CRISPR/Cas9-mediated gene editing of vacuolar ATPase subunit d mediates phytohormone biosynthesis and virus resistance in rice

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Vacuolar ATPases (V-ATPases) are proton pumps for proton translocation across membranes that utilize energy derived from ATP hydrolysis. OsV-ATPase subunit d (OsV-ATPase d) is part of an integral, membrane-embedded V0 complex in the V-ATPase complex. Whether OsV-ATPase d is involved in phytohormone biosynthesis and resistance in rice remains unknown. The knockout mutants of OsV-ATPase d in rice were generated using the CRISPR/Cas9 system, and mutation of OsV-ATPase d did not show any detrimental effect on plant growth or yield productivity. Transcriptomic results showed that OsV-ATPase d significantly increased JA and ABA biosynthesis and resistance against Southern rice black-streaked dwarf virus (SRBSDV), but it decreased resistance against Rice stripe virus (RSV) in rice. The data presented in this study reveal that OsV-ATPase d mediates phytohormone biosynthesis and virus resistance in rice and can be selected as a potential target for resistance breeding in rice.

KEYWORDS
CRISPR/Cas9, vacuolar ATPase subunit d, phytohormone biosynthesis, virus resistance, rice

Introduction

Rice (Oryza sativa) is one of the most important staple foods for more than half of the world’s population. However, many viral diseases have severely challenged rice production and have increased the risk to food security in many global areas (Jones and Dangl, 2006; Mandadi and Scholthof, 2013; Nicaise, 2014; Baulcombe, 2019). For example, Southern rice black-streaked dwarf virus (SRBSDV) is severely epidemic and has caused 30-50% rice yield
losses in southern China and Southeast Asia in the last decade (Hoang et al., 2011; Cheng et al., 2013; Zhang et al., 2013; Zhou et al., 2013;). Rice stripe virus (RSV) is one of the most destructive viruses affecting rice production, and it is widespread throughout East Asia, especially in China, Japan, and Korea (Hu et al., 2020). Although SRBSDV and RSV has been successfully controlled by international cooperation, it still exists in the majority of rice-producing areas of eastern China, with periodic outbreaks in a few rice-producing areas and the potential for additional widespread outbreaks (Lv et al., 2017; Alonso et al., 2019). One of the most effective strategies to prevent viral diseases is growing resistant or tolerant varieties; nevertheless, almost all cultivated rice varieties are susceptible to SRBSDV (Wang et al., 2017; Yu et al., 2017; Zhou et al., 2021).

CRISPR-Cas9 based genome editing is increasing concern as a promising technique to induce resistance against DNA and RNA viruses in crops, and also can be explored for a variety of agronomic traits in agriculture crops (Hinge et al., 2021). And CRISPR/Cas-based genome editing is an alternative method for accelerating rice improvement (Ma et al., 2015). The availability of rice reference genome sequences and the CRISPR/Cas9-editing system has made it possible to develop disease-resistant or disease-tolerant rice by precisely editing endogenous genes (Huang et al., 2012; Yang et al., 2013; Jackson, 2016; Wing et al., 2018; Zhao et al., 2020).

Vacuolar ATPases (V-ATPases) are proton pumps for proton translocation across membranes that utilize energy derived from ATP hydrolysis (Forgac, 2007; Mazhab-Jafari and Rubinstein, 2016). In eukaryotic cells, V-ATPases are multisubunit complexes that mediate the pH of many intracellular organelles, including vacuoles and endosomes (Forgac, 2007). V-ATPase has been reported to regulate defense against viral infection in mammals, and V-ATPase activity is critical in mammal-virus interactions (Hunt et al., 2011; Jang et al., 2018). The V-ATPases of endosomes play pivotal roles in the successful entry and release of the viral genome into the cytoplasm for most human viruses, including Human coronavirus NL63 (HCoV-NL63), influenza viruses, Zika virus (ZIKV), Dengue virus (DENV), and Sindbis virus (SINV) (Hunt et al., 2011; Jang et al., 2018). Nevertheless, the subunits of V-ATPase present in the vacuoles during plant-virus interactions have never been studied. The latest study demonstrated that V-ATPase also regulates plant-virus interactions. For the first time, BSMV replicase was reported to competitively bind to subunit B2 of V-ATPase to detach subunit B2 from the tonoplast into the cytosol, leading to impairment of the connection between the V1 and V0 complexes. This severed connection disrupted the formation of V-ATPase on the tonoplast, which inhibited the activity of V-ATPase, increased the vacuolar pH in plants, and promoted infection by BSMV and multiple viruses, including Lychnis ringspot virus (LSRV), Cucumber mosaic virus (CMV), or Potato virus X (PVX) (Yang et al., 2021). However, the other subunits of V-ATPase involved in plant-virus interactions have never been studied.

