MAPK kinase 10.2 promotes disease resistance and drought tolerance by activating different MAPKs in rice

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SUMMARY

Mitogen-activated protein kinase (MAPK) cascades, with each cascade consisting of a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK, have important roles in different biological processes. However, the signal transduction in rice MAPK cascades remains to be elucidated. We show that the structural non-canonical MAPKK, MPKK10.2, enhances rice resistance to Xanthomonas oryzae pv. oryzicola (Xoc), which causes bacterial streak disease, and increases rice tolerance to drought stress by phosphorylating and activating two MAPKs, MPK6 and MPK3, respectively. MPKK10.2-over-expressing (oe) plants showed enhanced resistance to both Xoc and drought, whereas MPKK10.2-RNA interference (RNAi) plants had increased sensitivity to both Xoc and drought. MPK10.2 physically interacted with MPK6 and MPK3, and phosphorylated the two MAPKs in vivo. Transcriptionally modulating MPKK10.2 influenced MPK6 phosphorylation during rice-Xoc interaction, and MPKK10.2-oe/MPK6-RNAi double mutants showed increased sensitivity to Xoc. MPKK10.2-oe/MPK3-RNAi double mutants showed survival rates similar to those of control plants, although the survival rates of MPKK10.2 transgenic plants changed after drought stress. These results suggest that MPKK10.2 is a node involved in rice response to biotic and abiotic responses by functioning in the cross-point of two MAPK cascades leading to Xoc resistance and drought tolerance.

Keywords: MAPK, bacterial streak, drought, phosphorylation, Oryza sativa.

INTRODUCTION

Mitogen-activated protein kinase (MAPK) cascades are evolutionarily conserved in eukaryotes. Each cascade comprises three central components, a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK. This type of cascade transduces extracellular signals into cellular responses through sequential phosphorylation. Signals can activate a MAPKKK, which in turn can activate a MAPKK by phosphorylating its serine (S) and/or threonine (T) residues in the S/T-X3-S/T (X represents any amino acid residue) motif. The MAPKK then activates a MAPK by phosphorylating its threonine and tyrosine (Y) residues in the T-X-Y motif, which can be divided into at least two types, the T-aspartic acid (D)-Y and T-glutamic acid (E)-Y motifs, in plants (Kyriakis and Avruch, 2012; Meng and Zhang, 2013). The activated MAPK phosphorylates other proteins to activate or repress their functions.

Plant MAPK cascades are involved in various physiological responses, including biotic and abiotic stresses. Plant genomes contain relatively less MAPKK genes compared with the numbers of MAPKK and MAPK genes. For example, the Arabidopsis genome has 60 MAPKKK, 10 MAPKK and 20 MAPK genes (Ichimura et al., 2002); the rice genome has 74 MAPKKK (MPKKK), eight MAPKK (MPKK) and 17 MAPK (MPK) genes (Hamel et al., 2006; Reyna and Yang, 2006; Rao et al., 2010; Yang et al., 2015). This characteristic implies that multiple signal flows converge at the MAPKK level.

Biotic and abiotic stresses are limiting factors in crop production. Although the importance of MAPK cascades in eukaryote signal communication has been well recognized, no single MAPK cascade has been unambiguously characterized in the rice response to stresses. However, a few MAPKKK and MAPK genes that function in rice biotic or abiotic responses have been identified. MPKKK1 (also known as EDR1) and MPKKK6 (also known as DSM1) were reported to regulate rice resistance to bacterial blight caused by Xanthomonas oryzae pv. oryzae (Xoo) and drought tolerance, respectively (Ning et al., 2010; Shen et al., 2011; Yang et al., 2015). MPK3 (LOC_Os03 g17700; also known as MPK5), the ortholog of Arabidopsis MPK3,
negatively regulates rice resistance to blast caused by *Magnaporthe oryzae* and positively regulates drought and submergence tolerance (Xiong and Yang, 2003; Singh and Sinha, 2016). MPK4 (also known as MPK6, the ortholog of Arabidopsis MPK4) and MPK17-1 (also known as MPK12, the ortholog of Arabidopsis MPK17) regulate rice resistance to Xoo infection (Shen et al., 2010; Seo et al., 2011).

