Two VQ Proteins are Substrates of the OsMPKK6-OsMPK4 Cascade in Rice Defense Against Bacterial Blight

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Abstract

Background: The plant-specific valine-glutamine (VQ) protein family with the conserved motif FxxxVQxLTG reportedly functions with the mitogen-activated protein kinase (MAPK) in plant immunity. However, the roles of VQ proteins in MAPK-mediated resistance to disease in rice remain largely unknown.

Results: In this study, two rice VQ proteins OsVQ14 and OsVQ32 were newly identified to function as the signaling components of a MAPK cascade, OsMPKK6-OsMPK4, to regulate rice resistance to Xanthomonas oryzae pv. oryzae (Xoo). Both OsVQ14 and OsVQ32 positively regulated rice resistance to Xoo. In vitro and in vivo studies revealed that OsVQ14 and OsVQ32 physically interacted with and were phosphorylated by OsMPK4. OsMPK4 was highly phosphorylated in transgenic plants overexpressing OsMPKK6, which showed enhanced resistance to Xoo. Meanwhile, phosphorylated OsVQ14 and OsVQ32 were also markedly accumulated in OsMPKK6-overexpressing transgenic plants.

Conclusions: We discovered that OsVQ14 and OsVQ32 functioned as substrates of the OsMPKK6-OsMPK4 cascade to enhance rice resistance to Xoo, thereby defining a more complete signal transduction pathway for induced defenses.

Keywords: VQ protein, MAPK, Bacterial blight, Phosphorylation, Oryza sativa

Background

Rice bacterial blight, caused by Xanthomonas oryzae pv. oryzae (Xoo), is one of the most serious diseases affecting rice (Oryza sativa) worldwide, resulting in significant damages in rice quality and yield (Nino-Liu et al. 2006; Jiang et al. 2020). Development of host plant immunity has been considered as one of the best choices available for achieving economical and sustainable management of bacterial blight. Rice resistance to Xoo is mediated by major disease resistance (MR) genes and quantitative trait loci (QTLs) (Kou and Wang 2010; Zhang and Wang 2013). Molecular characterization of these Xoo-resistance genes and related pathways are therefore essential for development of broad-spectrum, durable resistance.

The mitogen-activated protein kinase (MAPK) cascade is composed of a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK, and regulates plant defense response through sequential phosphorylation (Zhang et al. 2018). MAPKKK phosphorylates MAPKK, thereby leading to MAPK phosphorylation within the conserved Thr-X-Tyr activation motif. Several MAPK cascades have been identified in rice response to pathogen infection. OsMPKKKe-OsMPKK4/5-OsMPK3/6 cascade regulates chitin signaling in rice resistance to the blast fungus Magnaporthe oryzae (Wang et al. 2017). OsMPKKK11 and OsMPKKK18 also activate
OsMPK4-OsMPK3/6 cascade in chitin signal transduction (Yamada et al. 2017). OsMPK10.2-OsMPK6 cascade promotes rice resistance to *M. oryzae* and *X. oryzae* pv. *oryzicola* via the activation of salicylic acid (SA) transduction (Ueno et al. 2015; Ma et al. 2017, 2021). Additionally, several MAPKK and MAPK genes have been identified in regulating resistance to diseases in rice. *OsEDR1* (OsMPKK1) negatively regulates resistance to *Xoo* but elevates resistance to *M. oryzae* via the activation of ethylene synthesis (Shen et al. 2011). *OsMPK4* (the ortholog of *Arabidopsis* MPK4) and *OsMPK17–1* (the ortholog of *Arabidopsis* MPK17) contribute to resistance to *Xoo* infection (Shen et al. 2010; Seo et al. 2011). *OsMPK15* negatively regulates resistance to *M. oryzae* and *Xoo* (Hong et al. 2019). Furthermore, all the MAPKK, MAPK and MAP genes involved in rice–*Xoo* interactions have been extensively analyzed (Yang et al. 2015).

Valine-glutamine (VQ) motif containing proteins, a class of plant-specific protein with the conserved FxxxVQxLTG amino acid sequence (where “x” represents any amino acid) and thus being termed the VQ-protein family, play key roles in the defense signal transduction process in plants (Yuan et al. 2021). AtSIB1 (AtVQ23) and AtSIB2 (AtVQ16) positively regulate defenses against necrotrophic pathogens via interaction with AtWRKY33 in *Arabidopsis* (Lai et al. 2011). AtVQ10 positively regulates *Arabidopsis* resistance to *Botrytis cinerea* via interaction with AtWRKY8 (Chen et al. 2018). JAV1 (AtVQ22), which forms a complex with JAZ8-WRKY51 to repress jasmonic acid (JA) biosynthesis, is rapidly phosphorylated in a Ca<sup>2+</sup>/calmodulin-dependent manner after injury caused by insect herbivory, and in turn dissolves the interaction with JAZ8-WRKY51 to activate JA biosynthesis for plant defenses (Hu et al. 2013; Yan et al. 2018). OsVQ13 promotes rice resistance to *Xoo* by activating the OsMPK6-OsWRKY45 signaling pathway (Uji et al. 2019).

