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ORIGINAL ARTICLE

# *Sasa quelpaertensis* Nakai extract suppresses porcine reproductive and respiratory syndrome virus replication and modulates virus-induced cytokine production

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**Abstract** Although *Sasa quelpaertensis* Nakai, a dwarf bamboo, is known to exert a variety of beneficial effects on health, its antiviral effect remains to be elucidated. Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most devastating viral pathogens of swine and has a substantial economic impact on the global pork industry. Therefore, the present study was conducted to determine whether *Sasa quelpaertensis* Nakai extract (SQE) inhibits PRRSV infection in cultured porcine alveolar macrophages (PAMs). Our results demonstrated that SQE treatment suppressed the replication of PRRSV in a dose-dependent manner. The antiviral activity of SQE on PRRSV replication was found to be primarily exerted at early times postinfection. Treatment with SQE resulted in marked reduction of viral genomic and subgenomic RNA synthesis, viral protein expression, and progeny virus production. Notably, pro-inflammatory cytokine production in PAM cells infected with PRRSV was shown to be modulated in the presence of SQE. Taken together, our data indicate that SQE has potential as a therapeutic agent against PRRSV.

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## Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), a pathogenic macrophage-tropic arterivirus of pigs, is the etiological agent of acute respiratory illness in young piglets and reproductive failure in pregnant sows [2]. PRRSV is a small enveloped single-stranded positive-sense RNA virus that belongs to the family *Arteriviridae* of the order *Nidovirales* [9, 27, 33]. The PRRSV genome contains two large open reading frames (ORFs), 1a and 1b, which comprise the 5′ two-thirds of the viral genome and encode non-structural proteins (nsps). The remaining ORFs located in the 3′-terminal region code for structural proteins [22, 32]. The initial translation of ORF1a and ORF1b yields the 1a and lab replicase polyproteins, respectively, which are then proteolytically processed into at least 16 functional nsps, including viral RNA-dependent RNA polymerase (RdRp) [7, 12, 13, 36]. The RdRp-containing replication complex mediates genomic RNA replication and subgenomic (sg) mRNA transcription, eventually generating a nested set of 3′-coterminal sg mRNAs that are individually translated to produce structural proteins [22, 32].

PRRSV preferentially replicates in porcine alveolar macrophages (PAMs) and can establish persistent infection in lymphoid tissues of infected pigs that lasts for several months [3, 10, 11, 37]. As a result, PRRSV infection suppresses normal macrophage functions and immune responses, which may render pigs susceptible to secondary bacterial or viral infections, leading to more-severe disease than caused by either agent alone [4, 14, 15, 38]. Furthermore, a highly pathogenic PRRSV infection, which is characterized by high fever and high mortality in pigs of all ages, has recently emerged in China and other Asian countries and severely affected local pig husbandries [6,

34]. Although both killed and modified live vaccines against PRRSV have been developed to control the disease and are used in the global market, most commercial vaccines have problems related to their efficacy and safety. Despite the availability of the vaccine, PRRSV has still continued to plague most pork-producing nations, causing enormous financial losses for the swine industry worldwide [16, 28]. Thus, the lack of effective vaccines increases the need for new compounds against PRRSV infection.

*Sasa quelpaertensis* Nakai, known as Jeju-Joritdae, is a type of bamboo grass that is widely distributed on Mt. Halla on Jeju Island in South Korea. Its leaf extract possesses a number of health-promoting activities, including anti-ulcerogenic, anti-obesity, anti-inflammatory, and anticancer properties [17–19, 29, 35]. To date, there are still no reports on the effectiveness of *Sasa quelpaertensis* Nakai extract (SQE) against viral infections in human or veterinary subjects. In the present study, therefore, we tried to investigate the antiviral activity of SQE and its mechanism of action in target cells upon PRRSV infection. Treatment of target cells with SQE significantly impaired PRRSV replication. Further experiments revealed that SQE suppresses post-entry steps in the replication cycle of PRRSV, including viral genomic and sg RNA synthesis, viral protein expression, and virus production. The presence of SQE notably altered expression of cytokine genes in PRRSV-infected PAM cells, suggesting that SQE activity is involved in the modulation of inflammatory responses during viral infection. Collectively, our results suggest that SQE may be an excellent therapeutic option for PRRSV infection.

## Materials and methods

### Cells, viruses, reagents, and antibodies

PAM-KNU cells (immortalized PAM cell line; [31]) were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum (FBS; Invitrogen), antibiotic-antimycotic solution (100×; Invitrogen), 10 mM HEPES (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and nonessential amino acids (100×; Invitrogen) in the presence of 200 μg/ml G418 (Invitrogen). The cells were maintained at 37 °C in a humidified 5 % $CO_2$ incubator. PRRSV strain VR-2332 was propagated in PAM-KNU cells as described previously [25]. SQE was a kind gift from Yong Chool Boo (Kyungpook National University, Daegu, South Korea). It was prepared and dissolved in distilled water (DW) as described elsewhere [5, 20, 21]. The yield of SQE from dried *S. quelpaertensis* leaves (1 kg) was approximately 10 %. *p*-Coumaric acid was purchased from Sigma (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO). A monoclonal antibody (MAb; SDOW17) against the PRRSV N protein was purchased from Rural Technologies (Brookings, SD). Anti-β-actin antibody and horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### Cell viability assay

The cytotoxic effects of reagents on PAM-KNU were analyzed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma) detecting cell viability. Briefly, PAM-KNU cells were grown to a density of $1 \times 10^4$ cells/well in a 96-well tissue culture plate with SQE or *p*-coumaric acid treatment for 24 and 48 h. After incubation, 50 μl of MTT solution (1.1 mg/ml) was added to each well, and the samples were incubated for an additional 4 h. The supernatant was then removed from each well, after which 150 μl of DMSO was added to dissolve the formazan crystals produced from the MTT. The absorbance of the solution was measured at 540 nm using an enzyme-linked immunosorbent assay plate reader. All MTT assays were performed in triplicate.