Rice MPKs can be divided into four groups (Hamel et al., 2006). Group A consists of MPKK1 and MPK6. MPKK1 is involved in the salt signaling pathway via the activation of MPK4 (Wang et al., 2014). Overexpression of constitutively activated MPKK6 also enhances rice chilling and salt tolerance (Xie et al., 2012; Kumar and Sinha, 2013). To date, there is no research regarding MPKK3, which is the only member of Group B. Group C comprises MPK4 and MPK5. MPKK4-MPK3 and MPKK4-MPK6 (LOC_Os06 g06090; the ortholog of Arabidopsis MPK6) cascades are activated by chitin, a fungal pathogen-associated molecular pattern, and contribute to the synthesis of antimicrobial compounds (Kishi-Kaboshi et al., 2010). Group D consists of MPKK10.1, MPKK10.2 and MPKK10.3. Mutated MPKK10.2 can phosphorylate MPK6 in vitro, which then positively regulates WRKY45-mediated blast resistance (Ueno et al., 2015). These results suggest that MAPK cascades appear to have important roles in rice responses to biotic and abiotic stresses.

Rice bacterial streak, which is caused by biotroph *Xanthomonas oryzae pv. oryzae* (*Xoc*), is a disease in China that requires quarantine (Li and Wang, 2013). *Xoc* infects rice via the stomata or wound. The molecular mechanism of rice resistance to *Xoc* is poorly understood; however, the rice gene *xas5/TFIIA*;Δ^CORE^, which mediates passive and quantitative resistance to *Xoc*, has been reported recently (Yuan et al., 2016). Drought stress is an environmental disaster that can result in more than 50% yield loss of crops (Hu and Xiong, 2014). Although a few MAPK cascade genes have been identified as being involved in the rice response to drought stress, MAPK cascade signaling in the drought tolerance of rice has been rarely defined.

To understand the roles of MAPK cascades in the rice response to pathogens, we generated transgenic plants of several MPKKs and found that MPKK10.2 promoted rice resistance to *Xoc*. In addition, MPKK10.2 promoted rice tolerance to drought stress. Further genetic and biochemical studies have indicated that MPKK10.2 regulates resistance to *Xoc* and drought by phosphorylating and activating two MAPKs, MPK6 and MPK3, respectively.

**RESULTS**

**MPKK10.2 was transcriptionally induced by both biotic stress and abiotic stress**

To determine whether MPKK10.2 is involved in the rice response to biotic or abiotic stress, rice seedlings at the four-leaf stage were exposed to *Xoc* inoculation, drought stress and treatment with benzothiadiazole (BTH), which is an analog of defense hormone salicylic acid (SA) to biotrophic pathogens, or abscisic acid (ABA), which frequently contributes to plant tolerance to abiotic stresses. **MPKK10.2 expression** was highly induced by *Xoc* infection and drought stress, and it was also rapidly induced by BTH or ABA treatment (Figure 1a). These results suggest that **MPKK10.2 may be involved in rice responses to biotic stress and abiotic stress.**

**MPKK10.2 positively regulated rice resistance to *Xoc* and drought stress**

To further analyze the biological function of **MPKK10.2**, we suppressed and overexpressed **MPKK10.2** in rice variety Zhonghua 11 (WT). Twenty-eight independent **MPKK10.2**-suppressing transgenic plants (**MPKK10.2**-RNAi) and 15 independent **MPKK10.2**-overexpression transgenic plants (**MPKK10.2**-oe) were obtained. Some of these transgenic plants showed significantly decreased or increased expression of **MPKK10.2** (Figure S1a,b). To evaluate the specificity of the **MPKK10.2**-RNAi construct, the expressions of the other two genes in rice MPKKs group D, **MPKK10.1** and **MPKK10.3**, were examined in three **MPKK10.2**-RNAi plants (3, 8 and 11). The **MPKK10.2** gene shares 41.9% and 43.3% of its sequence identity with **MPKK10.1** and **MPKK10.3**, respectively. No suppression of the **MPKK10.1** gene or **MPKK10.3** gene was observed in **MPKK10.2**-RNAi plants.
Rice MPKK10.2-MPK3/6 signaling cascade
(Figure S1c). Hence, the progenies from three MPKK10.2-RNAi T0 plants (3, 8 and 11) and three MPKK10.2-oe T0 (6, 9 and 13) were further analyzed.

