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Betulinic Acid Delays Turnip Mosaic Virus Infection by Activating the Phytosulfokine Signalling Pathway in *Nicotiana benthamiana*

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Keywords: betulinic acid | defence response | phytosulphokine | PSK-PSKR signalling pathway | TuMV

ABSTRACT

Plant viral diseases pose a significant threat to agricultural production, and the availability of effective drugs against viral diseases remains limited. In this study, we discovered that betulinic acid (BA), a pentacyclic triterpenoid derived from plants, delays infection by turnip mosaic virus (TuMV) in *Nicotiana benthamiana*. Transcriptomic analysis revealed that BA treatment specifically induced the expression of *N. benthamiana* phytosulfokine 3 (NbPSK3), a plant pentapeptide hormone with diverse functions, while TuMV infection suppressed its expression. Further study demonstrated that NbPSK3 positively regulates antiviral defence against TuMV infection. Disruption of PSK signalling by targeting the membrane-bound PSK receptors (PSKRs) promoted viral infection. Additionally, exogenous sulphonated PSK (active form) treatment significantly delayed infection by TuMV in *N. benthamiana* compared to unmodified PSK peptides (dPSK, inactive form) or control treatments, while silencing the receptor *NbPSKR1* abolished the ability of PSK to inhibit TuMV infection. Moreover, the inhibition of TuMV infection by BA is dependent on the PSK-PSKR signalling pathway. Overall, these findings not only underscore the potential of BA as a promising and environmentally friendly agent for modulating plant viral diseases but also emphasise the role of the PSK signalling pathway in promoting at least partial resistance to TuMV, which might have interest for crop breeding.

1 | Introduction

In nature, plants are continuously exposed to a variety of pathogens, including fungi, bacteria, nematodes and viruses. Among these, plant viruses are particularly threatening to agricultural production, owing to the characteristics of their obligate-parasite lifestyle and limitations in prevention and curation of viral diseases; thereby, viral diseases can be known as 'plant cancer' (Scholthof et al. 2011; Tatineni and Hein 2023). Unfortunately, effective biological, chemical and other antiviral products for preventing and controlling plant viral disease are limited. Moreover, traditional strategies

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no longer suffice for modern agricultural needs (Jones and Naidu 2019). Recently, research has shifted toward exploiting plant-derived substances for controlling plant viral diseases (Zhao et al. 2017). These substances offer advantages such as safety, cost-effectiveness and environmental friendliness due to their degradation pathways. Plant-derived substances have already shown promising efficacy and potential applications in treating animal viruses, cancer and tumour diseases (Samec et al. 2020; Zou et al. 2021; Kłos and Chlubek 2022). Curcumin, the major bioactive in turmeric, can inhibit the replication of hepatitis B virus (HBV) and human immunodeficiency virus (HIV) (Wei et al. 2017; Butnariu et al. 2022). Butyric acid is a fatty acid derived from multiple vegetable sources and inhibits HBV replication and cell proliferation in hepatoma cells by inhibiting SIRT-1 expression (Pant et al. 2019). Betulinic acid (BA), also known as birchic acid, is a pentacyclic triterpene compound found in various plants including birch, eucalyptus and plane trees (Aswathy et al. 2022). BA can be extracted from natural sources or synthesised through oxidative methods using betulinol as a starting material (Lou et al. 2021). Research on BA has revealed a range of biological and pharmacological functions, including antitumour, anti-inflammatory and antiviral activities (Zhang et al. 2015; Jiang et al. 2021). For example, BA has demonstrated good antiviral effects against dengue virus (DENV), zika virus (ZIKV) and chikungunya virus (CHIKV) (Loe et al. 2020). Consequently, identifying plant-derived substances with significant inhibitory effects on plant viral infections is crucial for the sustainable management of plant viral diseases.

Phytosulfokine [PSK; Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln] is a plant peptide growth regulator that is widely distributed among higher plants (Sauter 2015). Mature PSK is derived from precursor proteins consisting of 80–120 amino acids, which are encoded by nuclear genes and are conserved across various species, including *Arabidopsis*, soybean, *Brassica napus*, *Asparagus officinalis*, maize and rice (Matsubayashi and Sakagami 1996; Matsubayashi et al. 1997; Yu et al. 2019). The PSK precursor protein undergoes sulphonylation by casein sulphotransferase (TPST) in the Golgi apparatus, after which it is transferred to the cytoplasmic ectodomain. There it is further cleaved by SBT protease to form mature PSK with biological function (Komori et al. 2009; Kaufmann and Sauter 2019). Mature PSK peptides are recognised at the cell surface by membrane-bound PSK receptors (PSKRs), which belong to the leucine-rich repeat receptor-like kinase (LRR-RLK) class. These receptors consist of an extracellular domain, a transmembrane domain and an intracellular domain (Kaufmann et al. 2017). The extracellular domain contains leucine-sequence repeats that bind PSK and recruit the co-receptor RLK (Matsubayashi et al. 2002; Ladwig et al. 2015). The intracellular domain is responsible for autophosphorylation or phosphorylation of downstream substrates, thereby transmitting extracellular signals (Hartmann et al. 2014, 2015). Notably, the recognition and biological activity of PSK are mainly dependent on PSKR1 (Zhang et al. 2018).

The PSK-PSKR signalling pathway plays a pivotal role in plant resistance to pathogen infection. For instance, PSK enhances the immunity of tomato plants to the necrotrophic pathogen *Botrytis cinerea* by increasing cytoplasmic Ca^{2+} concentration and activating auxin-mediated pathways (Zhang et al. 2018). In *Arabidopsis*, overexpression of *PSK2* and *PSK4* increases susceptibility to the bacterial pathogen *Pseudomonas syringae* pv. *tomato*, while it increases resistance to the fungal pathogen *Alternaria brassicicola* (Mosher et al. 2013). PSKR1, the primary receptor for PSK, is critical in the PSK-PSKR signalling pathway. In *Arabidopsis*, *PSKR1* mutants exhibit higher susceptibility to *A. brassicicola* but show enhanced resistance to *P. syringae* pv. *tomato* (Igarashi et al. 2012; Mosher et al. 2013). In tomato, silencing of *PSKR1* results in heightened susceptibility to *B. cinerea* (Zhang et al. 2018). In rice, *OsPSKR1* enhances resistance to rice leaf blight (*Xanthomonas oryzae*) by inducing the expression of *PR* genes (Yang et al. 2019). However, whether the PSK-PSKR signalling pathway regulates plant viral infections remains unclear.

Turnip mosaic virus (TuMV) is a single-stranded, positive-sense RNA virus belonging to the genus *Potyvirus* in the family *Potyviridae*. It infects a wide range of plant species (Yang et al. 2021), and diseases caused by TuMV pose a significant threat to the safe production of vegetable crops. Unfortunately, there are no effective strategies to control plant viral diseases. In this study, we demonstrated that BA delays the infection of TuMV in *Nicotiana benthamiana* by activating the PSK-PSKR signalling pathway. Overall, our study identifies BA as a novel antiviral agent for modulating plant viral diseases and reveals a novel antiviral pathway involving the PSK-PSKR signalling pathway.

2 | Results

2.1 | Exogenous Application of BA Delays TuMV Infection in *N. benthamiana*

To determine whether BA is effective for controlling plant viral infections, the roots of *N. benthamiana* plants were treated with 10 mg/L BA and subsequently inoculated with TuMV. At 5 days post-inoculation (dpi), BA treatment was found to significantly inhibit TuMV infection, as evidenced by reduced GFP-labelled TuMV accumulation (Figure 1a). Systemic leaves from the inoculated plants were then collected, and viral accumulation was analysed using reverse transcription-quantitative PCR (RT-qPCR) and western blotting assays. The results indicated that BA treatment significantly decreased the expression levels of TuMV genomic RNA and GFP protein compared to the control (Figure 1b,c). This result was confirmed in inoculated leaves (Figure S1), suggesting that BA positively contributes to delaying TuMV infection. We also monitored symptoms and viral accumulation at different time points after inoculation; by 15 dpi, the virus had spread throughout the entire plant, when no significant differences in viral accumulation were detected. To evaluate BA efficacy against other viruses, similar experiments were conducted using tobacco mosaic virus (TMV) and cotton leaf curl Multan virus (CLCuMuV). Exogenous application of BA was found to inhibit TMV and CLCuMuV infection as well (Figure S2). Collectively, these findings suggest that BA treatment may help to control a variety of viral infections, at least in the early stages.