### Immunofluorescence assay (IFA)

PAM-KNU cells grown on microscope coverslips placed in 6-well tissue culture plates were pretreated with SQE or *p*-coumaric acid for 1 h and mock infected or infected with PRRSV at a multiplicity of infection (MOI) of 1 in the presence of each compound. The virus-infected cells were allowed to grow in the presence of SQE or *p*-coumaric acid until 48 hpi, fixed with 4 % paraformaldehyde for 10 min at room temperature (RT), and permeabilized with 0.2 % Triton X-100 in PBS at RT for 10 min. The cells were blocked with 1 % bovine serum albumin (BSA) in PBS for 30 min at RT and then incubated with an N-specific MAb 7 for 2 h. After being washed five times in PBS, the cells were incubated for 1 h at RT with a goat anti-mouse secondary antibody conjugated with Alexa Fluor 488 (Molecular Probes, Carlsbad, CA), followed by counterstaining with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). The coverslips were mounted on glass microscope slides in mounting buffer (60 % glycerol and 0.1 % sodium azide in PBS), and cell staining was visualized using a fluorescent Leica DM IL LED microscope (Leica, Wetzlar, Germany).

### Western blot analysis

PAM-KNU cells were grown in 6-well tissue culture plates for 1 day and were mock infected or infected with PRRSV at an MOI of 1. At the indicated times, cells were harvested in 50 μl of lysis buffer (0.5 % TritonX-100, 60 mM β-

glycerophosphate, 15 mM ρ-nitro phenyl phosphate, 25 mM MOPS, 15 mM, $MgCl_2$, 80 mM NaCl, 15 mM EGTA [pH 7.4], 1 mM sodium orthovanadate, 1 μg/ml E64, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF) and sonicated on ice five times for 1 s each. Homogenates were lysed for 30 min on ice and clarified by centrifugation at 15,800×*g* (Eppendorf centrifuge 5415R, Hamburg, Germany) for 30 min at 4 °C. The protein concentrations of the cell lysates were determined by a BCA protein assay (Pierce, Rockford, IL). The cell lysates were mixed with 4× NuPAGE sample buffer (Invitrogen) and boiled at 70 °C for 10 min. The proteins were separated on a NuPAGE 4–12 % gradient Bis-Tris gel (Invitrogen) under reducing conditions and electrotransferred onto Immobilon-P membranes (Millipore, Billerica, MA). The membranes were subsequently blocked with 3 % powdered skim milk (BD Biosciences, Belford, MA) in TBS (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) with 0.05 % Tween-20 (TBST) at 4 °C for 2 h and reacted at 4 °C overnight with the primary antibody against PRRSV N or β-actin. The blot was then incubated with the secondary horseradish peroxidase (HRP)-labeled antibody at a dilution of 1:5,000 for 2 h at 4 °C. Proteins were visualized by addition of enhanced chemiluminescence (ECL) reagent (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. To quantify viral protein production, the band densities of PRRSV N proteins were quantitatively analyzed using a computer densitometer with the Wright Cell Imaging Facility (WCIF) version of the ImageJ software package (http://www.uhnresearch.ca/facilities/wcif/imagej/) based on the density value relative to β-actin gene.

### Time course of SQE treatment

PAM-KNU cells were infected with PRRSV at an MOI of 1 as described above. At -1, 0, 1, 2, 4, 6, 8, 10, 12, or 24 hpi, SQE was added to reach the indicated final concentration for the remainder of the time course experiment. The virus-infected and SQE-treated cells were further maintained, and the infection was terminated at 48 hpi by fixing the monolayers with 4 % paraformaldehyde for 10 min at RT. The fixed cells were subjected to IFA to assess the presence of PRRSV infection as described above.

### Virus internalization assay

An internalization assay was performed as described previously [8]. Briefly, PAM-KNU cells grown in 6-well culture plates were infected with PRRSV at an MOI of 1 at 4 °C for 1 h in the presence of SQE. Unbound viruses were then washed with PBS, and the cells were incubated at 4 °C or 37 °C in the presence of SQE for 1 h, followed by treatment with proteinase K (0.5 mg/ml) at 4 °C for 45 min to remove the bound but non-internalized virus particles. The PRRSV-infected cells were then serially diluted in RPMI and added to fresh PAM-KNU cell monolayers in 96-well tissue culture plates. At 48 h post-incubation, internalized viruses were titrated by IFA as described above, and the 50 % tissue culture infectious dose ($TCID_{50}$) was calculated.

### Quantitative real-time RT-PCR

PAM-KNU cells were incubated with SQE for 1 h prior to infection and then inoculated with PRRSV at an MOI of 1 for 1 h at 37 °C. The virus inoculum was subsequently removed and the infected cells were maintained in fresh medium containing SQE for 48 h. Total RNA was extracted from lysates of the infected cells at 48 hpi using TRIzol reagent (Invitrogen) and treated with DNase I (TaKaRa, Otsu, Japan) according to the manufacturer's protocols. The concentration of the extracted RNA was measured using a NanoVue spectrophotometer (GE Healthcare, Piscataway, NJ). Quantitative real-time RT-PCR was conducted using a Thermal Cycler Dice Real Time System (TaKaRa) with gene-specific primer sets as described previously [30]. The primers used in this study were described elsewhere [23, 24], and primer sequences are available on request. The RNA levels of viral and cytokine genes were normalized to that of mRNA for the porcine β-actin gene, and the relative quantities (RQ) of mRNA accumulation were determined using the $2^{-\Delta\Delta Ct}$ method [26]. To detect alteration of genomic RNA and sg mRNA levels in the presence of SQE during PRRSV infection, the results obtained using SQE-treated samples were compared with those obtained with vehicle-treated samples. To assess the effect of SQE on transcriptional activation of pro- and anti-inflammatory cytokines in virus-infected cells, the relative fold change of each cytokine gene was calculated and compared between virus-infected and mock-infected PAM cells and between vehicle-treated and SQE-treated virus-infected cells.