The MPKK10.2-RNAi plants showed increased susceptibility to Xoc compared with WT, and the increased susceptibility was significantly correlated ($P < 0.01$) with reduced MPKK10.2 transcripts in T1 families (Figure S2). The correlation coefficients of the lesion length and MPKK10.2 expression level were 0.912, 0.802 and 0.939 ($n = 20$, $P < 0.01$) for MPKK10.2-RNAi3, MPKK10.2-RNAi8 and MPKK10.2-RNAi11 T1 families, respectively. In contrast, the MPKK10.2-oe plants had reduced susceptibility to Xoc compared with WT, and the reduced susceptibility significantly correlated ($P < 0.01$) with the increased MPKK10.2 transcript in T1 families (Figure S3). The correlation coefficients of the lesion length and MPKK10.2 expression level were 0.826 ($n = 19$, $P < 0.01$), 0.876 ($n = 13$, $P < 0.01$) and 0.766 ($n = 18$, $P < 0.01$) for MPKK10.2-oe6, MPKK10.2-oe9 and MPKK10.2-oe13 T1 families, respectively. These results were further confirmed in T2 plants. The average lesion lengths caused by Xoc were 3.42 ± 0.48 cm for MPKK10.2-RNAi-positive plants and 2.04 ± 0.35 cm for MPKK10.2-oe-positive plants compared with 2.95 ± 0.41 cm for WT (Figure 1b). The negative transgenic plants showed a level of lesion length that was similar to that of WT. Furthermore, the average Xoc growth rate of rice leaves was 7.6-fold higher for MPKK10.2-RNAi-positive plants and 11.6-fold lower for MPKK10.2-oe-positive plants compared with WT (Figure 1c). These results suggest that MPKK10.2 promotes rice resistance to Xoc.

The MPKK10.2-RNAi lines were more sensitive to drought treatment, with approximately 3.1-fold lower survival rates after treatment compared with WT (Figure 1d). However, the MPKK10.2-oe lines showed enhanced drought tolerance, with 3.9-fold higher survival rates compared with WT (Figure 1d). These results suggest that MPKK10.2 also promotes rice tolerance to drought stress.

**MPK3 functioned downstream of MPKK10.2 in the rice response to drought**

Among 17 MAPK genes in the rice genome, only MPK3 has been reported to function in disease resistance and drought tolerance (Xiong and Yang, 2003). To clarify whether MPK3 functions downstream of MPKK10.2, we suppressed and overexpressed MPK3 in the same WT (Figure S4). The targeted region of MPK3 by the RNAI construct was the same as that previously reported (Xiong and Yang, 2003). In accordance with a previous report (Xiong and Yang, 2003), suppression of MPK3 (MPK3-RNAI) reduced rice tolerance to drought stress (Figure S5a), and overexpression of MPK3 (MPK3-oe) enhanced rice tolerance to drought stress (Figure S5b). However, both MPK3-RNAI and MPK3-oe plants showed a response to Xoc infection that was similar to that of WT (Figures S6 and S7). Further analysis showed that the MPK3 knockdown (MPK3-RI) plants (named MPK5-RI in the original articles) generated previously (Xiong and Yang, 2003; Xie et al., 2014) also exhibited a response similar to that of WT Nipponbare to Xoc (Figure S8). We then crossed MPKK10.2-oe lines with MPK3-RNAi lines, and the MPKK10.2-oe/MPK3-RNAI double mutants had significantly increased MPKK10.2 transcripts and suppressed MPK3 transcripts (Figure 2a). The double mutants showed survival rates similar to those of WT after drought treatment (Figure 2b), although the MPKK10.2-oe plants had a significantly higher survival rate than WT, and MPK3-RNAI plants had a significantly lower survival rate than WT after drought treatment (Figures 1d and S5a). In comparison, the double mutants showed a level of resistance to Xoc similar to that of MPKK10.2-oe plants (Figure 2c). These results suggest that, considering MAPK cascade signaling, MPK10.2 may regulate the rice response to drought stress through MPK3 and that it regulates the rice response to Xoc infection through another MAPK other than MPK3.