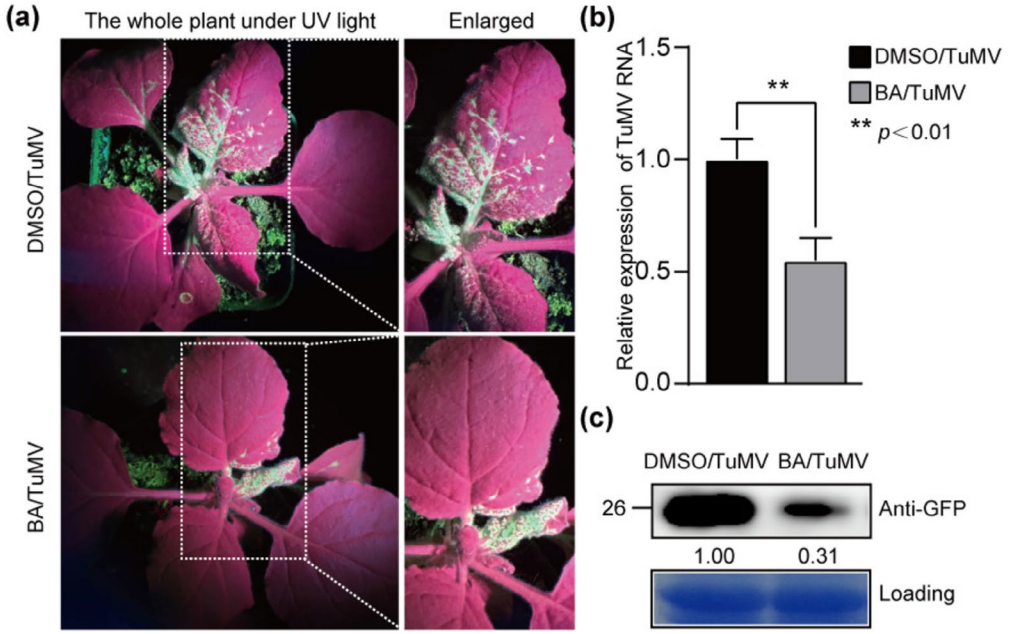

FIGURE 1 | Betulinic acid (BA) treatment inhibits TuMV-GFP infection. (a) BA treatment decreased the fluorescence intensity of TuMV-GFP. *Nicotiana benthamiana* plants were treated with 10 mg/L BA for 5 days and then inoculated with TuMV-GFP. At 5 days post-inoculation (dpi), the symptoms were observed under a UV lamp. (b) BA treatment significantly decreased the expression level of TuMV genomic RNA compared with dimethyl sulphoxide (DMSO)-treated plants. The relative expression level of TuMV genomic RNA was quantified by reverse transcription-quantitative PCR at 5 dpi with TuMV-GFP in BA- or DMSO-treated plants in the systemic leaves. *NbActin* was used as an internal reference gene. Statistical analyses were performed using Student's *t* test, $^{**}p < 0.01$. (c) The accumulation of TuMV-GFP protein significantly decreased in the BA-treated plants compared to the control. Western blot detection of the protein content in the systemic leaves of TuMV-GFP in BA- or DMSO-treated plants at 5 dpi. Total protein was extracted from TuMV-GFP-inoculated plants and western blot was performed using an anti-GFP antibody. The Coomassie brilliant blue-stained RuBisCO large subunit gel shows equivalent sample loadings. The results were reproduced in three independent experiments using 10 plants per treatment with similar results.

2.2 | *NbPSK3* Is a Target of TuMV Infection and BA Treatment

To elucidate the molecular mechanism underlying BA-mediated delays to TuMV infection in *N. benthamiana*, RNA sequencing was performed on leaves under four different treatments: CK_Mock (CK indicates dimethyl sulphoxide [DMSO] treatment), BA_Mock, CK_TuMV and BA_TuMV. Principal component analysis (PCA) showed that the three biological replicates of each treatment clustered together, indicating strong correlation within groups and significant differences between treatments (Figure S3). We identified a total of 592, 9135 and 598 differentially expressed genes in the comparisons of BA_Mock versus CK_Mock, CK_Mock versus CK_TuMV and CK_TuMV versus BA_TuMV (Figure 2a,b). Venn diagram analysis revealed that 3879 genes (3879/9135) were specifically up-regulated following TuMV infection (Figure 2a). A total of 285 genes were activated in the BA-treated plants compared to DMSO control plants (Figure 2b). Further analysis of gene response patterns under TuMV infection and BA treatment showed that 10 of the 5256 down-regulated genes induced by TuMV were up-regulated by BA, and 55 of the 3721 up-regulated genes induced by TuMV were down-regulated by BA (Figure 2c, Table S2).

Notably, the *NbPSK3* precursor gene with a critical role in plant resistance, exhibited significant differential expression (Figure 2d). Specifically, TuMV infection significantly down-regulated *NbPSK3*, whereas BA treatment significantly up-regulated *NbPSK3* expression, with the BA-induced up-regulation surpassing the suppression caused by TuMV (Figure 2d). This finding was corroborated by RT-qPCR assays (Figure 2e,f). Therefore, we hypothesised that the *NbPSK3* might play a critical role in the BA-mediated inhibition of TuMV infection.

2.3 | Silencing of *NbPSK3* Benefits TuMV Infection

To investigate the role of *NbPSK3* in TuMV infection, we used the tobacco rattle virus (TRV)-based gene silencing (VIGS) system to silence *NbPSK3*. Seven days after inoculation, plants inoculated with TRV-PDS (TRV-PDS as a positive control of the silencing effect; PDS: phytoene desaturase) exhibited significant photobleaching phenotypes. We then observed the phenotypes exhibited in *NbPSK3*-silenced plants and found that leaves of *NbPSK3*-silenced plants displayed curling, accompanied by shortened root systems, while no obvious growth defects were observed in the TRV-Luc-inoculated control plants (Figure S4a,c). RT-qPCR assays were performed to determine the amount of silencing of *NbPSK3*; the expression of *NbPSK3* in TRV-NbPSK3-inoculated plants was decreased to 24% of that in control plants, confirming effective silencing of *NbPSK3* (Figure S4b).

To assess the response of *NbPSK3*-silenced plants to TuMV infection, we conducted TuMV inoculation experiments using *NbPSK3*-silenced plants and control plants. At 5 dpi,