### Northern blot analysis

PAM-KNU cells were treated with SQE (5 mg/ml) for 1 h prior to infection, followed by PRRSV inoculation at an MOI of 1 for 1 h at 37 °C. The infected cells were maintained in fresh medium containing SQE or vehicle for 24 h. Total RNA was extracted from lysates of the infected cells at 24 hpi using TRIzol reagent and treated with DNase I as described above. Northern blotting was conducted using a NorthernMax Kit (Ambion, Austin, TX) according to the manufacturer's instructions. Samples of total RNA (5 μg)

were loaded onto a 1 % agarose gel and separated by electrophoresis, using a denaturing buffer system. The separated total RNA was then transferred to a BrightStar-Plus nylon membrane (Ambion) for 3 h and cross-linked by exposure to UV light for 5 min. Pre-hybridization was performed at 42 °C for 40 min, followed by detection using the genotype-specific probe for the 3′ UTR (5′-AATTTCGGCCGCATGGTTTTCGCCAATTAAATCTTACCCCCACACGGTCGC-3′) or porcine 16S rRNA (5′-GCGGTTGCACCATTTGGGTGTCCTG-3′). The blot was hybridized to biotin-labeled oligonucleotide probes in ULTRAhyb reagent at 42 °C overnight and washed twice with low-stringency blocking buffer and wash buffer. Target viral RNAs were detected using a BrightStar BioDetect Kit (Ambion) according to the manufacturer's protocols. The membrane was incubated with alkaline-phosphatase-conjugated streptavidin, followed by reaction with the chemiluminescent substrate CDP-STAR (Ambion). The overlaid films were exposed in a dark cassette box in a dark room, and quantitation was performed by densitometry of the corresponding bands as described above.

### Virus titration

PAM-KNU cells were infected with PRRSV along with SQE treatment as described above. The culture supernatant was collected at different time points (6, 12, 24, 36, and 48 hpi) and stored at −80 °C. The titer of PRRSV was measured by limiting dilution on PAM-KNU cells by IFA as described above and then quantified as the $TCID_{50}$ per ml.

### Statistical analysis

Student's *t*-test was used for all statistical analyses and *p*-values of less than 0.05 were considered statistically significant.

## Results

### Suppression of PRRSV replication by SQE

To investigate the antiviral activity of SQE, PRRSV was chosen because it is one of the most important viral pathogens that have an economic impact on the global pork industry. Based on the MTT assay, the highest concentration of SQE that was noncytotoxic was 5 mg/ml in PAM-KNU cells. PAM-KNU cells were pretreated with SQE at concentrations of 0.1 to 5 mg/ml or with DW as a vehicle control for 1 h prior to infection, and SQE was present during the entire period of infection. Virus production was initially measured by monitoring the cytopathic effect (CPE) after infection and confirmed by immunofluorescence at 48 hpi using an N-protein-specific MAb (Fig. 1). In vehicle-treated control cells, visible CPE appeared at 24 hpi and became prominent by 48 hpi, and N-specific staining was clearly evident in many cell clusters, indicating infection and the spread of the virus to neighboring cells. In contrast, SQE had an obvious inhibitory effect on virus propagation. As shown in Fig. 1A and B, SQE significantly decreased virus-induced CPE and viral gene expression at concentrations of ∼1 mg/ml. N protein staining revealed that the number of cells expressing viral antigen decreased during SQE treatment, resulting in a maximum of ∼80 % inhibition at a concentration of 5 mg/ml (Fig. 1A and B). Furthermore, the effective dose for inhibiting PRRSV infection by 50 % ($ED_{50}$) was determined to be about 3.05 mg/ml. Taken together, these results demonstrate that SQE efficiently suppresses the replication of PRRSV.

To determine the point at which SQE acted during PRRSV infection, PAM-KNU cells were treated independently with SQE at various time points postinfection. At 48 hpi, the level of PRRSV replication was measured indirectly using IFA to determine the number of cells expressing the N protein (Fig. 2). Treatment of cells with 5 mg/ml SQE at -1, 0, and 1 hpi resulted in a more than 80 % decrease in PRRSV-positive cells when compared to untreated controls. Addition of SQE between 2 and 12 hpi led to a 72 % to 33 % decrease in the number of PRRSV-infected cells. In contrast, no significant impairment of PRRSV propagation was observed when SQE was added at 24 hpi. These data indicate that the inhibitory effect of SQE was exerted mainly during the initial period of infection, suggesting that its action occurs at early time points after PRRSV infection.

### Inhibition of infectious viral progeny production by SQE

To further assess the effect of SQE on PRRSV replication, virus yield was determined during treatment with SQE. Viral supernatants were collected at 48 hpi and viral titers were measured. As shown in Fig. 3A, treatment with SQE inhibited the release of viral progeny in a dose-dependent manner. The peak viral titer was determined to be $10^{7.06}$ $TCID_{50}$/ml in the vehicle-treated control for PRRSV, but the addition of 5 mg/ml SQE decreased the titer of PRRSV to $10^{2.6}$ $TCID_{50}$/ml (>4 log reduction compared with the control). A growth kinetics study further demonstrated that the overall process of PRRSV replication was significantly delayed when cells were treated with SQE (Fig. 3B). Consequently, these findings revealed that SQE inhibits the optimal release of progeny virus from the natural host cells.