**MPK6 functioned downstream of MPKK10.2 in the rice response to Xoc infection**

To determine which MAPK was involved in MPKK10.2-mediated defense against Xoc, we analyzed the phosphorylation status of MAPKs in MPKK10.2 transgenic plants in vivo after Xoc infection using anti-pTEpY antibody, which is commonly used to detect plant-phosphorylated MAPKs on the conserved T-E-Y motif by MAPKKs (Yamaguchi et al., 2013; Willmann et al., 2014). A MAPK with a molecular mass of approximately 48 kD was highly phosphorylated after inoculation of Xoc in WT plants compared with non-inoculated plants (Figure 3a,b). Because the phosphorylated band was absent in MPK6 knockout mutant (mpk6; Figure 3a,b), which has a single nucleotide deletion in the coding region resulting in loss of kinase activity and highly reduced protein expression (Liu et al., 2015; Figure S9), the phosphorylated band was considered to correspond to MPK6. Further analysis showed that MPKK10.2-oe plants had more phosphorylated MPK6 molecules than WT before and after inoculation of Xoc (Figure 3a), whereas the MPKK10.2-RNAI plants had less phosphorylated MPK6 molecules than WT after inoculation of Xoc (Figure 3b). These results suggest that Xoc infection increases phosphorylated MPK6 and that MPKK10.2 promotes accumulation of phosphorylated MPK6 after Xoc infection.

To examine whether MPK6 was involved in the rice-Xoc interaction, we overexpressed MPK6 in Zhonghua 11 (Figure S10). The MPK6-oe plants showed reduced susceptibility to Xoc compared with WT, and the reduced susceptibility was significantly correlated ($P < 0.01$) with increased expression of MPK6 in T1 families (Figure S11). The correlation coefficients for lesion length and MPK6
expression level were 0.624 and 0.796 (n = 20, P < 0.01) for the MPK6-oe1 and MPK6-oe9 T1 families, respectively. This result was further confirmed in T2 plants. The lesion lengths caused by Xoc in MPK6-oe-positive plants were significantly shorter than those of WT (P < 0.01). The negative transgenic plants showed a lesion length similar to that of WT (Figure 3c). In addition, the Xoc growth rates were significantly lower (P < 0.01) in MPK6-oe plants and significantly higher in mpk6 plants compared with WT (Figure 3d). These results suggest that MPK6 promotes rice resistance to Xoc.

We next suppressed MPK6 in WT and two MPK10.2-oe lines (MPK10.2-oe6 and MPK10.2-oe9). The targeted region by the RNAi construct was the same as previously reported (Lieberherr et al., 2005). The MPK6-RNAi plants showed increased susceptibility to Xoc compared with WT,
and the increased susceptibility in MPK6-RNAi plants was significantly correlated ($P < 0.01$) with reduced MPK6 transcripts in T$_1$ families (Figure S12). The correlation coefficients for lesion length and MPK6 expression level were 0.827 and 0.942 ($n = 15$; $P < 0.01$) for the MPK6-RNAi44 and MPK6-RNAi62 T$_1$ families, respectively. Furthermore, the Xoc growth rates were significantly higher ($P < 0.01$) for MPK6-RNAi plants compared with WT (Figure 3e).

Although the MPKK10.2-oe plants showed reduced susceptibility to Xoc compared with WT, the MPKK10.2-oe/MPK6-RNAi plants showed increased susceptibility to Xoc compared with WT (Figure S13). In addition, the increased susceptibility of MPKK10.2-oe/MPK6-RNAi plants was significantly correlated ($P < 0.01$) with reduced MPK6 transcripts in T$_1$ families (Figure S13). The correlation coefficients for lesion length and MPK6 expression level were 0.984 ($n = 11$; $P < 0.01$) and 0.926 ($n = 12$; $P < 0.01$) for the MPKK10.2-oe6/MPK6-RNAi and MPKK10.2-oe9/MPK6-RNAi T$_1$ families, respectively. Furthermore, the Xoc growth rates were significantly higher ($P < 0.01$) for MPKK10.2-oe/MPK6-RNAi plants compared with WT, although MPKK10.2-oe plants showed lower Xoc growth rates (Figure 3f). These results suggest that MPKK10.2 regulates the rice response to Xoc infection through MPK6.

**MPK6 negatively regulated rice resistance to drought stress**

Further analysis was performed to examine the role of MPK6 in the rice response to drought stress. The MPK6-oe lines were more sensitive to drought treatment, with approximately 2.1-fold to 2.6-fold lower survival rates after treatment compared with WT (Figure 4a). However, the mpk6 mutant showed enhanced drought tolerance with 3.4-fold higher survival rates compared with the WT (Figure 4b). These results suggest that MPK6 is a negative regulator of rice tolerance to drought stress.