The pathogens Sarocladium oryzae and Pseudomonas fuscovaginae cause rice sheath rot and produce cyclic lipopeptides to inhibit membrane-bound H⁺-ATPase pumps in the rice plant, resulting in reduced abscisic acid (ABA), jasmonate acid (JA) and auxin levels and grain yield in rice (Peeters et al., 2020). This suggests that H⁺-ATPase is probably involved in regulating the plant hormone pathway. Plant hormones are pivotal for biotic and abiotic resistance, and rice hormones have diverse functions in rice resistance against different viruses (Yan and Xie, 2015; Xie et al., 2018; Zhang et al., 2020). Therefore, OsV-ATPase d might be an alternative target for gene editing by CRISP/Cas9 to enhance viral resistance in rice.

In this study, the function of OsV-ATPase d, which is a rice gene encoding the protein V-ATPase d that functions as subunit d of the membrane-embedded V0 complex of V-ATPase in rice plants, was characterized by using knockout rice lines. The results showed that knocking out OsV-ATPase d in rice had no detrimental impact and differently mediated resistance against the plant RNA viruses RBSDV and RSV and phytohormone biosynthesis in rice. To the best of our knowledge, this is the first report of a protein with a classical function as a proton pump for proton translocation during the regulation of vacuolar acidification being involved in different methods of plant defense against plant RNA viruses and the regulation of phytohormone biosynthesis.

Materials and methods

Plant growth and virus inoculation

Rice cultivar Oryza sativa L. japonica. Nipponbare (NIP) was used in this study. The cultivar Nipponbare is highly susceptible to SRBSDV and RSV (Xu et al., 2014; Yang et al., 2018b). NIP was used to produce transgenic rice. SRBSDV-infected plants were kindly provided by Professor Guohui Zhou and Tong Zhang (South China Agricultural University Guangzhou, China). Rice seedlings were grown in a greenhouse at 26 to 28°C with a 14-h light/10-h dark cycle under artificial light. Rice plants infected with SRBSDV and RSV were cultivated in an experimental field in Changsha and Nanjing, respectively, under natural long-day conditions. Viruliferous or virus-free planthoppers were reared on healthy rice seedlings (Wuyuig No. 3) in glass beakers at 25°C.

SRBSDV was transmitted by the white backed plant hopper (Sogatella furcifera) at approximately the 1.5-leaf-stage of rice seedling. To obtain viruliferous insects, nymphs were reared on virus-infected rice plants for 2 days, and viruliferous or virus-free nymphs were transferred to each experimental rice plant to feed for 3 days, after which the planthoppers were removed. The proportions of healthy plants were calculated 30 days after inoculation. The percentage of about 30 plants infected by virus (viral incidence) of each of triplication was determined following specific quantitative RT-PCR of samples of each plant using virus-specific primers (Table S1) and western blotting using SRBSDV P8 polyclonal antibody.

RVS was transmitted experimentally to rice plants by the small brown planthopper (Laodelphax striatellus) at approximately the 1.5-leaf-stage of rice seedling. To obtain viruliferous insects, nymphs were reared on virus-infected rice plants for 2 days, and viruliferous or virus-free nymphs were transferred to each experimental rice plant to feed for 3 days, after which the planthoppers were removed. The proportions of healthy plants were calculated 30 days after inoculation. The percentage of about 30 plants infected by virus (viral incidence) of each of triplication was determined following specific quantitative RT-PCR of samples of each plant using virus-specific primers (Table S1).
Vector construction and agrobacterium-mediated transformation in rice

The binary CRISPR/Cas9 vector pYLCRISPR/Cas9-MH and corresponding gRNA vector pYLgRNA-U3 were provided by Yaguang Liu at South China Agricultural University. Specific single guide RNAs (sgRNAs) targeted to OsV-ATPase d were selected and constructed and used to transform the rice cultivar NIP by Agrobacterium-mediated transformation as previously described (Ma et al., 2015). The transgenic rice lines were selected based on hygromycin resistance. The primers used for vector construction are listed in Supplementary Table S1.