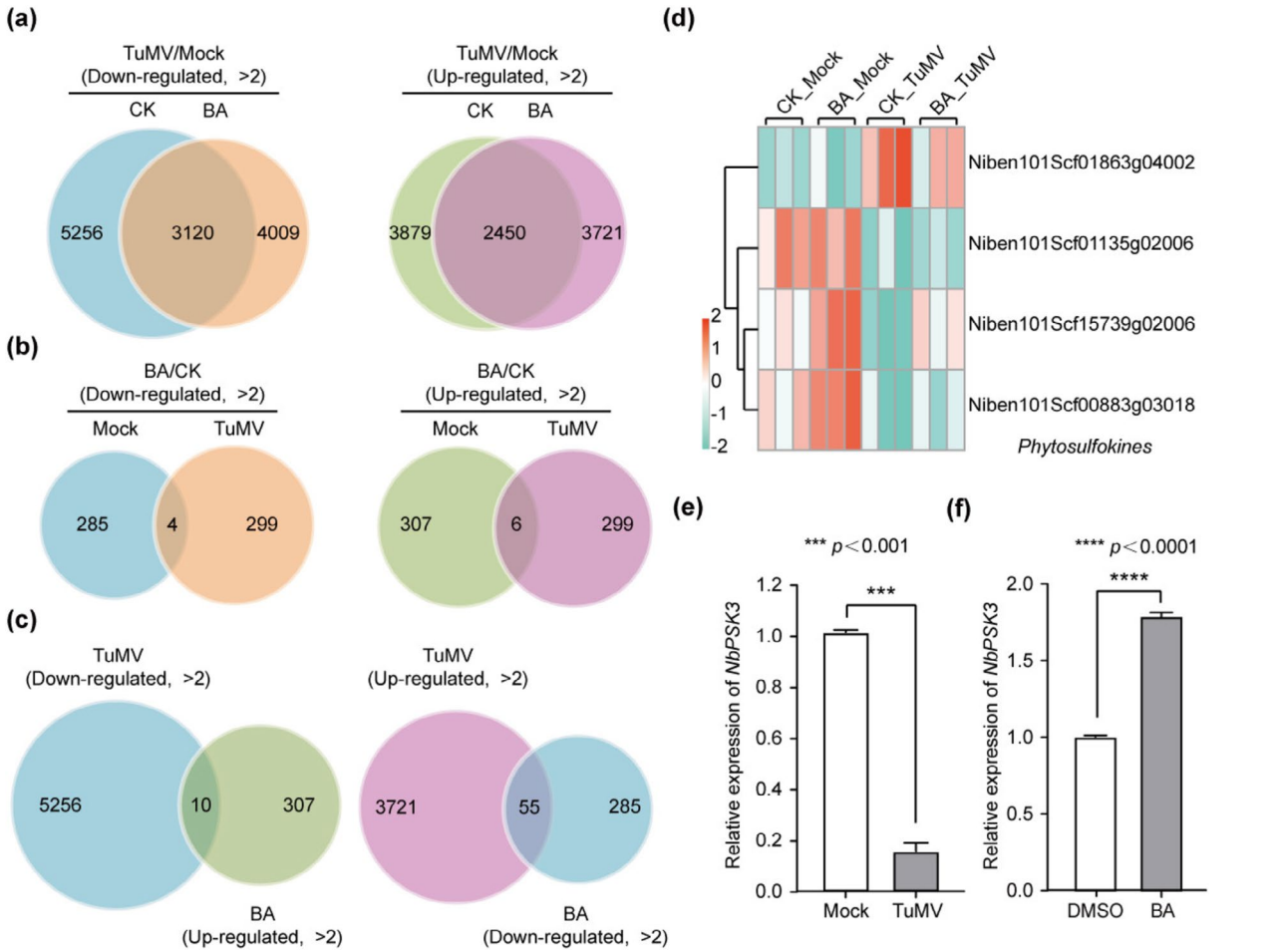

FIGURE 2 | Transcriptomic analysis of gene expression profiles under four different treatments (CK_Mock, BA_Mock, CK_TuMV and BA_TuMV). CK, dimethylsulphoxide treatment (DMSO); BA, betulinic acid treatment. (a–c) Venn diagrams showed the number of genes significantly up-regulated and down-regulated between different treatments. (d) Analysis of *NbPSK* precursor gene expression patterns in different treatment groups. (e) Reverse transcription-quantitative PCR (RT-qPCR) analysis of the relative expression level of the *NbPSK3* gene after infection by TuMV at 5 days post-inoculation (dpi). (f) RT-qPCR analysis of the relative expression level of the *NbPSK3* gene after BA treatment at 5 dpi. ***$p < 0.001$, ****$p < 0.0001$, significant differences with Student's *t* test. All experiments were repeated at least three times with similar results.

fluorescence observations showed a higher number of fluorescent spots on the systemic leaves of *NbPSK3*-silenced plants compared to control plants (Figure 3a). This suggests TuMV accumulation was greater in *NbPSK3*-silenced plants than in control plants. To quantify the TuMV accumulation, we performed RT-qPCR and western blotting assays to measure systemic levels of TuMV genomic RNA and GFP protein. The results indicated that TuMV accumulation was significantly higher in *NbPSK3*-silenced plants compared to control plants (Figure 3b,c). Similar results were confirmed in inoculated leaves (Figure S5a). The progress of TuMV infection also seemed to be slightly quicker in the *Arabidopsis atpsk3* mutant versus wild-type *Arabidopsis* (Figure S4g,h). These findings suggest that the disruption of *PSK3* promotes TuMV infection.

2.4 | Overexpression of *NbPSK3* Delays TuMV Infection

To further investigate the role of *NbPSK3* in TuMV infection, we used a PVX-based vector to overexpress *NbPSK3* in *N. benthamiana*. At 7 dpi, no obvious phenotypic differences were observed between the above-ground parts of PVX-NbPSK3- and PVX-inoculated plants, but it significantly promoted root length (Figure S4d,f). RT-qPCR analysis showed that the mean transcription level of *NbPSK3* in PVX-NbPSK3-inoculated plants was approximately 4-fold higher compared to PVX-inoculated control plants (Figure S4e). Following TuMV inoculation, we observed weaker fluorescence intensity on the systemic leaves of plants overexpressing *NbPSK3* compared to control plants (Figure 3d). Consistent with this, TuMV RNA and GFP protein were significantly lower in *NbPSK3*-overexpressing plants than in control plants (Figure 3e,f). Similar results were obtained in inoculated leaves (Figure S5b). These results collectively suggest that *NbPSK3* positively enhances plant delays to TuMV infection.

2.5 | BA-Induced Delay of TuMV Infection Relies on *NbPSK3*

We hypothesised that the hindering effects on TuMV infection progress induced by BA could rely on the activation of the PSK-PSKR signalling pathway. To test this hypothesis, we used the

TRV-based VIGS to silence the *NbPSK3* gene. At 5 dpi, we treated the plants with a BA solution continuously for 5 days. Systemic leaves of *N. benthamiana* plants were then collected for analysis. RT-qPCR results indicated that exogenous BA treatment did not affect the expression level of *NbPSK3* in *NbPSK3*-silenced plants (Figure S6). We then inoculated these plants with TuMV and examined TuMV accumulation at 5 days after TuMV inoculation. The fluorescence intensity of GFP-labelled TuMV in the systemic leaves of *NbPSK3*-silenced plants treated with BA or DMSO was higher than in control plants treated with BA. However, there was no significant difference in fluorescence intensity between *NbPSK3*-silenced plants treated with BA or DMSO (Figure 4a). Additionally, the RNA and protein levels of TuMV in BA/TRV-NbPSK3 and DMSO/TRV-NbPSK3 inoculated plants were analysed using RT-qPCR and western blotting assays, and the accumulation of TuMV was significantly higher in BA/TRV-NbPSK3 and DMSO/TRV-NbPSK3 plants than that in BA/TRV-Luc inoculated plants (Figure 4b,c). Similar results were obtained in inoculated leaves (Figure S7). These results collectively suggest that the inhibitory effect of BA on TuMV infection is dependent on NbPSK3.