**MPKK10.2 could interact with and phosphorylate MPK6 and MPK3**

To learn whether MPKK10.2 directly regulates MPK6 and MPK3, co-immunoprecipitation assays were performed in rice plants. Protein extracted from the leaves of WT, MPKK10.2-RNAi3, mpk6 and MPK3-RNAi4 plants without or exposed to Xoc infection or drought stress was immunoprecipitated with MPKK10.2-specific antibody. Without stress, MPKK10.2 was detected in the immunoprecipitated protein complex from WT, and MPK6 and MPK3 were not detected in the immunoprecipitated protein complex from WT (Figure 5a). After Xoc infection, MPKK10.2
and MPK6 were both detected in the immunoprecipitated protein complex from WT; however, MPK3 was not detected (Figure 5a). After drought stress, MPKK10.2 and MPK3 were both detected in the immunoprecipitated protein complex of WT; however, MPK6 was not detected (Figure 5a). In addition, MPK3 was not detected in the immunoprecipitated protein complex from mpk6 mutant exposed to Xoc infection and MPK6 was not detected in the immunoprecipitated protein complex from MPK3-RNAi plants exposed to drought stress (Figure S14). These results suggest that MPKK10.2 and MPK6 functioned in the same protein complex in the rice response to Xoc infection, and that MPKK10.2 and MPK3, but not MPKK10.2 and MPK6, were in the same protein complex in the rice response to drought stress.

To determine whether MPKK10.2 could phosphorylate MPK6 and MPK3, we expressed and purified these three proteins from bacteria. Both MPK6 and MPK3 could autophosphorylate and phosphorylate the myelin basic protein, a common substrate of MAPKs (Figure 5b). We then generated the kinase-inactive versions of MPK6 (MPK6K96R) and MPK3 (MPK3K65R) by substituting a conserved lysine (K) residue (K96 in MPK6 and K65 in MPK3) for arginine (R) in the ATP-binding domain. Both MPK6K96R and MPK3K65R lacked autophosphorylation activity, but they were phosphorylated by MPKK10.2 (Figure 5c). We also immunoprecipitated MPKK10.2 from the leaves of WT plants before and after Xoc infection or drought stress. The phosphorylation of MPK6K96R and MPK3K65R by immunoprecipitated MPKK10.2 was detected using the anti-pTEpY antibody. The results showed that immunoprecipitated MPKK10.2 (+IP:MPKK10.2) after Xoc infection and drought stress could phosphorylate MPK6K96R and MPK3K65R, respectively (Figure 5d,e). All these results suggest that MPKK10.2 may perform its function in Xoc resistance and drought tolerance via activating MPK6 and MPK3 through phosphorylation.

Xoc resistance and drought tolerance required MPK6-mediated SA signaling and MPK3-mediated ABA signaling pathways

It has been reported that SA treatment activates MPK6 but not MPK3 (Xiong and Yang, 2003; Ueno et al., 2015). To determine whether the SA signaling pathway contributed to MPK6-mediated Xoc resistance, SA-deficient transgenic rice (named NahG rice), which has a reduced SA level by expressing bacterial salicylate hydroxylase that degrades SA (Yang et al., 2004), was used for inoculation analysis. 

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The NahG rice showed increased susceptibility to Xoc compared with WT Nipponbare, and the Xoc growth rate was higher for NahG rice compared with WT (Figure 6a,b). Moreover, after BTH pre-treatment, both WT and NahG rice showed significantly reduced \( P < 0.01 \) lesion lengths compared with that treated with deionized water (Figure 6c). These results suggest that the SA signaling pathway contributed to Xoc resistance.

Next, MPK6 and MPK3 were detected in NahG rice after Xoc infection. Consistent with these results (Figure 3a,b), MPK6 was highly phosphorylated after inoculation of Xoc in WT (cultivar Nipponbare) compared with non-inoculated (a)
plants, whereas NahG rice had less phosphorylated MPK6 molecules than WT after inoculation of *Xoc* (Figure 6d).

The MPK6 expression level and the phosphorylation of immunoprecipitated MPK3 were normal in both WT and NahG rice before and after *Xoc* inoculation, as detected using the anti-MPK6 antibody and the anti-pTEpY antibody (Figure 6e,f), suggesting that MPK6 but not MPK3 phosphorylation is required in SA-mediated *Xoc* resistance.