Research in *Arabidopsis* has revealed that VQ proteins are phosphorylated by the MAPKs to regulate plant defense responses. AtMKSI (AtVQ21) is phosphorylated by AtMPK4 that is activated by pathogen infection, and subsequently releases from the AtMPK4-AtMKSI-AtWRKY33 complex to induce the expression of *PHYTOALEXIN DEFICIENT 3*, thereby resulting in the elevated resistance to disease (Andreasen et al. 2005; Qiu et al. 2008; Petersen et al. 2010). At least 10 VQ proteins (AtVQ4, AtVQ6, AtVQ9, AtVQ11, AtVQ13, AtVQ14, AtVQ19, AtVQ31, AtVQ32, AtVQ33) have been observed to be phosphorylated by AtMPK3/AtMPK6 (Pecher et al. 2014); however, the biological function of the phosphorylation remained largely unknown. The rice genome contains 40 VQ genes (Li et al. 2014a). So far, only OsVQ13 has been observed to mediate the biological process of rice defense responses (Uji et al. 2019). The MAPK-dependent regulatory mechanisms underlying the function of VQ proteins require further investigation.

In the present study, we characterized the functions of two VQ proteins, OsVQ14 and OsVQ32 (the homologs of AtVQ21) in rice defense responses. Our investigation confirmed positive regulation of both OsVQ14 and OsVQ32 in rice resistance to *Xoo*. In vitro and in vivo tests further revealed that OsVQ14 and OsVQ32 functioned as phosphorylation substrates of OsMPK4 to physically interact with OsMPK4. In vivo phosphorylation assays revealed that OsMPK4 was highly phosphorylated in the OsMPK6-overexpressing transgenic lines. Furthermore, the phosphorylated OsVQ14 and OsVQ32 were accumulated in OsMPK6-overexpressing transgenic plants, resulting in the elevated resistance to *Xoo*. The results demonstrated that OsVQ14 and OsVQ32 functioned as the substrates of the OsMPK6-OsMPK4 cascade to enhance rice resistance to *Xoo*.

**Results**

OsVQ14 and OsVQ32 Positively Regulate Rice Resistance to *Xoo*

Our previous research revealed that *Xoo* infection strongly induced the expression of OsVQ14 and OsVQ32 in rice (Li et al. 2014a). Two genes OsVQ14 and OsVQ32 herein were separately overexpressed in the rice variety Zhonghua 11 (wild type, WT) to further investigate their biological functions. Positive transgenic plants with high OsVQ14 or OsVQ32 expression showed significantly enhanced resistance (*P* < 0.01) to *Xoo* strain PXO347, with the lesion areas ranging from 17.4% to 31.5% in OsVQ14-oe plants and 4.8% to 24.6% in OsVQ32-oe plants, compared to 35.5% in the WT (Fig. S1). T<sub>1</sub> progenies derived from two OsVQ14-oe lines (19 and 36) and two OsVQ32-oe lines (24 and 30) were further examined with *Xoo*. Compared to the WT, the OsVQ14-oe plants and OsVQ32-oe plants exhibited the improved resistance to *Xoo* (Fig. 1a and b). The lesion areas were significantly correlated with the expression levels of OsVQ14 or OsVQ32. The correlation coefficients were −0.776 and −0.547 (*n* = 15, *P* < 0.01) in OsVQ14-oe19 and OsVQ14-oe36, and −0.828 and −0.553 (*n* = 15, *P* < 0.01 and *n* = 13, *P* < 0.05) in OsVQ32-oe24 and OsVQ32-oe30, respectively (Fig. 1a and b). The data suggested that the increased resistance was significantly correlated with elevated expression levels of OsVQ14 and OsVQ32. Furthermore, compared to WT at 6 to 15 days after inoculation, the reduction of growth rates of *Xoo* in rice leaves were 7.6- to 16.7-fold in OsVQ14-oe plants and 3.8- to 8.2-fold in OsVQ32-oe plants (Fig. 1c).
Overexpressing OsVQ14 and OsVQ32 enhanced rice resistance to Xoo. The asterisks "**" or "*" indicate a significant difference between transgenic plants and wild type (WT; Zhonghua 11) plants at $P < 0.01$ or $P < 0.05$, respectively. N, negative siblings segregated from the T1 families.

The enhanced resistance of the transgenic plants to Xoo is associated with OsVQ14 expression (a) and OsVQ32 expression (b) in two T1 families. Bars represent mean (3 to 5 leaves from one plant for lesion area, and 3 replicates for expression level) ± standard deviation (SD).

Analysis of the Xoo growth in leaves of OsVQ14-oE and OsVQ32-oE plants. Bars represent mean (3 leaves from 3 positive plants) ± SD. The significant difference was detected between transgenic plants and WT with the same treatment. cfu, colony-forming unit.