Genomic DNA extraction and mutation detection

The genomic DNA was extracted from young leaves of T0-T2 transgenic plants by CTAB reagent, which was then used to amplify specific fragments in the OsV-ATPase d gene using primers flanking two targeted sites (Table S1). PCR was conducted under the following conditions: 94°C for 5 min; 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min (35 cycles); and 72°C for 10 min as the final extension. PCR products were directly sequenced using the Sanger method by Sangon Biotech Company (Shanghai, China). The transgenic plants were also verified as Cas9-free with primers specific for Cas9 (Table S1) (Ma et al., 2015).

Measurement of agronomic traits of rice

Seeds from fully mature rice were collected and dried. Seeds from each rice plant were randomly selected. Seed weight was measured using an electronic balance. Plant height and spike length were measured with a steel ruler.

RNA library construction, sequencing, and analysis

Total RNA was isolated from leaves of 15-day-old rice with a plant RNA purification reagent kit (Invitrogen, USA). The concentration, quality, and purity of the RNA were detected with an Agilent 2100 Bioanalyzer RNA 6000 Nano kit (Agilent, USA). RNA libraries were constructed with a TruSeq™ RNA Sample Prep kit (Illumina, USA) and sequenced by OE Biotech Company (Shanghai, China) on an Illumina HiSeq 4000. The clean reads were mapped to the reference genome using HISAT2 (Kim et al., 2015), and DEGs were identified using DESeq (Fraze et al., 2014). GO enrichment and KEGG (Kanehisa et al., 2008) pathway enrichment analyses of DEGs were performed using R based on the hypergeometric distribution.

Total RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT–PCR)

Total RNA was isolated from rice leaf samples (100 mg tissue per sample) using TRIzol reagent (TIANGEN Biotech, Beijing, China). The concentration of total RNA in each sample was determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). cDNA was synthesized using 1 μg total RNA per 20 μL reaction using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Dalian, China). Quantitative RT-PCRs were performed on an ABI PRISM 7500 device using a SYBR Premix ExTaq RT–PCR Kit (Takara). Relative transcript levels were calculated by the 2−ΔΔCT method as previously described (Rao et al., 2013), and the rice ubiquitin gene (Os03g0234350) was used as an internal control. The primers for quantitative RT–PCR analysis are listed in Table S1.

Immunoblot assay of SRBSDV P8 protein

Assays of SRBSDV P8 proteins were performed as described previously (Yang et al., 2018b). Total protein was isolated from leaf tissues at 30 dpi and separated in 15% SDS–PAGE gels. The protein bands were blotted onto polyvinylidene difluoride (PVDF) membranes followed by protein detection using an SRBSDV P8 polyclonal antibody. Protein loading was estimated through Coomassie Brilliant Blue staining.

Quantification of endogenous phytohormones in tissue samples

The assay was determined following a previously published method (Novák and Floková, 2018) Rice leaf samples (50 mg each) were collected from the assayed plants at 30 dpi, ground individually in liquid nitrogen, and then homogenized in 1 ml extraction buffer containing isopropanol/H2O/hydrochloric acid (200:100:0.2). The crude leaf extracts were incubated at -20°C for 12 h and then ultrasonicated for 30 min in an ice bath, followed by the addition of 1 mL of dichloromethane and 1 μL of 300 ng/mL double internal standard samples (succinic acid - d4 and Lyso PC17:0). The organic phase was evaporated to dryness in vacuo, dissolved in 200 μl methanol/H2O (595, including 10 ng/mL 2-ct-phe), ultrasonicated for 3 min in an ice bath, and then centrifuged at 13 000 rpm for 10 min at 4°C. The supernatant of each sample was filtered with a 0.22 μm organic filter membrane and then tested by OE Biotech Company (Shanghai, China) using HPLC–MS (AB Exion coupled with AB Sciex Qtrap 6500+) with an ACQUITY UPLC HSS T3 chromatographic column (100 mm×2.1 mm, 1.8 μm).