Abscisic acid treatment induced and activated MPK3 but inactivated MPK6 (Xiong and Yang, 2003; Ueno *et al.*, 2015). Based on this, we detected MPK6 and MPK3 after drought stress in ABA-deficient mutant phs3-1, which has a G-to-C transition in the donor site of intron 6 of a carotenoid isomerase-like gene resulting in mis-splicing and reduced ABA level (Fang *et al.*, 2008). The phs3-1 mutant was more sensitive to drought stress compared with WT.
(cultivar Xiushui11; Du et al., 2010). After drought stress, the phosphorylation of MPK6 was decreased in WT, but normal in phs3-1 mutant (Figure 6g); MPK3 was highly induced in WT, but much less induced in the phs3-1 mutant (Figure 6h); the phosphorylation of immunoprecipitated MPK3 was enhanced in WT, but normal in phs3-1 mutant (Figure 6i). These results suggest that the expression and phosphorylation of MPK3 but the dephosphorylation of MPK6 is necessary in ABA-mediated drought tolerance.

DISCUSSION

The present results suggest that rice MPKK10.2 positively regulates bacterial resistance and drought tolerance via phosphorylating and activating two distinct MAPks, MPK6 and MPK3. These results facilitate our understanding of the tight control of MAPK cascades in the biological processes of rice.

**MPKK10.2 possesses the typical MAPKK kinase activity**

Rice MPKks of group D, including MPKK10.2, belong to structural non-canonical MPKks because they do not have the typical S/T-X_S/T-M motif of most plant MAPKks that are phosphorylation sites of MAPKKks (Hamel et al., 2006; Figure S15). Therefore, it is not clear whether MPKK10.2 can function as a canonical MAPKK to phosphorylate and activate MAPks. Although a previous study reported that mutated MPKK10.2 (named OsMKK10-2D in the original article), which has constitutively activated kinase activity, can phosphorylate MPK6 (Ueno et al., 2015), it is unknown whether the WT MPKK10.2 possesses such ability. Here, we provide the evidence that WT MPKK10.2 is activated by Xoc infection and drought stress, and can phosphorylate MPK6 and MPK3 on the T-E-Y motif as the phosphorylation site of canonical MAPKKs (Figures 3a,b and 5c-e).

**MPKK10.2 is a cross-point in rice responses to biotic and abiotic stresses**

There are much less MPKK genes than MPKKK and MPK genes in the rice genome, suggesting that each MPKK may be phosphorylated by multiple MPKKks and that each MPK may phosphorylate multiple MPks during signal transduction. Therefore, each MPKK could be involved in the regulation of diverse physiological activities. An example is MPKK6, which positively regulates rice responses to both chilling stress and salt stress (Xie et al., 2012; Kumar and Sinha, 2013). However, no MPKK has been reported to regulate both biotic stress and abiotic stress in rice. The present results indicate that MPKK10.2 enhances rice resistance to both Xoc infection and drought stress. The MPKK10.2 gene was transcriptionally activated by Xoc infection, drought stress, SA analog and ABA (Figure 1a). MPKK10.2 kinase activity was also induced by Xoc infection and drought stress (Figure 5d,e). SA is known to regulate the plant defense to biotrophic pathogens (McDowell and Dangl, 2000), including Xoc (Figure 6a-c), and ABA is frequently involved in plant tolerance to abiotic stresses (Zhu, 2002), including rice drought tolerance (Du et al., 2010). MPK6 and MPK3 were required for SA-mediated Xoc resistance and ABA-mediated drought tolerance, respectively (Figure 6d-i). We suggest that Xoc infection and drought stress induce accumulation of SA and ABA, respectively, and that increased SA and ABA levels may result in the activation of unknown MPKKks, which in turn activate MPKK10.2 by phosphorylating the latter (Figure 7). Activated MPKK10.2 likely promotes resistance to Xoc through phosphorylating and activating MPK6, and likely promotes drought tolerance through phosphorylating and activating MPK3 (Figure 7). Therefore, MPKK10.2 is a node in the rice response to biotic stress and abiotic stress by functioning in the cross-point of two MAPK cascades leading to Xoc resistance and drought tolerance, respectively.

How rice MPKK10.2 distinguishes different stimuli and modulates distinct but proper output is a principal question. A co-immunoprecipitation experiment showed that MPKK10.2 co-immunoprecipitated with MPK6, but not MPK3, after Xoc infection, and with MPK3, but not MPK6, after drought stress (Figures 5a and S14). Therefore, one explanation is that different scaffold proteins may function in MPKK10.2-involved cascades. Each scaffold protein that binds components of one MPKK10.2-involved MAPK cascade together could restrict the signaling that occurs only in a certain protein complex to avoid erroneous response. For example, the Arabidopsis MKK4/MKK5-MPK3/MPK6 cascade regulates the immune response and stomatal development and patterning (Asai et al., 2002; Wang et al., 2017).
Negative role of MPK6 in rice response to drought stress