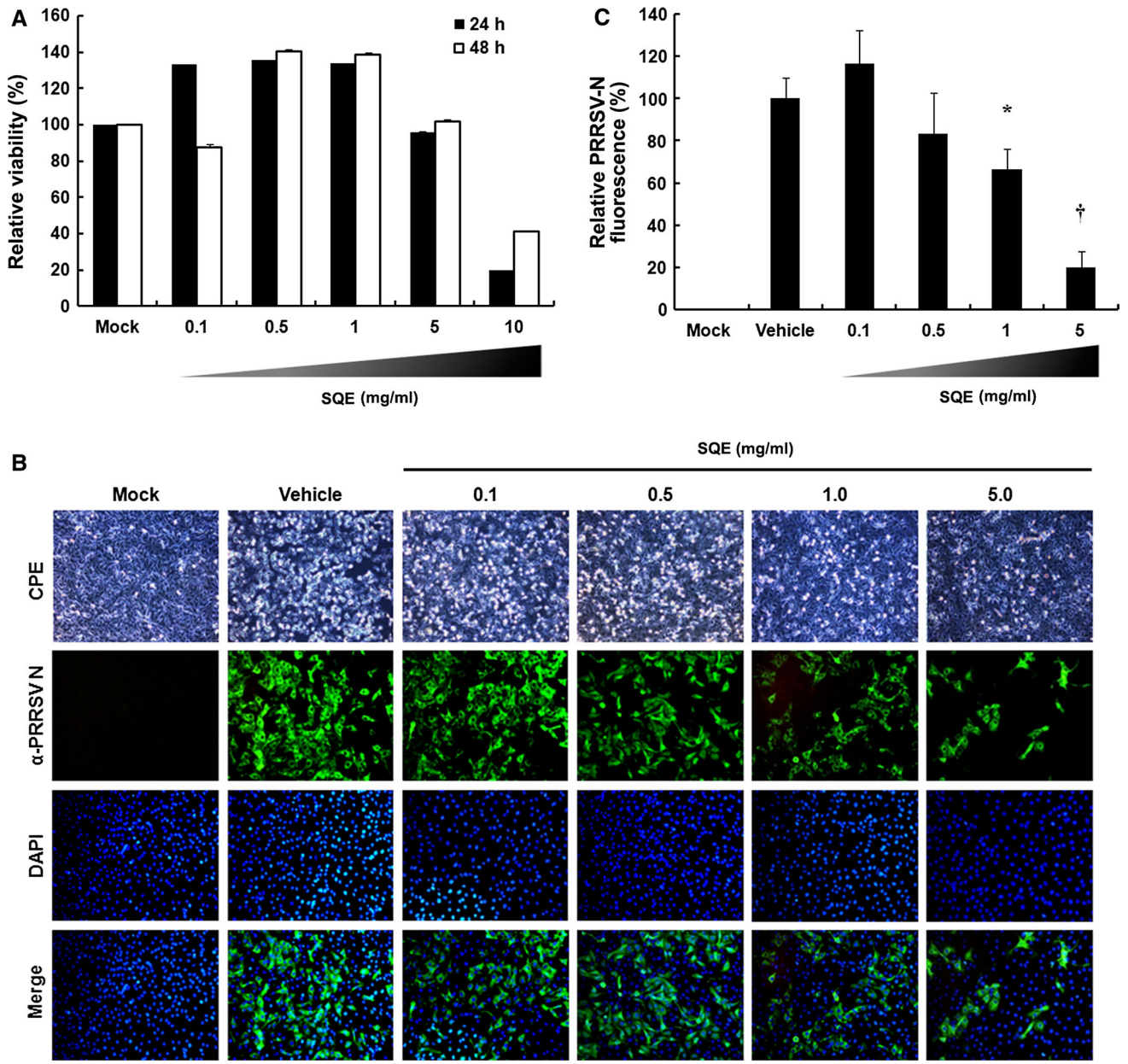



**Fig. 1** Effect of SQE on the replication of PRRSV. (**A**) PAM-KNU cells were incubated with various concentrations of SQE for 24 h prior to the MTT assay, and cytotoxicity of SQE was determined by the MTT assay. (**B**) PAM-KNU cells were preincubated with SQE at the indicated concentrations for 1 h prior to infection and were mock infected or infected with PRRSV at an MOI of 1. The virus-infected cells were then maintained for another 48 h in the presence of vehicle or SQE. Virus-specific CPEs were observed daily and were photographed at 48 hpi using an inverted microscope at a magnification of 100× (first row of panels). For immunostaining, infected cells were fixed at 48 hpi and incubated with an MAb against the N protein of PRRSV, followed by Alexa Green–conjugated goat anti-mouse secondary antibody (second row of panels). The cells were then counterstained with DAPI (third row of panels) and examined using a fluorescent microscope at 200× magnification. (**C**) Production of virus in the presence of SQE was measured by counting the cells expressing N proteins, using IFA. Five fields at 200× magnification were counted for each condition, and the total number of cells per field as determined by DAPI staining was similar in all fields. Values are representative of the mean of three independent experiments, and error bars represent standard deviations. *, $P = 0.001$ to 0.05; †, $P < 0.001$

### Effect of SQE on the virus internalization process

To evaluate which steps of the replication cycle of PRRSV were targeted by SQE, we started focusing on the earliest step, virus entry. To address this issue, an internalization assay was performed as described previously [8]. PAM-KNU cells were inoculated with PRRSV at 4 °C for 1 h to allow virus attachment and further maintained either at

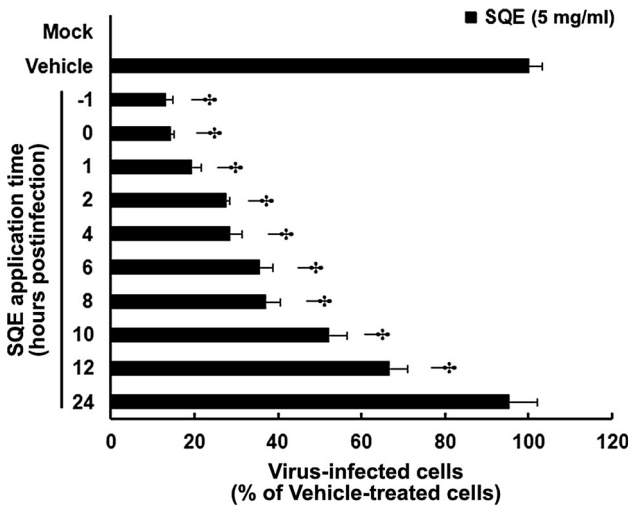
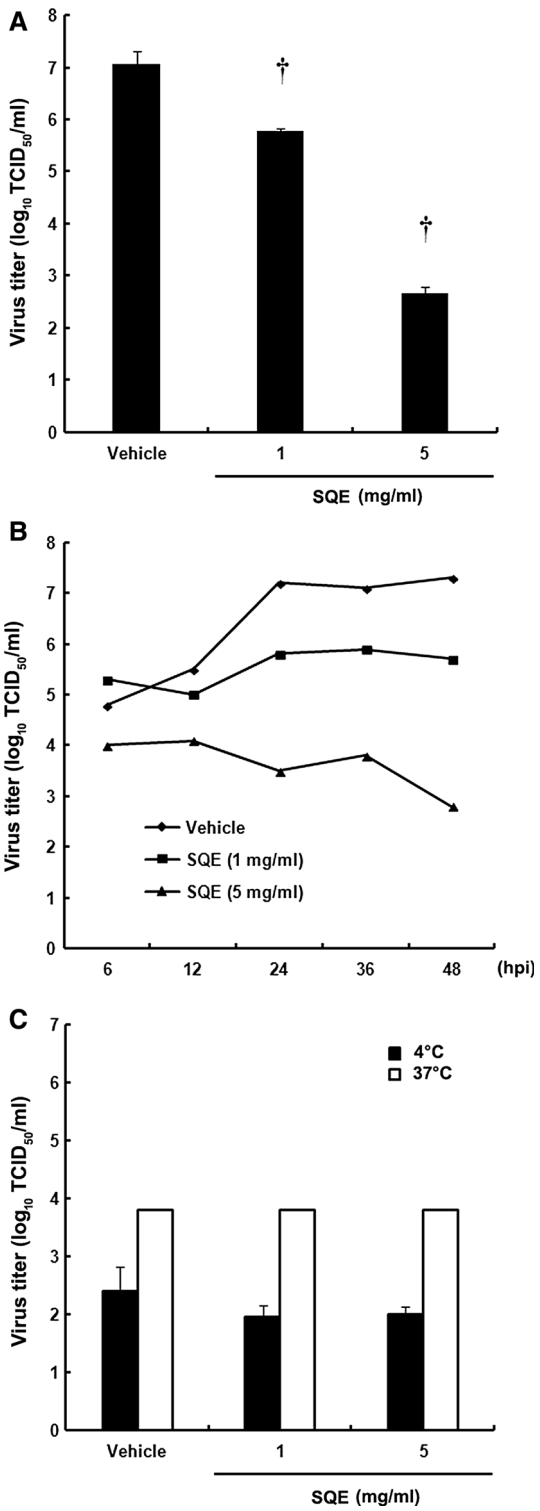