Statistical analysis

All the experiments conducted in this study were performed in triplicate to quintuple. The results of the experiments are presented as the means of three to five independent experiments ± their standard deviations (SDs). Statistical analyses were performed using DPS 19.05 software (Tang and Zhang, 2013) and Student’s t tests (Krzywinski and Altman, 2013).

Results

CRISPR/Cas9-engineered mutations in OsV-ATPase d had no effect on rice agronomic traits

In this study, CRISPR/Cas9-based genome-editing technology was employed to edit OsV-ATPase d in Nipponbare (Oryza sativa
L. cv. japonica, NIP), which is highly susceptible to SRBSDV and rice stripe virus (RSV) (Zhang et al., 2019). Two guide RNAs were designed to target the first exon of OsV-ATPase d by CRISPR Design (http://cripsr.mit.edu) (Figure 1A). Specific single guide RNAs (sgRNAs) targeted to OsV-ATPase d were selected and constructed by universal primers (Table S1) and used to transform the rice cultivar NIP by Agrobacterium-mediated transformation. Five independent T0 lines were found to carry homozygous mutations in OsV-ATPase d. From the T1 segregation population, two CAS9-free homozygous mutants with knock-out of OsV-ATPase d (hereafter named line 2 and line 5) were identified. Conventional Sanger sequencing verified that a “G” deletion resulted in a frameshift mutant and three nucleotide site mutations in line 2, and a “C” insertion resulted in a frameshift mutant with a “G” deletion in line 5 (Figure 1B).

The growth trial of editing line 2 and line 5 grown in pots under greenhouse conditions showed normal growth with no morphological differences when compared to wild-type plants at 60 days of age (Figures 2A-C). No adverse effect was observed regarding the yield differences when compared to wild-type plants at 60 days of age in greenhouse conditions showed normal growth with no morphological differences when compared to wild-type plants at 60 days of age (Figure 1B).

The transcriptome profile of more genes was upregulated in OsV-ATPase d knockout rice

To gain insight into the functional profiles of OsV-ATPase d in rice, the transcriptomic response (dataset was permanently deposited in GenBank with accession number: PRJNA753714) of editing line 5 plants was comparatively analyzed with that of wild-type plants. The transcriptomic sequencing yielded approximately 277.25 M total clean reads, with a mapping ratio of 91.73% - 92.48% after quality control. The average number of clean reads obtained from editing line 5 and NIP samples was approximately 46.86 M and 45.56 M, respectively (Table S2). A global analysis of mapped reads in GenBank with accession number: PRJNA753714) of editing line 5 rice, the transcriptomic response (dataset was permanently deposited in GenBank with accession number: PRJNA753714) of editing line 5 plants was comparatively analyzed with that of wild-type plants. The transcriptomic sequencing yielded approximately 277.25 M total clean reads, with a mapping ratio of 91.73% - 92.48% after quality control. The average number of clean reads obtained from editing line 5 and NIP samples was approximately 46.86 M and 45.56 M, respectively (Table S2). A global analysis of mapped reads

As shown in Figure 4, the high correlation coefficient ($R^2 = 0.85$) of the expression fold changes of selected genes from qRT–PCR was compared with those obtained from RNA-Seq, which confirmed that the RNA-Seq data are reproducible.

Knockout of OsV-ATPase d increased endogenous ABA and JA biosynthesis in rice

Transcriptomic analysis showed that OsV-ATPase d is involved in plant hormone mediation; thus, the plant hormones in editing line 5 and the wild type were then quantified by ultrahigh-performance liquid chromatography-triple quadrupole mass spectrometry (UPLC–MS/MS). As expected, OsV-ATPase d was indeed involved in mediating plant hormone biosynthesis. Compared with wild-type
plants, editing line 5 showed significantly increased JA and ABA biosynthesis, but there was no effect on the biosynthesis of five other plant hormones, including 1-aminocyclopropanecarboxylic acid (ACC), indoleacetic acid (IAA), salicylic acid (SA) and trans-zeatin (tZ) (Table 2).