MPK6 was negatively involved in rice response to drought stress (Figure 4), and its repressed activity is necessary in ABA-dependent drought response (Figure 6g), while in SA-mediated disease resistance, MPK6 activity is required (Figure 6). The antagonistic interaction between SA- and SA-mediated disease resistance, MPK6 activity is required (Figure 6). The antagonistic interaction between SA- and SA-mediated disease resistance, MPK6 activity is required (Figure 6). The antagonistic interaction between SA- and SA-mediated disease resistance, MPK6 activity is required (Figure 6). The antagonistic interaction between SA- and SA-mediated disease resistance, MPK6 activity is required (Figure 6).

Unique role of MPK3 in rice responses to biotic stresses

In addition to promoting rice drought adsorption, MPK3 negatively regulates the rice defense against various pathogens, including bacterial pathogens _Burkholderia glumae_ and _Xanthomonas oryzae pv. oryzae_, and fungal pathogen _M. oryzae_ (Xiong and Yang, 2003; Seo _et al._, 2011). However, our results suggest that MPK3 was not involved in the rice response to Xoc infection (Figures S6 and S7). A MAPK cascade gene can regulate different responses to different pathogens. One example is rice EDR1, a MAPKKK. The _edr1_ knockout mutant showed enhanced resistance to _X. oryzae pv. oryzae_ but increased susceptibility to _M. oryzae_ (Shen _et al._, 2011). Knockdown of rice MPK6 using RNAi did not influence the rice response to the blast pathogen (Lieberherr _et al._, 2005), but caused increased susceptibility to _Xoc_ (Figures 3e and S12). The Arabidopsis _mpk3_ (the ortholog of rice MPK3) knockout mutant showed reduced susceptibility to bacterial pathogen _Pseudomonas syringae pv. tomato_ DC3000 (Frei _et al._, 2014), but enhanced susceptibility to powdery mildew _Golovinomyces cichoracearum_, a fungal pathogen (Zhao _et al._, 2014). Rice MPK3 is another example.

A previous study reported that rice CPK18, a calcium-dependent protein kinase, negatively regulates rice resistance to _M. oryzae_ through phosphorylating and activating MPK3 (Xie _et al._, 2014), indicating that the MPK3-mediated defense may include the MAPKK-independent pathway.

EXPERIMENTAL PROCEDURES

Rice materials

Rice varieties Zhonghua 11, Nipponbare and Xiushui 11 belong to the japonica (_Oryza sativa ssp. japonica_) subgroup of Asian cultivated rice. The _mpk6_ knockout mutant, which has a single nucleotide deletion in the sixth exon resulting in the loss of kinase activity, has the genetic background of Zhonghua 11 (Liu _et al._, 2015). NahG rice, which has reduced the SA level by expressing bacterial salicylate hydroxylase that degrades SA, has the genetic background of Nipponbare (Yang _et al._, 2004). The _phs3-1_ mutant, which has a G-to-C transition at the donor site of intron 6 of a carotenoid isomerase-like gene resulting in mis-splicing and reduced ABA level, has the genetic background of Xiushui 11 (Fang _et al._, 2008). MPK3 knockdown (MPK3-Ri) plants (named MPK5-Ri in the original articles; Xiong and Yang, 2003; Xie _et al._, 2014) have the genetic background of Nipponbare.

Vector construction and rice transformation

The full-length and fragments of cDNAs MPK10.2, MPK3 and MPK6 were amplified from Zhonghua 11 using the primers listed in Table S1. For construction of the overexpressing vector, the amplified cDNA was inserted into the pU1301 vector (Cao _et al._, 2007). For construction of the RNA interference (RNAi) vector, the polymerase chain reaction (PCR) product was inserted into the pDS2301 vector, which was constructed based on the pDS1301 vector (Yuan _et al._, 2007) and contains a G418 antibiotic selection marker. Agrobacterium-mediated transformation was performed according to protocol (Lin and Zhang, 2005). For construction of the protein expression vectors, the open reading frames (ORFs) of target genes were cloned into the pET28a vector (New England Biolabs, USA) and pMAL-c2x vector (New England Biolabs, USA).