**Fig. 2** Suppression of PRRSV propagation by SQE at early times postinfection. PAM-KNU cells were pretreated with vehicle and SQE (5 mg/ml) and were mock infected or infected with PRRSV. At the indicated times after infection, SQE was added to achieve a final concentration of 5 mg/ml. At 48 hpi, virus-infected cells were fixed, and virus infectivity was determined by counting the cells expressing N proteins, using IFA. Data are representative of the mean of three independent experiments, and error bars represent standard deviations. †, $P < 0.001$

4 °C or at 37 °C to permit virus internalization in the presence of SQE. The samples were then treated with proteinase K to remove the remaining viruses from the cell surface. The serially diluted infected cells were subsequently subjected to an infectious center assay on uninfected PAM-KNU cell monolayers, and virus titers were measured 2 days later by IFA. As shown in Fig. 3C, the titer of PRRSV was virtually the same in cells treated with SQE or without SQE at 37 °C and was determined to be $10^{3.8}$ TCID50/ml, indicating no differences between those cells. In contrast, only minimal viral production of about $10^2$ $TCID_{50}$/ml was observed in PRRSV-infected cells maintained at 4 °C, which was likely due to inefficient removal of the bound virus. These results indicated that SQE has no inhibitory effect on the virus entry process.

## Inhibition of viral protein expression by SQE treatment

Following the uncoating process, like all positive-sense RNA viruses, the genome of PRRSV is released into the cytoplasm and immediately serves as a template for viral translation by a cellular cap-dependent mechanism. Early viral translation produces replicase polyproteins that are posttranslationally cleaved into nsps by viral or cellular proteinases. Subsequently, the replicase proteins mediate *de novo* synthesis of viral RNA, including genomic RNA replication and sg mRNA transcription. The structural proteins of PRRSV are translated later from their




respective sg mRNA transcripts. Thus, to investigate inhibitory effects of SQE on post-entry steps of the PRRSV life cycle, we first investigated whether viral protein translation was affected by SQE. To accomplish this, PAM-KNU cells were treated with SQE for 1 h prior to infection, and the drug was allowed to remain during

◀**Fig. 3** Reduction of viral progeny production by SQE treatment. (**A**) PAM-KNU cells were pretreated with SQE for 1 h and were mock infected or infected with virus. SQE was present in the medium throughout the infection. At 48 hpi, virus-containing supernatants were collected and virus titers were determined. (**B**) Growth kinetics of PRRSV on PAM-KNU cells treated with SQE, using the same experimental conditions as in panel **A**. At the indicated time points postinfection, culture supernatants were harvested and virus titers were measured. (**C**) A virus-internalization assay was conducted by infecting PAM-KNU cells with PRRSV at an MOI of 1 at 4 °C for 1 h. After washing with cold PBS, infected cells were maintained in the presence or absence of SQE, either at 4 °C or 37 °C, for an additional hour. Bound but non-internalized virus particles were removed by treatment with proteinase K. The infected cells were then serially diluted and plated onto PAM-KNU cells in 96-well tissue culture plates. At 2 days post-incubation, internalized viruses were titrated, using IFA for detection, and the 50 % tissue culture infectious dose ($TCID_{50}$) was calculated. Results are expressed as the mean values from triplicate wells, and error bars represent standard deviations. †, $P < 0.001$

infection and subsequent incubation. The expression level of the PRRSV N protein in the presence or absence of SQE was evaluated at 48 hpi by western blot analysis. The production of the PRRSV N protein was drastically diminished during SQE treatment (Fig. 4). Densitometric analysis of the western blot revealed that the intracellular expression of the viral protein was reduced by SQE treatment, with a maximum of about 90 % inhibition at a concentration of 5 mg/ml (Fig. 4). This marked reduction was probably not the result of a nonspecific decrease in translation, since Ponceau S staining indirectly indicated that the overall expression levels of cellular proteins remained similar following treatment (data not shown). Taken together, these results demonstrated the inhibitory effects of SQE specifically against viral protein expression during PRRSV replication.