Genes involved in ABA and JA biosynthesis and signal transduction were upregulated in OsV-ATPase d knockout rice

To provide mechanistic insights into the effects of OsV-ATPase d on mediating ABA and JA biosynthesis in rice, the expression levels of key genes involved in the biosynthetic and signal transduction pathways of ABA and JA biosynthesis were retrieved from the transcriptomic dataset. As shown in Figure 5, the expression of the key genes OsNCED4 (LOC_Os07g0154201) and OsNCED5 (LOC_Os12g0617250) in ABA biosynthesis and of OsSWEET15 (LOC_Os02g0513100) in the ABA signal transduction pathway (Teng et al., 2014; Yang et al., 2018a; Huang et al., 2019; ) were significantly upregulated in OsV-ATPase d knockout rice. The expression of OsPAO7 (LOC_Os09g0368500), which is involved in ABA catabolism (Liu et al., 2014), was significantly downregulated in OsV-ATPase d knockout rice. Figure 6 also shows that the expression of the key gene OsAOS3 (LOC_Os02g0218700) in the JA biosynthetic pathway (Haga and Iino, 2004) was significantly upregulated in OsV-ATPase d knockout rice, and OsJAZ5 (LOC_Os07g0153000) and OsCM-LOX1 (LOC_Os12g0559934) involved in JA signal transduction were the negative regulators of JA biosynthesis (Wang et al., 2008; Singh et al., 2015; ) and were significantly downregulated. Taken together, these results suggest that OsV-ATPase d is involved in the synergistic regulation of JA/ABA biosynthesis.

### TABLE 1 Agronomic traits of the knock-out mutants of OsV-ATPase d and the wild type.

| Sample | Spike length (cm) | Number of spikelets | grain number per spike | 1000-grain weight (g) |
|--------|-------------------|---------------------|------------------------|-----------------------|
| line 2 | 20.86 ± 0.52a     | 10.60 ± 0.81a       | 116.80 ± 5.22a         | 19.66 ± 1.45a         |
| line 5 | 20.26 ± 0.56a     | 9.67 ± 0.67a        | 114.33 ± 1.45a         | 19.76 ± 1.18a         |
| NIP    | 19.37 ± 0.47a     | 9.67 ± 0.33a        | 104.00 ± 4.04a         | 20.91 ± 0.91a         |

Letters indicate significantly different values using the Student’s t test (p < 0.05).
Knockout of OsV-ATPase d mediates virus resistance in rice

Transcriptomic and plant hormone biosynthesis analysis showed that OsV-ATPase d may mediate resistance in rice. Three replicates of editing line 5 were evaluated for resistance against SRBSDV and RSV. SRBSDV disease symptom observations showed that at 30 dpi, the NIP plants showed more severe stunting upon SRBSDV infection (Figure 6A), and the SRBSDV disease incidence and accumulation of SRBSDV virions in the wild-type plants were significantly higher than those in the editing line 5 plants (Figures 6B, C). In contrast, the editing line 5 plants displayed higher susceptibility to RSV than the wild-type plants (Figures 6D-F). Further analyses showed that editing line 5 showed no significant effect on virus-transmitting vector infestation. These results indicated that OsV-ATPase d can differentially regulate root resistance to SRBSDV and RSV infection.