Analysis of the response of OsVQ14-oE and OsVQ32-oE plants to different Xoo strains. Bars represent mean (3 plants, with each plant having 3 to 5 leaves for lesion area) ± SD.
OsVQ14-oe and OsVQ32-oe plants were further inoculated with five other Xoo strains (PXO61, PXO71, PXO99, PXO341, and Zhe173). As shown in Fig. 1d, the lesion areas of OsVQ14-oe and OsVQ32-oe plants were significantly reduced compared to the WT ($P < 0.01$), indicating that the OsVQ14-oe and OsVQ32-oe plants were significantly resistant to all five Xoo strains.

We further generated knock-out (KO) mutations of OsVQ14 and OsVQ32 in the WT using CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9). Two target sites were selected for each VQ gene (Fig. S2a and c). The T$_1$ lines of two homozygous KO mutants for each VQ gene were inoculated with Xoo (Fig. S2b and d). As shown in Fig. 2a, the lesion areas in two homozygous OsVQ14-KO lines (58 and 118) were similar to those in the WT. However, the lesion areas in two homozygous OsVQ32-KO lines (88 and 91) were significantly greater than those in the WT ($P < 0.01$) (Fig. 2a). In addition, the Xoo growth rates in homozygous OsVQ32-KO plants were 1.6- to 5.0-fold higher than those in the WT at 9 to 15 days after inoculation (Fig. 2b). Next, we generated the OsVQ14-KO/OsVQ32-KO double mutant by crossing the OsVQ14-KO plants with the OsVQ32-KO plants. The OsVQ14-KO/OsVQ32-KO double mutant showed increased susceptibility to Xoo, resulting in significantly increased lesion area and Xoo growth compared with WT ($P < 0.05$) (Fig. 2c and d).

To confirm whether CRISPR/Cas9 caused off-target mutations in OsVQ32-KO plants, the genome-wide potential off-target sites were analyzed using the CRISPR-P website (http://cbi.hzau.edu.cn/crisprp) (Liu et al. 2017) (Table S1). The sequencing results verified that none of the potential off-target sites contained any DNA

![Fig. 2](image_url)  
Knocking out OsVQ32 reduced rice resistance to Xoo. The asterisks ‘**’ or ‘*’ indicate a significant difference between transgenic plants and wild type (WT; Zhonghua 11) at $P < 0.01$ or $P < 0.05$, respectively. 

a Analysis of the response of OsVQ14-KO and OsVQ32-KO plants to Xoo inoculation. Bars represent mean (3 to 5 plants, with each plant having 3 leaves for lesion area) ± standard deviation (SD). 
b Analysis of the Xoo growth in leaves of OsVQ32-KO plants. Bars represent mean (3 leaves from 3 positive plants) ± SD. 
c Analysis of the response of OsVQ14-KO/OsVQ32-KO double mutants to Xoo inoculation. Bars represent mean (3 leaves from one plant for lesion area) ± standard deviation (SD). 
d Analysis of the Xoo growth in leaves of OsVQ14-KO/OsVQ32-KO double mutants. Bars represent mean (3 leaves from 3 positive plants) ± SD. The significant difference was detected between transgenic plants and WT with the same treatment. cfu, colony-forming unit.
mutations (Fig. S3), indicating that the OsVQ32-KO phenotypes were unlikely contributed by off-target mutations in this study. Taken together, these results revealed that both OsVQ14 and OsVQ32 positively regulate rice resistance to Xoo infection.

**OsVQ14 and OsVQ32 Interact with OsMPK4**

Previous studies demonstrated that AtVQ21 (AtMKS1) is the substrate of AtMPK4 in *Arabidopsis* (Andreasson et al. 2005; Qiu et al. 2008). Because OsVQ14 and OsVQ32 are homologs of AtVQ21 (Figs. S4 and S5) and OsMPK4 (the ortholog of *Arabidopsis* MPK4) is instrumental in rice resistance to *Xoo* infection (Shen et al. 2010), we performed yeast two-hybrid (Y2H) analysis to detect whether OsVQ14 and OsVQ32 interact with OsMPK4. The results showed that both OsVQ14 and OsVQ32 strongly interacted with OsMPK4 in yeast cells (Fig. 3a-c). To further specify which domains of OsVQ14 and OsVQ32 were involved in the interactions, we performed Y2H analysis using two N-terminal deletions, two C-terminal deletions, and a VQ domain (Fig. 3a and b). The data showed that the C-terminal deletions of OsVQ14 and OsVQ32, designated as OsVQ14(32)-dC1 and OsVQ14(32)-dC2 (both containing domain I that containing the putative MAPK docking domain), interacted with OsMPK4 (Fig. 3c). However, no interaction was observed between the N-terminal deletions (designated as OsVQ14(32)-dN1, OsVQ14-dN2, and OsVQ14(32)-dNC) and OsMPK4, and only a weak interaction was detected between OsVQ32-dN2 and OsMPK4 (Fig. 3c).