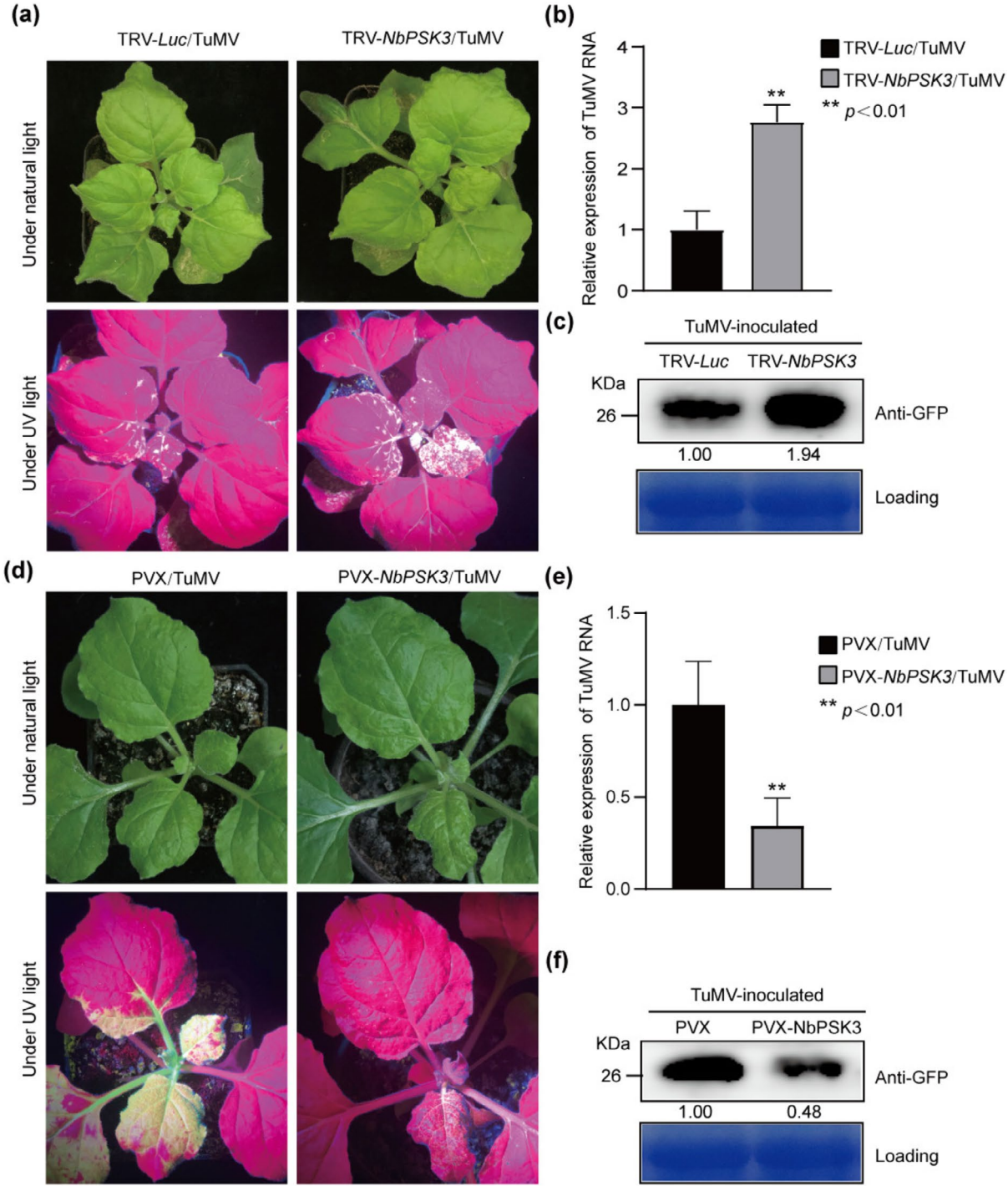

FIGURE 3 | Legend on next page.

FIGURE 3 | *NbPSK3* positively modulates *Nicotiana benthamiana* delays to TuMV-GFP infection. (a) Silencing *NbPSK3* promotes TuMV-GFP infection. The fluorescence intensity of GFP in the systemic leaves of the *NbPSK3*-silenced plant was significantly higher compared with that in the non-silenced control plants at 5 days post-inoculation (dpi). (b) The relative expression levels of TuMV genomic RNA significantly increased in the systemic leaves of *NbPSK3*-silenced plants compared to the non-silenced control plants at 5 dpi. The expression of TuMV genomic RNA was quantified by reverse transcription-quantitative PCR (RT-qPCR) at 5 dpi in *NbPSK3*-silenced and non-silenced plants systemic leaves. $^{**}p < 0.01$ significant difference. (c) The accumulation of TuMV-GFP protein significantly increased in the systemic leaves of *NbPSK3*-silenced plant compared to the non-silenced control plants. Western blot analysis of TuMV-GFP protein content in the *NbPSK3*-silenced plant compared to the non-silenced control plants at 5 dpi. (d) Overexpression of *NbPSK3* significantly inhibits TuMV-GFP infection. The fluorescence intensity of GFP in the systemic leaves of *NbPSK3*-overexpressing plants was significantly weaker than that in control plants at 5 dpi. (e) The relative expression levels of TuMV genomic RNA significantly decreased in the systemic leaves of *NbPSK3*-overexpressing plants compared to the control plants at 5 dpi. The expression of TuMV genomic RNA was quantified by RT-qPCR in the systemic leaves of *NbPSK3*-overexpressing and control plants at 5 dpi. *NbActin* was used as an internal reference gene. Statistical analyses were performed using Student's *t* test, $^{**}p < 0.01$. (f) The accumulation of TuMV-GFP protein significantly decreased in the systemic leaves of *NbPSK3*-overexpressing plant compared to the control plants. The accumulation of TuMV-GFP protein was detected by western blot at 5 dpi. The Coomassie brilliant blue-stained RuBisCO large subunit gel showed an equal amount of protein loaded in each well. The results were reproduced in three independent experiments using 10 plants per treatment with similar results.

2.6 | Exogenous Application of PSKα Hinders Progress of TuMV Infection

To further elucidate the role of PSK in regulating TuMV infection, we synthesised sulphonated and unmodified PSK polypeptides (which are small peptides derived from PSK3 and designated as PSKα and dPSK). Leaves of *N. benthamiana* plants were sprayed with either 10 μM PSKα, dPSK or water (control) 12 h before TuMV inoculation. At 5 dpi, fluorescence observations revealed that PSKα treatment significantly inhibited TuMV infection (Figure 5a). Additionally, the accumulation levels of TuMV RNA and GFP protein were significantly reduced in the systemically infected leaves of plants treated with PSKα compared to those treated with dPSK or water (Figure 5b,c). Similar results were obtained in inoculated leaves (Figure S5c). These results suggest that sulphonated PSK is functional in defence against TuMV.

2.7 | *NbPSKR1* Is Essential for PSK3-Mediated Delays to TuMV Infection

The recognition and biological function of PSK predominantly rely on its receptors, particularly PSKR1 (Matsubayashi et al. 2002). To uncover the NbPSKR1 function in plant immunity against TuMV infection, we silenced *NbPSKR1* expression in *N. benthamiana* using TRV-based VIGS. At 7 dpi, the silencing efficiency was assessed via RT-qPCR. Expression of *NbPSKR1* was significantly down-regulated to approximately 40% of that in control plants (Figure S8). Despite this silencing, the growth and development of *N. benthamiana* plants were unaffected by *NbPSKR1* silencing. Following TuMV infection, stronger fluorescence signals were observed in the systemic leaves of *NbPSKR1*-silenced plants compared to control plants at 5 days after TuMV inoculation (Figure 6a). Additionally, TuMV coat protein (CP) RNA and GFP protein levels were higher in *NbPSKR1*-silenced plants than in control plants (Figure 6b,c). Similar results were obtained in inoculated leaves (Figure S9a). These findings suggest that silencing *NbPSKR1* promotes TuMV infection.

In *N. benthamiana*, two homologues encode PSK receptor proteins, namely NbPSKR1 and NbPSKR2, with 56.1% amino acid sequence similarity between them. To reveal the role of NbPSKR2 in response to TuMV infection, we used TRV-based VIGS to silence *NbPSKR2*. As shown in Figure S10a, TuMV accumulation in *NbPSKR2*-silenced plants was similar to that in control plants (Figure S10a). Western blot analyses of GFP revealed no significant differences in their levels between *NbPSKR2*-silenced and control plants (Figure S10c). These results indicate that NbPSKR2 is not essential for plant resistance to TuMV infection, consistent with previous studies (Zhang et al. 2018).

To examine the role of *NbPSKR1* in PSKα-mediated delays to TuMV infection, we pretreated both *NbPSKR1*-silenced and control plants with PSKα before TuMV inoculation. The results showed that *NbPSKR1* silencing led to a loss of PSKα's ability to hinder TuMV infection, as evidenced by higher fluorescence intensity and virus accumulation levels in the systemic leaves of *NbPSKR1*-silenced plants compared to control plants (Figure 6d–f). Similar results were obtained in inoculated leaves (Figure S9b).