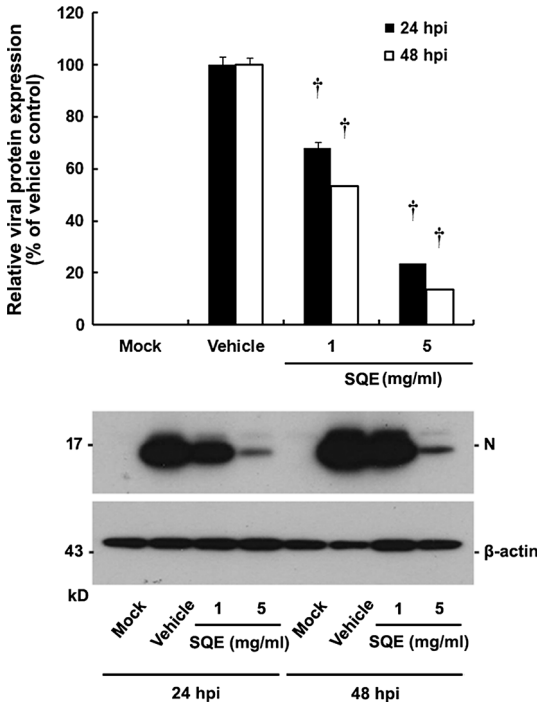


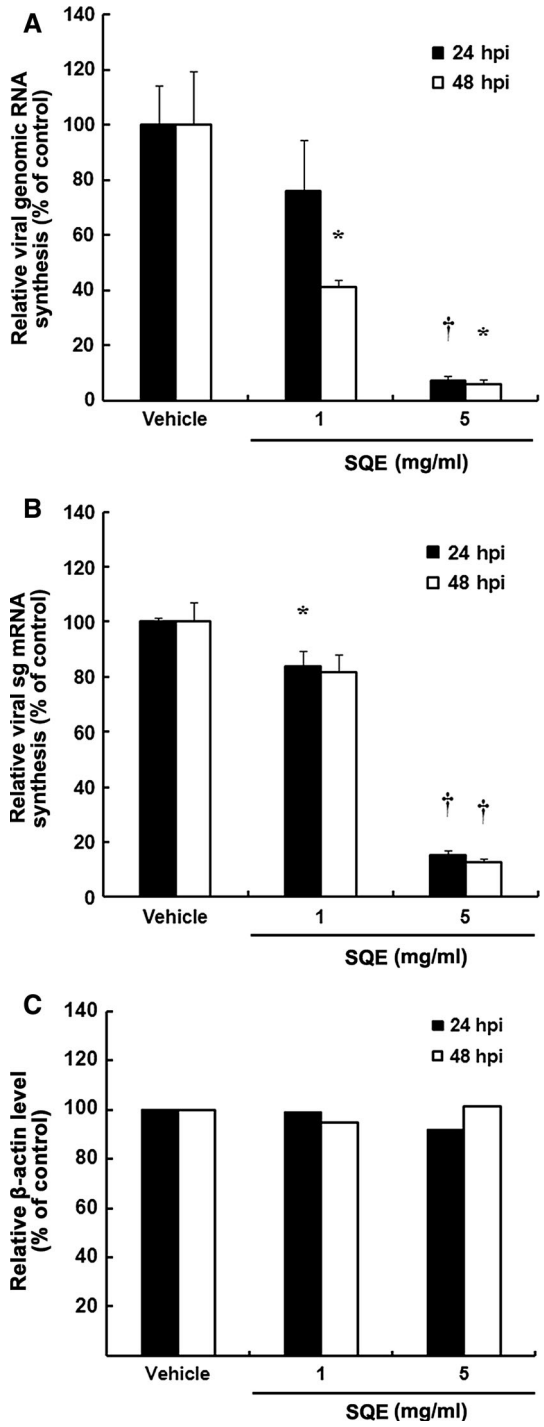

**Fig. 4** Inhibition of viral protein translation by SQE treatment. SQE-treated PAM-KNU cells were mock-infected or infected with PRRSV for 1 h and were further cultivated in the presence or absence of SQE. At 24 and 48 hpi, cell lysates were collected, resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted using a PRRSV-specific antibody that recognizes the PRRSV N protein. The blot was also reacted with mouse MAb against β-actin to verify equal protein loading. The amount of each viral protein was estimated by densitometry in terms of its band density relative to that of β-actin, and SQE-treated sample results were compared to vehicle-control results. Values are representative of the mean from three independent experiments, and error bars denote standard deviations. †, $P < 0.001$

## Suppression of PRRSV transcription by SQE

For positive-strand RNA viruses, viral nonstructural proteins are required for viral RNA replication and transcription. Thus, it is conceivable that the impaired viral protein expression was caused by suppression of viral RNA synthesis. Since PRRSV infection produces two RNA entities, genomic and subgenomic, we investigated whether SQE specifically affects genome replication and sg mRNA transcription. To answer this question, the relative levels of genomic RNA and sg mRNA were measured by quantitative real-time strand-specific RT-PCR in the presence or absence of SQE upon PRRSV infection. As shown in Fig. 5, SQE treatment resulted in a maximal reduction in the synthesis of PRRSV genomic RNA and sg mRNA of 90 % and 80 %, respectively, at a concentration of 5 mg/ml, when compared with untreated infected cells. The decrease in viral RNA levels after the addition of SQE was not due to nonspecific inhibition of transcription, as the mRNA level of the internal β-actin control remained unchanged in all samples (Fig. 5C). The impairment of PRRSV transcription in the presence of SQE was confirmed by northern blot analysis (Fig. 6). In the absence of SQE, PRRSV RNA synthesis was distinctly observed (lane 2). A >50 % reduction of RNA-synthesizing activity occurred in the presence of 1 mg of SQE per ml (lane 3), while viral RNA synthesis was almost completely abolished in the presence of 5 mg of SQE per ml (lane 4). Altogether, these results indicated that SQE specifically suppresses the synthesis of PRRSV genomic RNA and sg mRNA.

## Alteration of inflammatory responses by SQE during PRRSV infection

Since SQE has been reported to have anti-inflammatory properties, we examined whether SQE affects the transcriptional activation of immune-response genes upon PRRSV infection to determine whether this contributes to




◀**Fig. 5** Inhibition of viral RNA transcription by SQE. PAM-KNU cells pretreated with SQE were mock infected or infected with PRRSV for 1 h and were incubated in the presence of SQE. Total cellular RNA was extracted at 24 hpi (black bars) and 48 hpi (white bars), and viral genomic RNA and sg mRNA were amplified by quantitative real-time RT-PCR. Viral positive-sense genomic RNA (**A**) and sg mRNA (**B**) were normalized to mRNA for porcine β-actin mRNA (**C**), and relative quantities (RQ) of mRNA accumulation were evaluated. SQE-treated sample results were compared with untreated results. Values are representative of the mean from three independent experiments, and error bars denote standard deviations. *, $P = 0.001$ to 0.05; †, $P < 0.001$