The interactions of OsVQ14 and OsVQ32 with OsMPK4 were further confirmed in rice plants. OsMPK4 was immunoprecipitated from the protein complex extracted from rice leaves, and OsVQ14 or OsVQ32 was detected in immunoprecipitated protein extracts obtained from OsVQ14-oe or OsVQ32-oe transgenic plants instead of OsVQ14-KO or OsVQ32-KO plants (Fig. 3d). These results suggested that OsMPK4 physically interacts with OsVQ14 and OsVQ32 in vivo.

**OsMPK4 Phosphorylates OsVQ14 and OsVQ32**

MAPK primarily functions to recognize and phosphorylate target substrates on serine (S) or threonine (T) residues, followed by proline (Tanoue and Nishida 2003). Amino acid sequence analysis showed that OsVQ14 and OsVQ32 carried six potential MAPK phosphorylation sites (S37, T61, S137, S147, S153, and S164) and eight sites (S7, S55, S117, S141, S157, S163, S181, and S183) (Fig. 4a and b), respectively. The potential MAPK phosphorylation residues (S or T) were then cumulatively substituted with alanine (A) (Fig. 4a and b) for in vitro phosphorylation assays with the proteins purified from the bacterium. The results showed that trigger factor (TF) and His-tagged OsVQ14 and OsVQ32 (TF-His-OsVQ14 and TF-His-OsVQ32) were strongly phosphorylated by His-tagged OsMPK4 (His-OsMPK4) (Fig. 4c and d). In OsVQ14, simultaneous substitutions of S37 and S164 (OsVQ14SA) did not affect the phosphorylation process, whereas further substitution of T61 (OsVQ14A) was abolished almost all phosphorylation. As S137 failed to be replaced by A, the other five residues were substituted to generate OsVQ14SA, in which all phosphorylation had already been abolished (Fig. 4c). In OsVQ32, simultaneous substitutions of S7, S55 and S117 (OsVQ32A) did not affect the phosphorylation process, whereas further substitution of S141 (OsVQ32SA) abolished most phosphorylation, and the substitutions of all eight residues (OsVQ32AA) abolished phosphorylation entirely (Fig. 4d). These results suggest that OsMPK4 can phosphorylate OsVQ14 and OsVQ32, and that the T61 in OsVQ14 and S141 in OsVQ32 are essential for successful phosphorylation.

The constitutively active (CA) version of OsMPK4 (where aspartic acid at 198 and glutamic acid at 202 were replaced by glycine and alanine, respectively) (Berriri et al. 2012) was generated to determine OsMPK4-mediated OsVQ14 and OsVQ32 phosphorylation in vivo. In vitro phosphorylation assay showed that the His-OsMPK4CA exhibited obviously increased kinase activity toward His-TF-OsVQ14 than His-OsMPK4WT (Fig. 4e). OsMPK4CA was then co-expressed with OsVQ14WT or OsVQ14T61A (where T61 was replaced by A) in tobacco cells. OsVQ14WT or OsVQ14T61A was immunoprecipitated and analyzed by immunoblot with anti-phospho-serine (anti-pS) antibody, while OsVQ32WT or OsVQ32S141A (where S141 was replaced by A) was co-expressed with OsMPK4CA, OsVQ32WT was strongly phosphorylated as detected by anti-phospho-threonine (anti-pT) antibody. The results showed that OsVQ14WT but OsVQ14T61A was strongly phosphorylated by OsMPK4CA (Fig. 4f). Similarly, when OsVQ32WT or OsVQ32S141A (where S141 was replaced by A) was co-expressed with OsMPK4CA, OsVQ32WT was strongly phosphorylated as detected by anti-phospho-serine (anti-pS) antibody, while OsVQ32S141A was only weakly phosphorylated (Fig. 4f). The results suggest that OsMPK4 in vivo phosphorylates OsVQ14 and OsVQ32 mainly on T61 and S141, respectively.
non-substituted OsVQ14-oe and OsVQ32-oe plants (Fig. 5a and b). The protein levels of OsVQ14 and OsVQ32 in those overexpression transgenic lines were also confirmed by immunoblot assays (Fig. 5c and d). These results verified that OsMPK4-mediated OsVQ14 and OsVQ32 phosphorylation is required for rice defense to Xoo.