3 | Discussion

Plant viral diseases are one of the most significant categories of plant diseases, which have caused considerable damage to agricultural production. The prevention and control of viral diseases remain a global challenge in agriculture (Jones and Naidu 2019). While chemical pesticides are effective in managing fungal and bacterial diseases, they are less effective against viral diseases. Plant-derived pesticides offer promising alternatives due to their environmental safety, specificity, non-target biosafety and reduced likelihood of drug resistance. Numerous plant-derived antiviral substances have been identified with potential for development. For instance, extracts from *Thuja orientalis* significantly enhance resistance to cucumber mosaic virus (CMV) (Elbeshehy et al. 2015). Alkaloids derived from *Hosta plantaginea* exhibit inhibitory activity against TMV (Wang et al. 2007). Similarly, brucelicin-D from *Brucea brucea* shows a strong inhibitory effect on TMV infectivity (Shen et al. 2008). Alphamomorcharin, a member of the plant ribosomal inactivating proteins (RIPs) family, has demonstrated antiviral, antibacterial and antitumour activities in both animals and plants (Cao et al. 2015; Zhu et al. 2020).

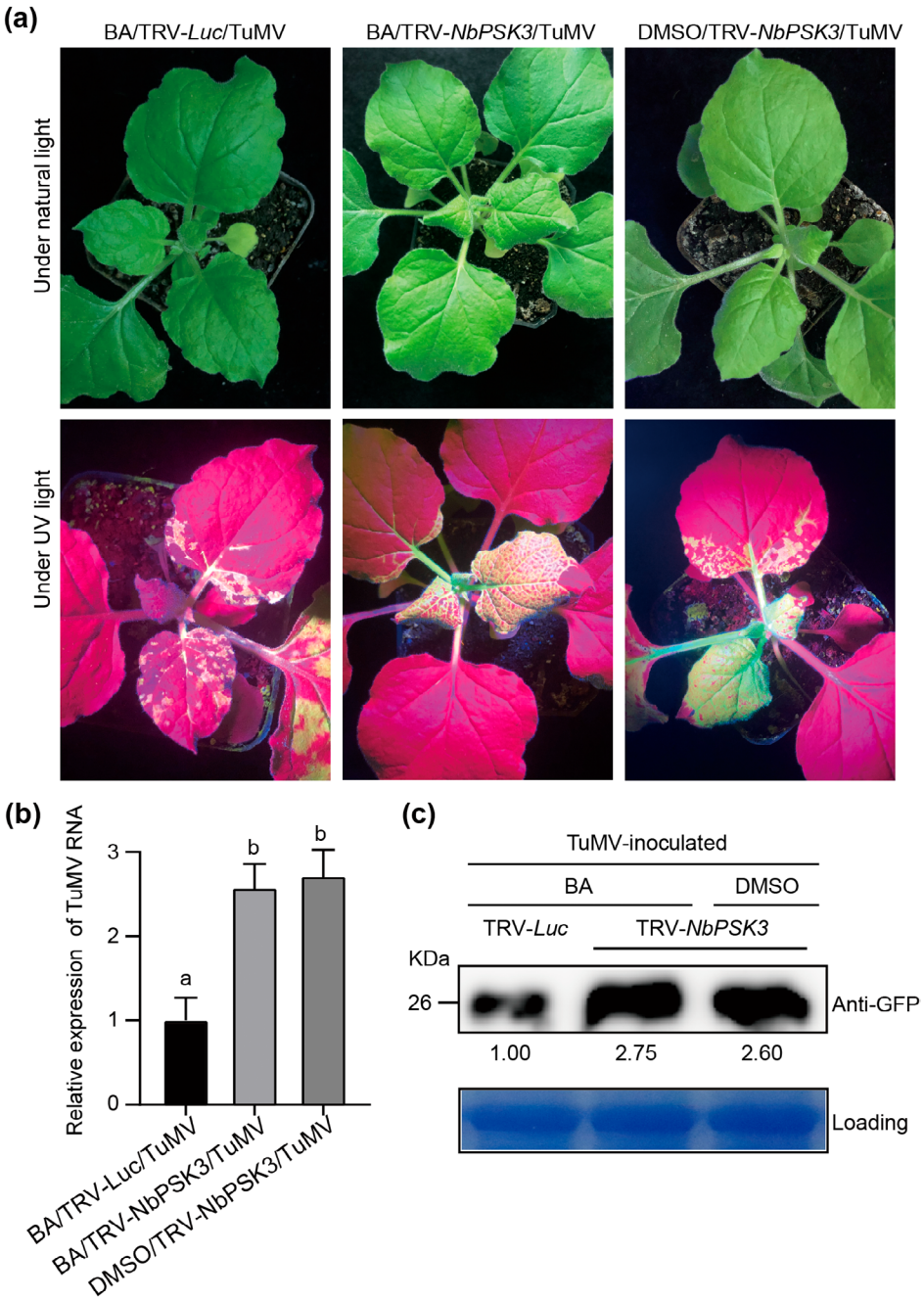

FIGURE 4 | Betulinic acid (BA) inhibition of TuMV infection is dependent on *NbPSK3*. (a) BA lost its inhibition effect on TuMV-GFP after silencing of the *NbPSK3* gene. In the systemic leaves of *NbPSK3*-silenced plants, the fluorescence intensity of GFP in both BA and dimethyl sulphoxide (DMSO) treatment was significantly stronger than that in non-silenced plants. (b) The relative expression levels of TuMV genomic RNA were significantly higher in the systemic leaves of BA/TRV-NbPSK3 and DMSO/TRV-NbPSK3 plants than that in BA/TRV-Luc inoculated plants at 5 days post-inoculation (dpi). The expression of TuMV genomic RNA was quantified by reverse transcription-quantitative PCR at 5 dpi in the systemic leaves of BA/TRV-NbPSK3, DMSO/TRV-NbPSK3 and BA/TRV-Luc plants. *NbActin* was used as an internal reference gene. Different letters above the bars indicate significant differences ($p < 0.05$). (c) The accumulation of TuMV-GFP protein were significantly higher in the systemic leaves of BA/TRV-NbPSK3 and DMSO/TRV-NbPSK3 plants than that in BA/TRV-Luc inoculated plants. Western blot detection of the protein content of TuMV-GFP in BA/TRV-Luc-, BA/TRV-NbPSK3- and DMSO/TRV-NbPSK3-inoculated plants at 5 dpi. The Coomassie brilliant blue-stained RuBisCO large subunit gel showed an equal amount of protein loaded in each well. All experiments were repeated at least three times using 10 plants per treatment with similar results.

BA, a natural compound widely found in *Betula* plants, is known for its broad range of biological and pharmacological properties (Aswathy et al. 2022). While the antitumour and antiviral activity of BA in mammals has been extensively studied, our research indicates that BA may hinder infection by TuMV, TMV and CLCuMuV in *N. benthamiana* plants. This suggests that BA holds significant potential for application in the control of plant viral diseases. Subsequent studies will focus on the ability of BA