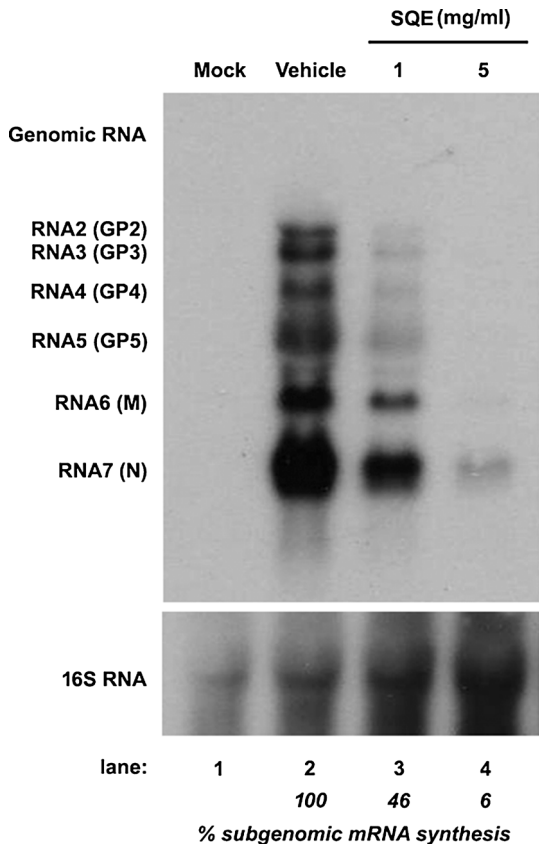



**Fig. 6** Suppression of viral RNA synthesis by SQE treatment. PAM-KNU cells were treated with SQE, followed by PRRSV inoculation, as indicated above the lanes. Total RNA was extracted from lysates of the infected cells at 24 hpi and subjected to northern blot analysis using genotype-specific biotin-labeled oligonucleotide probes against the 3′ UTR. Porcine 16S rRNA was used as a control to correct for variations in loading during viral RNA quantification. The amount of viral sg mRNAs was quantified by densitometry and normalized to that in PRRSV-infected, untreated control cells (100 %). The positions of the genomic RNA and sg mRNAs are indicated next to the gel

its antiviral activity. We found that numerous immune-related genes regulated by PRRSV infection were significantly altered by treating the cells with SQE when compared with results obtained with vehicle-treated virus-infected cells (Fig. 7). Among these, upregulation of IL-1α, IL-6, IL-8, IL-15, TNF-α, and AMCF-1 mRNA levels by PRRSV infection was dramatically reduced in the presence of SQE. However, chemokine genes that are modulated by PRRSV, including MCP-1 and RANTES, were synergistically elevated by SQE treatment. More interestingly, unchanged or slightly increased mRNA levels of interferon regulatory factors (IRFs), Toll-like receptors (TLRs), and antiviral genes including Mx1 and ISG-15 were

significantly increased in PRRSV-infected PAM cells treated with SQE compared with vehicle-treated infected cells. These results indicated that SQE modulates the cytokine genes for inflammatory responses during PRRSV infection.

## Discussion

PRRSV is not only a devastating viral pathogen in the pig population, but it can also be used as an animal virus model to study nidoviruses that are important in human and veterinary medicine. Despite tremendous efforts to control disease caused by this virus, PRRSV infection has continuously plagued pig-producing countries and has had a tremendous economic impact on the global swine industry. This is partially due to the absence of efficient vaccines capable of conferring full protection against heterologous viral infection as well as antiviral agents for treating PRRSV. Although SQE is widely known to possess several health-promoting properties [17, 18], its antiviral activity and mode of action on DNA and RNA viruses are currently unknown. The present study demonstrated that SQE exerts an adequate antiviral effect against PRRSV *in vitro*, as determined by the $ED_{50}$ for viral replication (antiviral activity) of approximately 3.05 mg/ml. Treatment of cells with SQE resulted in significant inhibition of post-entry steps during the replication of PRRSV, as demonstrated by reduced progeny production, diminished viral protein expression, and reduced synthesis of genomic RNA and sg mRNA. Furthermore, a growth kinetics experiment indicated an approximately 4-log reduction in the titer of PRRSV in the presence of SQE at 24 h after infection, and the titer continued to remain at a similar level 48 h after infection. Taken together, our data indicate that SQE potently elicits antiviral activity against PRRSV in natural target cells.

Compounds from medicinal plants or natural sources are of interest for their potential to inhibit a variety of viral infectious diseases and cancer. Bamboo grasses have been used as traditional medicines throughout Asia, and modern studies have reported their beneficial health effects. *Sasa quelpaertensis* Nakai is among these and is well known as Jeju-Joritdae in South Korea. SQE, its leaf extract, has a number of biological properties, including anticancer and anti-inflammatory effects. Since PRRSV infection modulates inflammatory cytokine expression in PAM cells, the antiviral activity of SQE may result predominantly from its anti-inflammatory effect. To elucidate potential mechanism(s) responsible for the antiviral effect of SQE on PRRSV, we first tested whether SQE affects the induction of cytokine genes by PRRSV in immortalized PAM cells. The present study provided evidence that various cytokines induced in PRRSV-infected cells are transcriptionally modulated in the presence of SQE. Our results indicate that treatment with SQE robustly reduced infection-induced expression of pro-inflammatory cytokines, including IL-1α, IL-6, IL-8, IL-15, and TNF-α. In contrast, SQE resulted in a synergic elevation in gene expression levels of chemokines. Notably, the induction of IRFs, TLRs, and genes involved in the antiviral immune response was specifically enhanced in SQE-treated virus-infected cells. PRRSV is known to possess the ability to escape host defense mechanisms by interfering with innate immune responses to favor its survival and spread in the natural host. Thus, SQE appears to disrupt one of the pivotal viral mechanisms of immune evasion by modifying the expression of immune-related genes to facilitate virus infection, resulting in the suppression of PRRSV replication. However, we were unable to identify which chemical constituent(s) in SQE is responsible for the regulation of immune-response gene expression that leads to its antiviral activity upon PRRSV infection. Although *p*-coumaric acid is known to be a major component of SQE (23.71 mg/g) and has diverse pharmacological properties, including antimicrobial, anticancer, and antiulcer activities [19, 20], this chemical was incapable of significantly impairing the replication of PRRSV, even at the highest noncytotoxic concentration of 100 μM (Fig. A1). It is conceivable that other minor constituents such as tricin, with a content in SQE of 0.028 mg/g, may possess the biological characteristics that give SQE its antiviral activity. Indeed, recent studies have demonstrated inhibitory effects of synthesized tricin or its derivative on viral infections [1, 39]. In addition to PRRSV, our experiment was further extended to other nidoviruses to confirm the antiviral activity of SQE. Porcine epidemic diarrhea virus (PEDV) is one of pathogenic coronaviruses of pigs. Like PRRSV, it belongs to the order *Nidovirales*, whose members all have a similar genome organization and replication strategy. Thus, we attempted to assess whether SQE also affects PEDV infection but found that it had no significant inhibitory effect on PEDV infection *in vitro* (Fig. A2). This observation might be explained by the tropism of each virus, since PRRSV is a porcine macrophage-tropic arterivirus causing acute respiratory illness, whereas PEDV is a pathogenic enterocyte-tropic coronavirus of swine causing acute enteritis and watery diarrhea. Although SQE failed to block PEDV infection, it still has potential as an antiviral agent against other coronaviruses of human and livestock, for which the modulation of immune-response cytokine gene production in the natural host is related to their replication.