OsMPKK6 Functions Upstream of the OsMPK4-OsVQ14/OsVQ32 Cascade in Rice Defense to Xoo

To identify OsMPK4 interacting proteins, we immunoprecipitated OsMPK4 from OsMPK4-oe transgenic plants with anti-OsMPK4 antibody, and then performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Five peptides were identified for OsMPKK6 (Fig. S6). It is therefore speculated that OsMPKK6 phosphorylates and activates OsMPK4 in the rice–Xoo interaction. Y2H analysis revealed that OsMPKK6 interacted with OsMPK4 in yeast cells (Fig. 6a). Further, OsMPKK6 was detected in immunoprecipitated proteins obtained with anti-OsMPK4 antibody from OsMPK4-oe but not OsMPK4-RNAi plants (Fig. 6b). In vitro phosphorylation assay showed that His-OsMPK6 strongly phosphorylated His-OsMPK4K72R or MBP-OsMPK4K72R (the kinase-inactive version of OsMPK4, which was generated by substituting a conserved lysine (K) (K72) residue in the ATP-binding domain for arginine (R)) in a dose-dependent manner (Fig. 6c). These results suggested
that OsMPKK6 physically interacts with and phosphorylates OsMPK4.

To determine whether OsMPKK6 contributes to rice resistance to Xoo, we overexpressed and suppressed OsMPKK6 in WT. The OsMPKK6-oe transgenic plants with high accumulation of OsMPKK6 showed increased resistance to Xoo compared to that of WT, as indicated by the reduced lesion area and Xoo growth (Fig. 6d and e); while the OsMPKK6-suppressed transgenic plants (OsMPKK6-RNAi) with reduced accumulation of OsMPKK6 showed disease levels similar to that of WT (Fig. 6d), suggesting that OsMPKK6 functions redundantly with other MAPKKs to promote rice resistance to Xoo.

To determine whether OsMPKK6 activates OsMPK4, we immunoprecipitated OsMPK4 and assessed its phosphorylation status using the anti-pTEpY antibody, which is widely used to detect MAPKs that have been phosphorylated and activated by MAPKKs (Ma et al. 2017; Willmann et al. 2014). OsMPK4 phosphorylation was increased in WT and OsMPKK6-oe plants after the inoculation with Xoo compared with that in non-inoculated plants (Fig. 6f). However, compared to the WT, OsMPK4 phosphorylation was obviously enhanced in OsMPKK6-oe plants before and after Xoo infection (Fig. 6f), suggesting that OsMPKK6 promotes OsMPK4 activation in vivo.
We then analyzed OsVQ14 and OsVQ32 phosphorylation in OsMPKK6 transgenic plants. Immunoblot with anti-pT and anti-pS antibody showed that immunoprecipitated OsVQ14 and OsVQ32 were strongly phosphorylated in OsMPKK6-oe transgenic plants compared with that in the WT, both before and after inoculation of Xoo. However, OsVQ14 and OsVQ32 phosphorylation in OsMPKK6-RNAi plants was similar to that of WT (Fig. 6f). The results suggested that OsMPKK6 promotes OsVQ14 and OsVQ32 phosphorylation in vivo.

Our previous study revealed that overexpression of OsMPK4 increased the expression of SA-signaling genes (Shen et al. 2010). We wanted to determine whether OsMPKK6- and OsVQ14/32-mediated rice immunity...
were also involved in SA signaling pathway. As shown in Fig. 6g, compared to the WT, the transcripts of SA-related genes (ICS1, PAL1, and PR10/PBZ1) were highly increased in OsMPKK6- and OsVQ14/32-oe plants, normal in OsMPKK6-RNAi and OsVQ14-KO plants, but reduced in OsVQ32-KO plants before and after Xoo infection. In addition, the transcripts of these genes were slightly higher in OsVQ14T61A-oe and OsVQ32S141A-oe plants.

Fig. 6 OsMPKK6 promoted OsMPK4-OsVQ14/OsVQ32 cascade phosphorylation in the rice response to Xoo. The asterisks ** indicate a significant difference between transgenic plants and WT plants at P < 0.01. a Interaction of OsMPK4 with OsMPKK6 in yeast. The interactions were assessed by growing yeast cells on synthetic defined premixes (SD) medium lacking (−) leucine (L), tryptophan (W), histidine (H), and adenine (A). AD, activation domain; BD, DNA-binding domain. Co-transformation of BD-S3 and AD-RecT was used as the positive control while co-transformation of BD-Lam and AD-RecT was used as the negative control. b Interaction of OsMPK4 with OsMPKK6 in rice plants was analyzed by co-immunoprecipitation assay. Total protein was extracted from rice leaves and anti-OsMPK4 antibody was used for the immunoprecipitation (IP). c Phosphorylation assays of OsMPK4K72R by OsMPKK6 in vitro. Auto., Autoradiograph; CBB, Coomassie brilliant blue staining. d Analysis of the response of OsMPKK6 transgenic plants to Xoo infection. Bars represent mean (5 plants, with each plant having 3 to 5 leaves for lesion area) ± SD. e Analysis of the Xoo growth in rice leaves. Bars represent mean (3 leaves from 3 positive plants) ± SD. The significant difference was detected between transgenic plants and WT with the same treatment. cfu, colony-forming unit. f Analyses of the phosphorylation of OsMPK4, OsVQ14 and OsVQ32 in rice plants before and after Xoo infection. 0 h, immediately before Xoo inoculation. IP, immunoprecipitated. g Analyses of the expression of salicylic acid (SA)-signaling genes before (0 h) and after (4 h) Xoo infection. Bars represent the mean (three replicates) ± SD.
plants compared with that in WT before and after Xoo infection (Fig. 6g). Together, the results suggest that OsMPKK6-OsMPK4-OsVQ14/32 form a cascade in SA-involved rice resistance to Xoo.