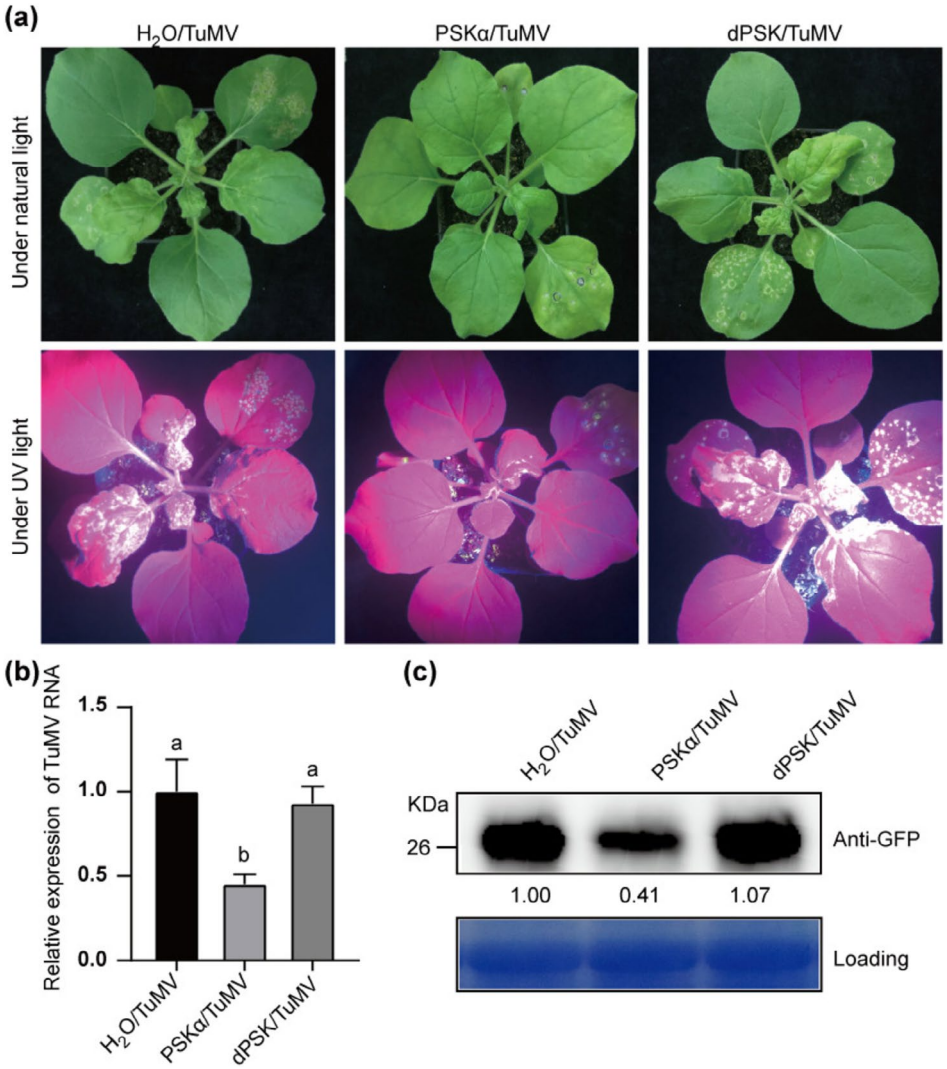

FIGURE 5 | Sulphonated phytosulfokine (PSKα) conferred immunity against TuMV in *Nicotiana benthamiana*. (a) PSKα-treated *N. benthamiana* plants displayed delays against TuMV infection. *Nicotiana benthamiana* plants leaves were sprayed with 10 μM either PSKα, unmodified PSK (dPSK) or water (control) 12 h before the TuMV inoculation, and the pictures were photographed at 5 days post-inoculation (dpi). (b) The relative expression levels of TuMV genomic RNA were significantly reduced in the systemically infected leaves of plants treated with PSKα compared to those treated with dPSK or water. The expression of TuMV genomic RNA was quantified by reverse transcription-quantitative PCR in the systemic leaves of plants treated with PSKα compared to those treated with dPSK or water at 5 dpi. *NbActin* was used as an internal reference gene. Different letters above the bars indicate significant differences ($p < 0.05$). (c) The accumulation levels of TuMV-GFP protein were significantly reduced in the systemically infected leaves of plants treated with PSKα compared to those treated with dPSK or water. The accumulation of TuMV-GFP protein was detected by western blot at 5 dpi. The Coomassie brilliant blue-stained RuBisCO large subunit gel showed an equal amount of protein loaded in each well. All experiments were repeated at least three times using 10 plants per treatment with similar results.

to control viral diseases in other important economic crops. BA exhibits a range of biological activities, has low toxicity and a high safety index, presenting excellent prospects for application development. Currently, research on BA is primarily conducted in the laboratory, and further studies are needed to investigate its stability in the field. Additionally, the poor water solubility of BA is a factor limiting its effective use. Using BA as a parent structure, structural modifications to develop compounds with better solubility and enhanced activity will be a focus of future research.

Plant polypeptides, a novel class of plant hormones, play crucial roles in plant growth, development and stress resistance (Czyzewicz et al. 2013; Segonzac and Monaghan 2019). PSK, a peptide hormone as an endogenous signalling molecule, exhibits a range of biological functions including promoting plant growth, development and defence against environmental stress (Song et al. 2017; Förderer and Chai 2023). In *Arabidopsis*, the PSK precursor gene *AtPSK* is activated in response to wounding, implying that PSK signalling is involved in the regulation of plant damage responses (Matsubayashi et al. 2006). Previous research has shown that fungal activators E-Fol, elf18 or necrotrophic phytopathogenic fungi can induce the expression of both PSK precursor and PSK receptor genes (Loivamäki et al. 2010). In our study, we observed that the expression level of the *NbPSK3* gene was significantly suppressed upon TuMV infection, indicating a different response pattern of *PSK* genes to various pathogens. Beyond that, the functions of the PSK-PSKR signalling pathway in different

