produce large quantities of protein, making it suitable for future production processes. The results of this study also pave the way for utilizing yeast expression systems in African swine fever virus research.

The findings of this study are just the beginning of our efforts toward producing subunit vaccines to combat the African swine fever virus through yeast expression systems. In subsequent experiments, we will conduct studies on the expression of the recombinant proteins of these target genes.

CONCLUSION

Currently, African Swine Fever is causing significant economic damage globally,

directly impacting the supply of pork and the income of pig farms. The devastation caused by this disease necessitates effective preventive measures to protect pig herds from the invasion of ASFV. In recent years, many studies have focused on subunit vaccines as a promising approach to combat ASFV. Our research has successfully generated a recombinant *P. pastoris* GS115 strain containing plasmids pPIC9K-p49, pPIC9K-p72, pPIC9K-pE199L, pPIC9K-pE248R, and pPIC9K-CD2v. Building on this foundation, we are conducting studies to express these recombinant proteins to develop a vaccine against ASFV. The objective is to evaluate the effectiveness of the vaccine in preventing ASFV invasion, especially in the context of the ongoing complex ASF outbreak in Vietnam. The results of this research are expected to play a crucial role in protecting pig herds and reducing the economic losses caused by African Swine Fever.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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