Discussion
In the present study, we found that OsMPKK6 (the homolog of AtMPKK1, AtMPKK2, and AtMPKK6) interacted with and activated OsMPK4 (the ortholog of AtMPK4) in rice resistance to Xoo (Fig. 6a-c, f), resembling the phenomenon observed in Arabidopsis whereby AtMPKK1/AtMPKK2 or AtMPKK6 activates AtMPK4 in plant defense signaling (Lian et al. 2018; Qiu et al. 2008). These results implied that the immunity-related MAPK signaling pathway is highly conserved between rice and Arabidopsis, providing additional evidence for the conservation of MAPK cascades among eukaryotes (Zhang et al. 2018). The VQ motif-containing proteins OsVQ14 and OsVQ32, the homologs of AtVQ21 (Figs. S4 and S5), functioned as the substrates of the OsMPKK6-OsMPK4 cascade (Figs. 4, 5 and 6) to promote rice resistance to Xoo (Figs. 1 and S1). These results partly corroborated the findings of previous studies concerning Arabidopsis, that AtMPK4, activated by AtMPKK1/AtMPKK2, phosphorylated AtVQ21 to promote defense response (Andreasson et al. 2005; Qiu et al. 2008). Therefore, our results highlighted a conserved defense-mediated MAPK-VQ cascade between rice and Arabidopsis.

As the MAPK-VQ cascade was revealed by the present study to be instrumental in defense signaling transduction, the mechanism by which VQ proteins transmit defense signals emerges as the next topic of focus. Previous results indicated that 29 of the 34 VQ proteins identified in Arabidopsis exhibited transcriptional activity in plant cells (Li et al. 2014b), implying that VQ proteins function as transcriptional regulators to transmit the defense signals. However, WRKY transcription factors were more frequently identified as substrates of plant MAPK cascades (Ishihama and Yoshioka 2012; Bigeard and Hirt 2018); for example, WRKY46 functioned as a substrate of the MPK3 to enhance basal plant defense in Arabidopsis (Sheikh et al. 2016), OsWRKY45 was identified as the downstream target of OsMPK6 for the positive regulation of rice defense response against M. oryzae (Ueno et al. 2015). These results raised some key questions: if both VQ and WRKY are downstream transcription regulators of MAPK cascades, which perform transcriptional reprogramming following signal perception of environmental stresses, and via which mechanisms? Although limited evidence has shown that MPK3/6-targeted VQ proteins interacted with WRKY proteins, thereby affecting the transcriptional activities of the latter to modulate defense gene transcription (Pecher et al. 2014), further research is required to fully understand the underlying mechanisms.

MAPKs phosphorylate their substrates to post-translationally regulate the functions of proteins, thereby contributing to the signaling of multiple environmental stresses and developmental processes (Bigeard and Hirt 2018). Thus, the identification of MAPK substrates will assist significantly in achieving a better understanding of the underlying signaling mechanisms. In the present study, we discovered that two VQ proteins OsVQ14 and OsVQ32 were substrates of OsMPK4 (Figs. 3 and 4), which was highly phosphorylated by OsMPKK6 in vitro and in vivo (Fig. 6c and f). Overexpression of OsVQ14 or OsVQ32 enhanced rice resistance to Xoo (Fig. 1). Moreover, overexpression of OsMPKK6 enhanced rice resistance to Xoo and simultaneously increased the phosphorylation of OsVQ14 and OsVQ32 (Fig. 6d-f). The results provide not only novel molecular insight into the overall regulatory map of defense signal transduction, but also evidence for breeding new disease-resistant rice varieties via manipulation of these defense-related genes.

Conclusion
We identified a signaling cascade, OsMPKK6-OsMPK4-OsVQ14/32, that positively regulated rice resistance to Xoo. Upon Xoo infection, OsMPK4 was phosphorylated and activated by OsMPKK6 to phosphorylate OsVQ14 and OsVQ32 mainly at T61 and S141, respectively, thereby increasing SA-involved rice resistance to Xoo.

Materials and Methods
Plant Materials
All the transgenic plants in this study are in the genetic background of Zhonghua 11, which belongs to the japonica/Geng (Oryza sativa ssp. japonica/geng) subgroup of Asian cultivated rice. The OsMPK4-RNAi and OsMPK4-oe plants (OsMPK4 was named OsMPK6 in the original article) have been previously described (Yuan et al. 2007; Shen et al. 2010).