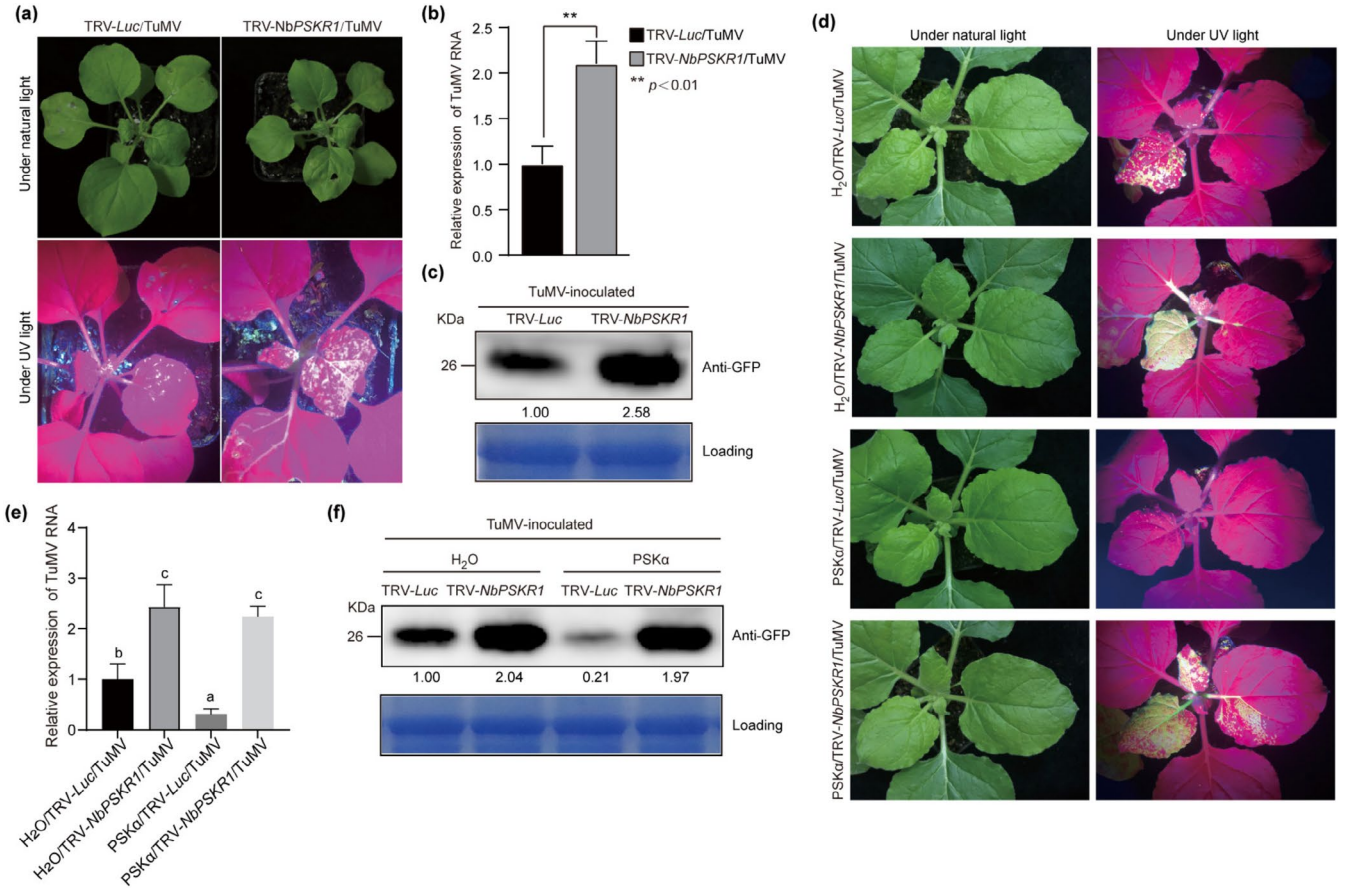

FIGURE 6 | *NbPSKR1* plays a negative role in TuMV-GFP infection. (a) Silencing *NbPSKR1* promoted TuMV-GFP infection. Fluorescence observation revealed that the fluorescence intensity of GFP in the systemic leaves of *NbPSKR1*-silenced plant was significantly higher than that in control plants at 5 days post-inoculation (dpi). (b) The relative expression levels of TuMV genomic RNA significantly increased in the systemic leaves of *NbPSKR1*-silenced plant compared to the non-silenced control plants at 5 dpi. The expression of TuMV genomic RNA was quantified by reverse transcription-quantitative PCR (RT-qPCR) at 5 dpi in *NbPSKR1*-silenced and non-silenced plants systemic leaves. $^{**}p < 0.01$ significant difference. (c) The accumulation of TuMV-GFP protein significantly increased in the systemic leaves of *NbPSKR1*-silenced plant compared to the non-silenced control plants. Western blot analysis of TuMV-GFP protein content in the *NbPSKR1*-silenced plant compared to the non-silenced control plants at 5 dpi. (d) Sulphonated phytosulfokine (PSKα) lost its inhibition effect on TuMV-GFP after silencing of the *NbPSKR1* gene. In the systemic leaves of *NbPSKR1*-silenced plants, the fluorescence intensity of GFP was no significant difference in PSKα and water treatment. (e) The relative expression levels of TuMV genomic RNA were significantly higher in the systemic leaves of water/TRV-NbPSKR1, and PSKα/TRV-NbPSKR1 plants than that in water/TRV-Luc inoculated plants at 5 dpi. The expression of TuMV genomic RNA was quantified by RT-qPCR at 5 dpi in the systemic leaves of water/TRV-Luc-, water/TRV-NbPSKR1- and PSKα/TRV-NbPSKR1-inoculated plants. *NbActin* was used as an internal reference gene. Different letters above the bars indicate significant differences ($p < 0.05$). (f) The accumulation of TuMV-GFP protein was significantly higher in the systemic leaves of water/TRV-NbPSKR1 and PSKα/TRV-NbPSKR1 plants than that in water/TRV-Luc-inoculated plants at 5 dpi. Western blot detection of the protein content of TuMV-GFP in Hwater/TRV-Luc-, water/TRV-NbPSKR1- and PSKα/TRV-NbPSKR1-inoculated plants at 5 dpi. The Coomassie brilliant blue-stained RuBisCO large subunit gel showed an equal amount of protein loaded in each well. All experiments were repeated at least three times using 10 plants per treatment with similar results.

host–pathogen models are poles apart. For instance, exogenous PSK treatment or overexpression of PSK precursor genes in *Arabidopsis* enhances sensitivity to *P. syringae* pv. *tomato* (Mosher et al. 2013). Similarly, in *Zinnia*, PSKα treatment inhibits defence-related genes, such as *PR1*, chitinase and several genes involved in salicylic acid (SA) biosynthesis, thereby attenuating plant stress responses (Motose et al. 2009). Conversely, exogenous PSK treatment improves tomato resistance to *B. cinerea*, while silencing the tomato PSK synthetic gene *PSK3/3L* significantly reduces plant resistance (Zhang et al. 2018). In our study, we found that exogenous BA treatment significantly upregulated the expression of the *NbPSK3* gene, and this effect of BA induction outweighed the inhibitory effect of TuMV. This finding is partially consistent with previous studies, indicating that the PSK signalling pathway plays an active role in delays against plant virus infections. Further research revealed that BA-mediated inhibition of TuMV propagation is dependent on *NbPSK3*. This suggests that BA-induced up-regulation of *NbPSK3* gene expression delays TuMV infection in *N. benthamiana* plants (Figure 7). Previous studies have demonstrated that PSK signalling is linked to the production of reactive oxygen species (ROS) (Kou et al. 2020) and Ca^{2+} signalling (Zhang et al. 2018), which are important molecules in plant immunity (Köster et al. 2022). However, the mechanisms underlying how *NbPSK3* enhances plant delays to virus infection require further investigation.

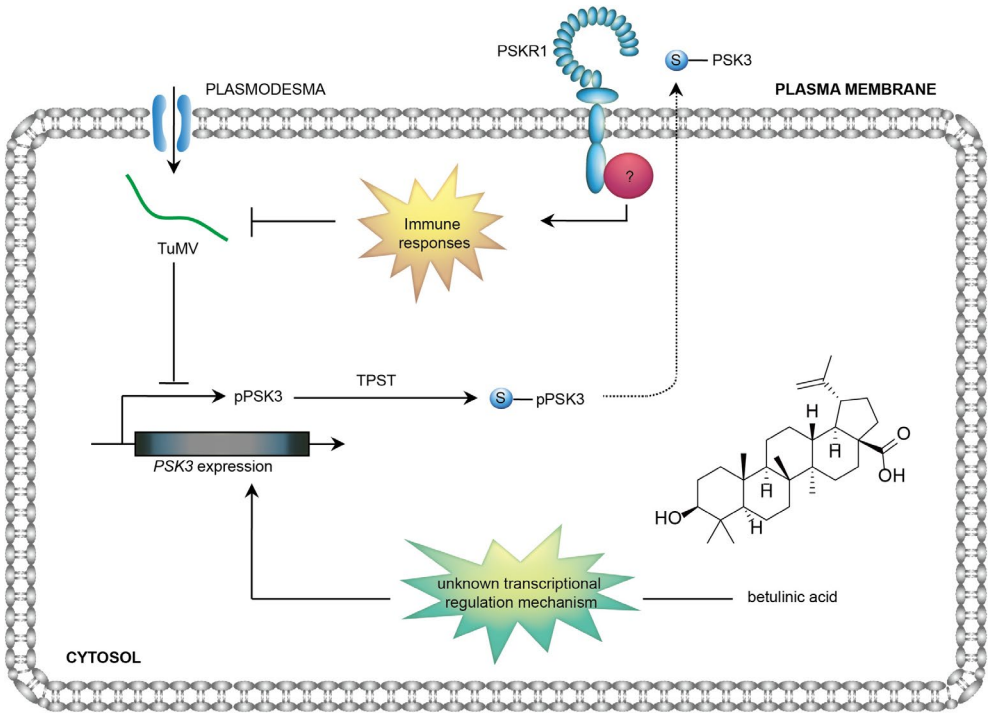

FIGURE 7 | Proposed model. In *Nicotiana benthamiana* plants, exogenous betulinic acid (BA) treatment specifically induced up-regulated expression of the *NbPSK3* gene, activated the PSK-PSKR signalling pathway and delayed TuMV infection.