Discussion

In this study, we showed that knocking out a rice gene encoding V-ATPase d, subunit d of the membrane-embedded V0 complex of many intracellular organelles, including tonoplasts, vacuoles and endosomes, which is crucial for the fate of functional proteins from viruses. In plant–virus interactions, the extensive or conserved pathway of plant viruses inhibits the activity of V-ATPase, which results in an increase in the vacuolar pH and impairs the capacity of degradation proteins encoded by plant viruses in many intracellular organelles in plants. This reduced capacity promotes infection by many plant viruses, including BSMV, LRSV, CMV, and PVX (Yang et al., 2021). The V-ATPase activity is also critical in mammalian endosomes play pivotal roles in the successful entry and release of the viral genome into the cytoplasm for most human viruses, including HCoV-NL63, influenza viruses, ZIKV, DENV, and SINV (Hunt et al., 2011; Jang et al., 2018). The V-ATPases of mammalian endosomes play pivotal roles in the successful entry and release of the viral genome into the cytoplasm for most human viruses, including HCoV-NL63, influenza viruses, ZIKV, DENV, and SINV (Hunt et al., 2011; Jang et al., 2018; Kao et al., 2018; Milewska et al., 2018). For the first time, this study provided a novel and third plant–virus interaction mechanism in which subunit d of V-ATPase in rice differentially mediates plant RNA viruses, which is reminiscent of previous document that plants with Rice black-streaked dwarf virus (RBSDV) infection were more resistant to subsequent challenge by SRBSDV, but more susceptible to RSV.

TABLE 2 Quantitative determination of endogenous plant hormones by a UPLC-MS/MS system.

| Sample | ABA (ng/g) | ACC (ng/g) | IAA (ng/g) | JA (ng/g)  | SA (ng/g) | tZ (ng/g) |
|--------|------------|------------|------------|------------|-----------|-----------|
| line 5 | 18.37 ± 0.95a | 222.56 ± 9.67a | 12.39 ± 0.12a | 204.05 ± 5.74a | 3157.16 ± 121.79a | 17.47 ± 4.17a |
| NIP   | 10.71 ± 0.62b | 213.41 ± 75.42a | 9.58 ± 1.43a | 124.23 ± 14.75b | 3672.21 ± 940.16a | 17.47 ± 4.17a |

Letters indicate significantly different values using the Student’s t test (p < 0.05).
(Zhang et al., 2019). The distinct resistance against different plant RNA viruses probably through synergistic regulation of JA/ABA biosynthesis (Table 2 and Figure 6). It is well documented that both JA and ABA are crucial for resistance against plant RNA viruses (Xie et al., 2018; Hu et al., 2020; Yang et al., 2020; ). Activating JA pathway would enhance the resistance to RSV in rice (Yang et al., 2020), however ABA negatively modulates plant defense against RBSDV infection by suppressing JA biosynthesis (Xie et al., 2018). Subunit d of V-ATPase in rice is also involved in mediating ABA, JA and auxin biosynthesis (Forgac, 2007). Previous studies have demonstrated that auxin-enhanced H⁺-pumping lowers the cell wall pH, activates pH-sensitive enzymes and proteins within the wall, and initiates cell-wall loosening and extension growth (Hager, 2003). Auxin can also decrease apoplastic pH regulated by V-ATPase to increase the expansion of conical cells in the flowers of angiosperm species (Dang et al., 2020). However, the interaction between ABA/JA and V-ATPase has received little attention and remains ambiguous. Therefore, uncovering the detailed molecular mechanisms of subunit d of V-ATPase in the rice-RBSDV/RSV interaction with synergistic regulation of JA/ABA biosynthesis would provide novel insight into the function of V-ATPase in plant-RNA virus interactions.
Conclusions

Altogether, the OsV-ATPase d knockout mutant of rice showed different levels of resistance to important viruses, SRBSDV and RSV, and did not show any detrimental effects on plant growth or yield productivity. This study indicates that OsV-ATPase d can be selected as a potential target for resistance breeding in rice. This study also paves the way for uncovering the novel molecular mechanisms of V-ATPase functioned in the rice-SRBSDV/RSV interaction and modulating plant hormones, which likely to dig out potential genes for viruses resistant breeding by CRISP/Cas9 system.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

QL, XL, XY, methodology and investigation. TZ, YXL, YZ, and YLan, methodology and formal analysis. DZ and LZ, conceptualization and funding acquisition. LL, SZ, and YLiu, project administration, visualization, funding acquisition, writing – review and editing. All authors contributed to the article and approved the submitted version.

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Funding

This work was supported by the National Natural Science Foundation of China (31501609), and the National Key Research and Development Project (No. 2016YFD0200809).

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1122978/full#supplementary-material
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