Rice Transformation
The full-length cDNAs of OsVQ14, OsVQ32, and OsMPKK6 were amplified from Zhonghua 11 using the primers listed in Table S2, and inserted into the transformation vector pU1301 (Cao et al. 2007) to construct the overexpressing vector. The cDNA fragment of OsMPKK6 was amplified using the primers listed in Table S2 and inserted into pDS1301 vector (Yuan et al. 2007) to construct the RNA interference (RNAi) vector of OsMPKK6. Two CRISPR gene-targeting units for each VQ gene were designed to construct CRISPR gene-editing vectors using the website CRISPR-P (http://cbi.hzau.edu.cn/crispr/) (Liu et al. 2017), then amplified
using gene-specific primers (Table S2) and inserted into vector pCXUN-Cas9 (He et al. 2017). All vectors were introduced into the Agrobacterium tumefaciens strain EHA105 via electroporation. Agrobacterium-mediated transformation was achieved with the calli derived from mature embryos of Zhonghua 11 (Lin and Zhang 2005).

Pathogen Inoculation
Rice plants were inoculated with 6 Xoo strains (one Chinese Xoo strain Zhe173 and 5 Philippine Xoo strains PXO61, PXO71, PXO99, PXO341, and PXO347) using the leaf-clipping method at the booting stage (Chen et al. 2002) to investigate proteins interaction in rice plants. Total proteins were extracted from rice leaves with extraction buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8.0), 5 mM EGTA (pH 7.0), 5 mM Na3VO4, 10 mM NaF, 50 mM β-glycerophosphate, 10% glycerol, 1 mM PMSF, and complete EDTA Free protease inhibitor cocktail) (Roche, China), and precleared with protein A/G agarose mixture (Roche, China) for 2 h at 4 °C. The cleaned proteins were then transferred and incubated with antibody overnight at 4 °C, and added protein A/G agarose mixture for further incubation for 3 h at 4 °C. The immunocomplex was washed three times with wash buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 5 mM Na3VO4, 50 mM β-glycerophosphate, 0.1% Tween 20, 1 mM PMSF, and complete EDTA-free protease inhibitor cocktail). Finally, the samples were boiled with 5× SDS-PAGE loading buffer for 5 min and subjected to immunoblot analysis.

Protein Point Mutation
PCR-mediated site mutagenesis was performed with site-directed Mutagenesis Kit (Sangon, China) to introduce point mutations into the open reading frames (ORFs) of OsVQ14, OsVQ32, and OsMPK4 using the primers listed in Table S2, in accordance with the manufacturer’s protocol.

Protein Expression, Purification and Phosphorylation Assays in Vitro
To express recombinant proteins in E. coli, the ORFs of OsMPKK6, OsMPK4, and OsMPK4A were cloned into the pET28 vector (Invitrogen, USA), the ORF of OsMPK4K72R was cloned into the vectors pET28 and pMAL-c2x (New England Biolabs, USA), and the ORFs of OsVQ14, OsVQ32, and their mutation versions were cloned into the pCOLD-TF vector (Takara, China). The E. coli cells were cultivated in Lysogeny Broth containing 100 µg/mL ampicillin or 50 µg/mL kanamycin at 37 °C and shaken for 2 to 3 h until the required optical density was reached (OD600 to 0.6–1.0). Expression was induced by adding 0.1% Isopropyl β-D-thiogalactopyranoside for 16–18 h at 16 °C. Proteins were purified for a direct phosphorylation in vitro in the presence of γ-32P-ATP, as previously described (Ma et al. 2017).

Immunoprecipitated Protein Phosphorylation Assay
To express proteins in tobacco (Nicotiana benthamiana) cells, plasmids were transformed into tobacco plants via A. tumefaciens strain GV3101-pPM90. The immunoprecipitated protein phosphorylation assay was performed as
described previously (Ma et al. 2017). Briefly, total proteins were extracted from tobacco or rice leaves with extraction buffer as described above. Target protein was immunoprecipitated using its specific antibody and protein A/G agarose. The immunocomplex was washed twice with wash buffer 1 (extraction buffer containing 150 mM NaCl, 0.1% Tween 20), twice with wash buffer 2 (extraction buffer containing 500 mM NaCl, 0.1% Tween 20), and once with wash buffer 3 (extraction buffer containing 0.1% Tween 20). Western blot analysis was then performed to detect protein phosphorylation and protein levels using phospho-specific antibodies (anti-pTEpY antibody and anti-pT antibody, Cell Signaling Technology; anti-pS antibody, Abcam) and non-phospho antibodies (anti-OsMPKK6 and anti-OsMPK4 antibodies that were produced using 6× His-tagged OsMPKK6 and OsMPK4 as antigens, respectively; anti-OsVQ14 and anti-OsVQ32 antibodies that were produced using TF- and His-tagged OsVQ14 and OsVQ32 as antigens, respectively), respectively.