As a short peptide regulator, PSK relies on its receptor PSKR to transmit signals. PSKR is conserved in plants such as *Arabidopsis* and tomato, both of which encode two PSKR orthologues, PSKR1 and PSKR2. The binding affinity and rate of PSKR1 for PSK are stronger than PSKR2, indicating that PSK perception largely depends on PSKR1 (Zhang et al. 2018). PSKR1 plays distinct roles in defence against different types of pathogens. For example, overexpression of *PSKR1* in *Arabidopsis* decreases resistance to bacterial leaf spot but enhances the plant's defence against the fungal disease black spot (Mosher et al. 2013). In rice, overexpression of *PSKR1* enhances the host ability to defend against rice white leaf blight (Yang et al. 2019). In this study, we found that silencing *NbPSKR1* was beneficial to TuMV infection, indicating that *NbPSKR1* played a positive role in defending against virus infection. Currently, understanding the molecular mechanisms through which PSKR1 regulates plant immunity has made some progress. *Arabidopsis pskr1* knockout mutants show reduced susceptibility to downy mildew infection, and this reduced susceptibility is dependent on functional SA signalling (Rodiuc et al. 2016). In tomato, PSKR1 transduces the signal into the cytoplasm by initiating cytosolic Ca^{2+} influx and activates auxin-mediated pathways that enhance tomato resistance to *B. cinerea* (Zhang et al. 2018). Additionally, the PSKR1 protein is directly targeted by the ubiquitin/proteasome degradation pathway. For instance, PUB12 and PUB13 can target PSKR1 for ubiquitination, and inhibiting PSKR1 ubiquitylation enhances plant resistance to *B. cinerea* (Hu et al. 2023). Overall, our study not only identifies BA as a novel plant-derived antiviral agent with potential for further exploration in viral disease management but also elucidates the role of the NbPSK3–NbPSKR1 signalling pathway in enhancing plant delays to viral infection. Although existing research has shown that essential cellular signalling pathways, including Ca^{2+}, ROS, and phytohormones, are involved in the PSK–PSKR-mediated delays to pathogen infection, the underlying mechanism through which the NbPSK3–NbPSKR1 pathway regulates virus infection requires further investigation.

In summary, our study suggests that BA hinders the progress of TuMV infection in *N. benthamiana* by activating the PSK–PSKR signalling pathway. These results not only suggest that BA may be a promising and environmentally friendly substance against plant viruses but also identify the value of PSK signalling in the crop improvement of TuMV resistance.

4 | Experimental Procedures

4.1 | Plant Growth and Virus Inoculation

Nicotiana benthamiana and *Arabidopsis* plants (*Arabidopsis* mutant *atpsk3* was obtained from Fuzhou AraShare Biotechnology Co., the type of mutation is the insertion of a T base) were grown in an artificial climate chamber at 25°C with a 16-h light/8-h dark cycle. Plants were inoculated with TuMV, TMV and CLCuMuV by *Agrobacterium* infiltration following the procedures described previously (Gaguancela et al. 2016). All inoculation experiments were repeated at least three times.

4.2 | BA And PSK Treatment

To investigate the impact of BA treatment on TuMV, TMV and CLCuMuV infection, BA was dissolved in DMSO to a concentration of 10 mg/mL and then diluted to 10 mg/L in sterile distilled water immediately before use. A control solution of 0.1%

DMSO was also prepared. For treatment, 5 mL of the solvent was applied to the roots of *N. benthamiana* plants once a day for 5 consecutive days, followed by inoculation with TuMV, TMV and CLCuMuV.

To investigate the effect of PSK treatment on TuMV infection, we commissioned Qiangyao Biotechnology Co. Ltd. to synthesise sulphonated and unmodified PSK peptides (PSKα and dPSK, respectively), and these peptides were dissolved in sterile water to a working concentration of 10 mM. *N. benthamiana* plants at the 4- to 6-leaf stage were sprayed with PSKα, dPSK or double-distilled water as a control. TuMV was inoculated 12 h after treatment.

4.3 | Total RNA Extraction and RT-qPCR

Total RNA was extracted from *N. benthamiana* leaf tissues using the RNAiso Plus reagent according to the manufacturer's protocol (Dalian). For reverse transcription, 1 μg of total RNA was treated with RNase-free DNase and incubated at 42°C for 2 min to completely remove genomic DNA. The RNA was then reverse transcribed to cDNA using the 5× HiScript III qRT Super Mix (Vazyme) with random primers. The cDNA concentration was normalised to 100 ng/μL using a spectrophotometer. qPCR was performed with the AceQ qPCR SYBR Green Master Mix (Vazyme) following the manufacturer's instructions. The expression level of the *NbActin* gene was used as an internal reference. Relative gene expression levels were analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All experiments were repeated three times with 10 plants per treatment. The primer sequences used in RT-qPCR are listed in Table S1.

4.4 | Protein Preparation and Western Blotting Assays

Total proteins were extracted from leaf tissue using protein extraction buffer (Biotime) and separated by electrophoresis on 10% SDS-PAGE gels. The proteins were then transferred to methanol-pre-activated PVDF membrane. The membrane was sealed with 5% skimmed milk solution, followed by incubation with anti-GFP monoclonal antibodies for 2 h at room temperature. Afterward, the residual antibodies were washed off using Tris-buffered saline with Tween 20 (TBST) solution, and the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody for 1 h. Finally, protein was detected by incubating the membrane in an ECL solution and visualising the signals using the Bio-Rad chemical imaging system. Quantitative analysis of protein expression levels in western blotting assays was analysed by ImageJ software.

4.5 | RNA Sequencing Analysis

At 5 dpi, *N. benthamiana* leaf samples from four different treatments (CK_Mock, BA_Mock, CK_TuMV and BA_TuMV) collected and the extracted total RNA was sent to tNovogene Bioinformatics Technology Company (Beijing, China) for RNA sequencing using the Illumina HiSeq 2000 platform. Sequencing reads were mapped to the *N. benthamiana* genome (*N. benthamiana* draft genome v. 2.6.1.) using Bowtie software. HTSeq v. 0.6.1 was used to count the number of reads mapped to each gene, and the relative gene expression levels were normalised as fragments per kilobase of transcript per million mapped reads (FPKM). Genes with a false discovery rate (FDR) < 0.05 and $\log_2$ fold-change $\geq |1|$ were considered significantly differentially expressed.

4.6 | VIGS Assays

To silence *NbPSK3*, *NbPSKR1* and *NbPSKR2* genes in *N. benthamiana*, specific sequences (approximately 150 bp) targeting *NbPSK3*, *NbPSKR1* and *NbPSKR2* were amplified and cloned into the TRV2 vector, generating TRV2-*NbPSK3*, TRV2-*NbPSKR1* and TRV2-*NbPSKR2* constructs, respectively. The recombinant plasmids were transformed into *Agrobacterium tumefaciens* GV3101. The recombinant strains were grown overnight at 28°C, then collected by centrifugation and resuspended in infiltration buffer (2 mM acetosyringone, 10 mM $MgCl_2$, 100 mM MES in distilled water) to an OD_{600} of 1.0 and inoculated at room temperature for 2–3 h. The agrobacterium culture containing TRV1 was mixed with an equal volume of *Agrobacterium* culture harbouring TRV2-NbPSK3 (TRV-NbPSK3), TRV2-NbPSKR1 (TRV-NbPSKR1) and TRV2-NbPSKR2 (TRV-NbPSKR2). TRV2-Luc (referred to as TRV-Luc) served as control. The mixture was then infiltrated into the leaves of 3- to 5-leaf-stage *N. benthamiana* plants using a sterile syringe. At 7 dpi, systemic leaves were harvested and the silencing efficiency of targeted genes was assessed by RT-qPCR. Plants with targeted gene overexpression were subsequently used for virus inoculation experiments.

4.7 | Virus-Based Gene Overexpression Assays

For the potato virus X (PVX)-based gene overexpression assay, the full-length sequence of *NbPSK3* (225 bp) was amplified and cloned into the PVX vector to generate PVX-NbPSK3. The PVX empty vector and PVX-NbPSK3 were each transformed into *A. tumefaciens* GV3101 (pSoup). Plants with targeted gene overexpression were subsequently used for virus inoculation experiments.

4.8 | Statistical Analysis

Differences were analysed using a Student's *t* test, and all analyses were conducted using GraphPad Prism 9 software. A *p*-value < 0.05 was considered statistically significant.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.