In conclusion, our findings described here demonstrate that SQE at subcytotoxic doses effectively prevents the replication of PRRSV in natural host cells. Additionally, SQE treatment significantly altered PRRSV-induced

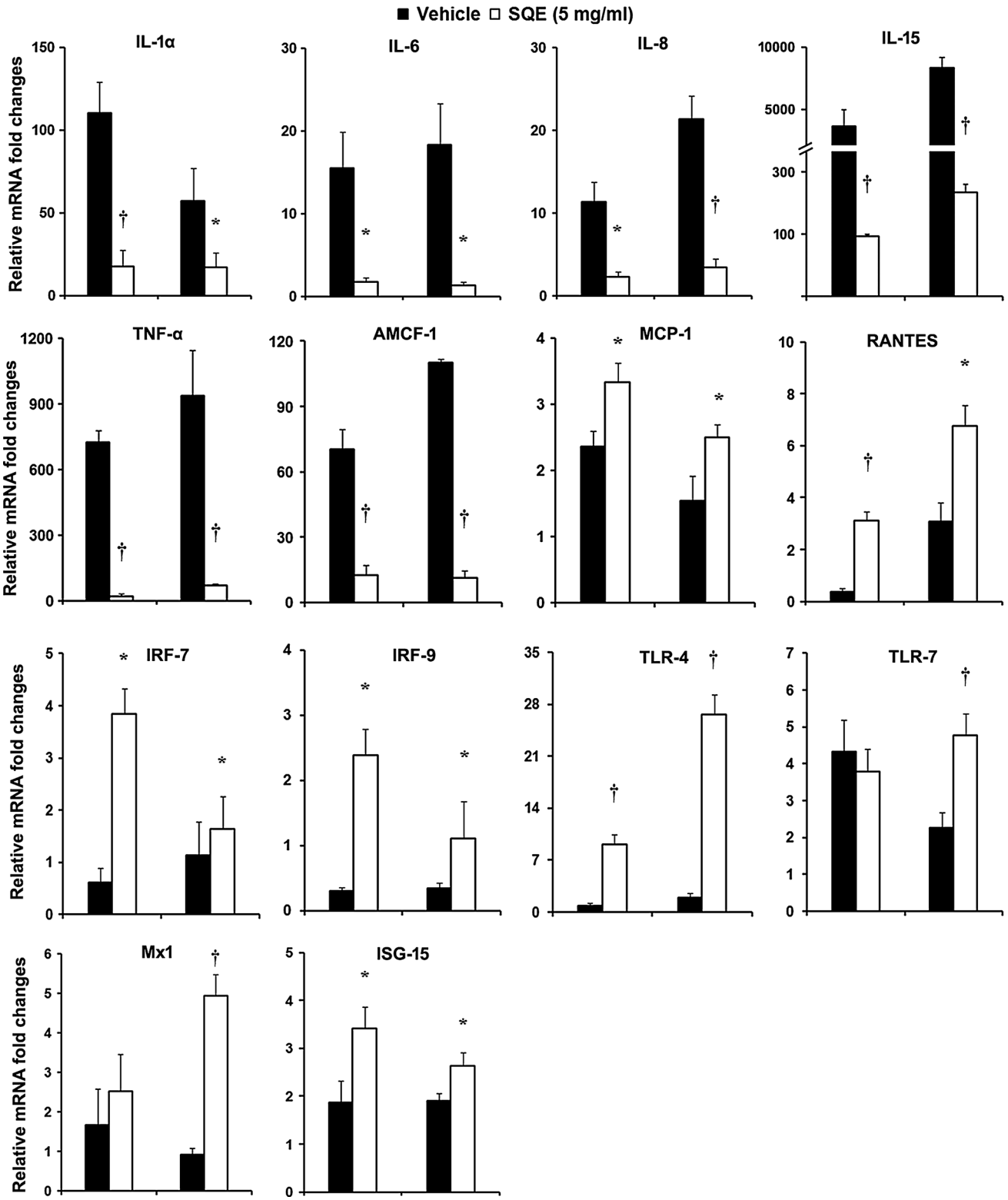



**Fig. 7** Regulation of immune-related genes by SQE during PRRSV infection. In the presence of vehicle or SQE (5 mg/ml), PAM-KNU cells were mock infected or infected with PRRSV at an MOI of 1. Total RNA was extracted from lysates of the infected cells at 24 hpi (left bar set) and 48 hpi (right bar set). The mRNA level of each cytokine gene was assessed by quantitative real-time RT-PCR and normalized to that of porcine β-actin. The relative fold difference between mock-infected and virus-infected cells (y-axis) was then calculated for each gene and compared between untreated and SQE-treated virus-infected cells. Data are representative of the mean values from three independent experiments carried out in duplicate, and error bars represent standard deviations. *, $P = 0.001$ to 0.05; †, $P < 0.001$

cytokine production in PAM cells. Therefore, we propose that one of the modes of action of SQE to elicit an antiviral effect against PRRSV is to regulate the expression of immune-related genes that are associated with the ability of PRRSV to escape the host innate immune responses. Although further *in vivo* studies are needed to evaluate the efficacy and safety of SQE, the results presented here indicate that it is a good candidate as an antiviral agent against PRRSV.

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