Phylogenetic Tree Construction
The proteins used for phylogenetic tree construction were listed in Tables S3 and S4 (Cheng et al. 2012; Li et al. 2014a). Unrooted tree was constructed using MEGA-X with neighbor-joining method based on Dayhoff model (Kumar et al. 2018). The gaps or missing data were treated as partial deletion with coverage cutoff at 50%. Bootstrap method with 1000 bootstrap replications was used to test the phylogeny. An online tool iTOL (Interactive Tree of Life, https://itol.embl.de/) was used to annotate the tree (Letunic and Bork 2019).

Statistical Analysis
The significance of the differences between the control and treatment was analyzed using the pair-wise t-test function installed in the Microsoft Office Excel program. The correlations between the disease and gene expression level was analyzed using the Pearson correlation method with GraphPad Prism 5 software.

Abbreviations
VQ: Valine-glutamine; MAPK: Mitogen-activated protein kinase; MAPKK: Mitogen-activated protein kinase kinase; MAPKKK: Mitogen-activated protein kinase kinase kinase; Xoo: Xanthomonas oryzae pv. oryzae; MR: Major disease resistance; QTTL: Quantitative trait loci; CRISPR/Cas9: Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9; Y2H: Yeast two-hybrid; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; RT-qPCR: Reverse transcription quantitative PCR; ORF: Open reading frames

Additional Files

Additional file 1: Fig. S1. Overexpressing OsVQ14 and OsVQ22 enhanced rice resistance to Xoo. Bars represent mean (3 to 5 leaves of lesion area for each plant, and 3 replicates for expression level) ± standard deviation (SD). The asterisks *** or ** indicate a significant difference between transgenic plants and wild type (WT; Zhonghua 11) plants at P < 0.01 or P < 0.05, respectively. N: negative transgenic plants.

Fig. S2. The positions of CRISPR/Cas9 system target sites in two VQ genes and sequencing results of transgenic plants. The protospacer adjacent motif (PAM) (CCN) is shown in bold and underlined. The dashed lines indicate base pairs deletion. Zhonghua 11 (WT) is the background of transgenic plants. Rectangles “I” and “VQ” represent domain I and VQ domain, respectively. a The two CRISPR/Cas9 system target sites (TS) in OsVQ14. b Sequencing results of OsVQ14-KO plants. “…” (51bp) “… means there is 51 base pairs and “… (17 aa) “… means there are 19 amino acids. c The two CRISPR/Cas9 system target sites (TS) in OsVQ22. d Sequencing results of OsVQ22-KO plants. “…” (30bp) “… means there are 30 base pairs and “… (10 aa) “… means there are 10 amino acids.

Fig. S3. The sequencing results of off-target sites of target site 1 (a) and 2 (b) in OsVQ32-KO88, OsVQ32-KO91, and the WT. The protospacer adjacent motif (PAM) (CCN) are in bold and underlined. The putative off-target sites are indicated with rectangles. Fig. S4. Phylogenetic tree of VQ proteins in Arabidopsis and rice. This phylogenetic unrooted tree was constructed using MEGA-X with neighbor-joining (NJ) method based on Dayhoff model. The gaps or missing data treatment was set as Partial deletion with coverage cutoff at 50%. Bootstrap method with 1000 bootstrap replications was used to test the phylogeny. An online tool iTOL (Interactive Tree of Life, https://itol.embl.de/) was used to annotate the tree. Only those values greater than 40% are displayed. The transcripts ID encoding VQ proteins in rice and Arabidopsis are listed in Table S3 and Table S4, respectively. Fig. S5. Sequence alignment of AtVQ21, OsVQ14 and OsVQ32. The abbreviations, dC1, dC2, dNC, dN1, dN2, on the top left and top right of triangles represent the start and end amino acids of each truncated proteins, respectively. Domains I is shown by underline. Amino acids deletions in mutant plants are labeled by dotted lines. Amino acids identical in three proteins are shaded, and residues similar in two proteins are pink. The putative nuclear localization sequences are red. Putative MAP kinase phosphorylation sites (S/T/P) are underlined. Asterisks indicate the highly conserved FxxqVqxLtx (x: any amino acid) sequences. The amino acids of truncated OsVQ14 and OsVQ32 are indicated by black and red colored triangles, respectively, which are corresponding to the truncated OsVQ14 and OsVQ32 in Fig. 3a and b. Fig. S6. Amino acid sequences of OsMPKK6. The boxed text indicates peptides found by mass spectrometry.

Additional file 2 Table S1. The putative off-target sites of target site 1 in OsVQ22-KO plants. Table S2, Primers used in vector construction, gene expression analysis, and detection of positive transgenic plants.

Table S3. Rice VQ genes used in phylogenetic tree. Table S4. Arabidopsis VQ genes used in phylogenetic tree.

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Authors’ Contributions
NL, ZY, HM, and SW designed the research; NL, ZY, JL, WX, XQ, YK, and HM performed experiments; QZ, XL, and JX provided field management and experiments support; NL, HM, and SW wrote the manuscript. All authors read and approved the final manuscript.

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Availability of Data and Materials
All data generated or analyzed during this study are included in this published article and its supplementary information files.
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