Pathogen inoculation

Plants were inoculated with _Xoc_ strain RH3 using the needle stab method during the seedling or booting stage (Tao _et al._, 2009). Lesion lengths were measured 2-3 weeks after inoculation. Xoc growth in rice leaves was determined by counting colony-forming units (Sun _et al._, 2004). The inoculation of transgenic plants with RH3 was biologically repeated at least twice with similar results, and only one replicate was presented.

Chemical treatment

Abscisic acid (Sangon, China) was first dissolved in a few drops of ethanol and then diluted with autoclaved deionized water to make 0.1 mM solution. BTH (Sigma-Aldrich, USA) was first dissolved in
dimethyl sulfoxide and then diluted with autoclaved deionized water to make 0.5 mM solution. Rice seedlings at the four-leaf stage were sprayed with the solution containing 0.02% (v/v) Tween 20 (Sangon, China) to increase adhesion or with the autoclaved deionized water containing 0.02% (v/v) Tween 20 as a control. Xoc inoculation was performed 24 h after BTH or the deionized water spray.

Drought stress

Stress was generated as reported previously (Tao et al., 2011). In brief, transgenic and control plants growing in the same pot were kept in a greenhouse with light strength maintained at 12,000–14,000 lux and with a 14 h light/10 h dark cycle at 25°C until the five- to six-leaf stage. The plants were withheld from water until all leaves were wilted; then, they were recovered by the provision of water for 7–9 days. Survival rates were recorded. Drought stress assay was biologically repeated three–six times.

Gene expression

Quantitative reverse transcription PCR (qRT-PCR) was conducted as described previously using gene-specific primers (Table S2; Qiu et al., 2007). The expression of the rice actin gene was used as an internal control to standardize the RNA sample for each qRT-PCR.

Point mutation

To introduce point mutations in ORFs of MPK6 and MPK3, PCR-mediated site mutagenesis was performed using the primers listed in Table S3. A site-directed Mutagenesis Kit (Sangon, China) was used according to the manufacturer’s protocol.

Recombinant protein expression, purification and in vitro phosphorylation assay

To express protein in Escherichia coli BL21 (DE3), the ORFs of MPKK10.2, MPK6 and MPK3 were cloned into the pET28a vector (Invitrogen, USA) and pMAL-c2x vector (New England Biolabs, USA). Expression and purification of the recombinant proteins were performed according to the manufacturer’s protocols.

The in vitro phosphorylation analysis was performed as described previously (Ning et al., 2011). In brief, proteins were incubated at room temperature in reaction buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 10 mM MnCl2, 1 mM dithiothreitol (DTT), 0.1 mM ATP and 5 μCi γ-32P-ATP for 30 min. The reaction was stopped by adding 5 × sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer containing 250 mM Tris-HCl (pH 6.8), 10% SDS (W/V), 0.5% bromphenol blue (W/V), 50% glycerol and 50 mM DTT. The samples were boiled for 3–5 min before being separated on SDS-PAGE. The gel was then dried on filter papers and exposed to Fuji X-ray film. Each phosphorylation analysis was biologically repeated two–three times with similar results, and only one replicate was presented.

Antibody production and Western blot analysis

Rabbit polyclonal antibodies against MPKK10.2, MPK6 and MPK3 were produced in Abclonal Technology (Wuhan, China). Full-length MPKK10.2, MPK6 and MPK3 recombinated with 6 × His tag in the amino terminus (N-terminus) expressed in E. coli BL21 (DE3) were used as antigens.

For Western blot analyses, total proteins were extracted from rice leaves in extraction buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8.0), 5 mM EGTA (pH 7.0), 5 mM Na2VO4, 10 mM NaF, 50 mM β-glycerophosphate, 10% glycerol, 2 mM DTT, 1 mM PMSF, and complete EDTA Free protease inhibitor cocktail (Roche, China)]. The protein concentration of the extract was quantified as described previously (Shen et al., 2010). Phosphorylated MAPK analyzed by Western blot technology using phospho-p44/42 ERK1/2 (Thr202/Tyr204) antibody (anti-pTeP-Y; Cell Signaling Technology, USA) was performed according to the manufacturer’s protocol. Protein expression analysis was performed as described previously (Yuan et al., 2016). Each Western blot analysis was biologically repeated at least twice with similar results, and only one replicate was presented.

Co-immunoprecipitation analysis

For co-immunoprecipitation experiments, total proteins were extracted from 10 g rice leaves of wild-type (WT), MPKK10.2-RNAi3, mpk6 and MPK3-RNAi4 plants before and after Xoc infection and drought stress in extraction buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8.0), 5 mM EGTA (pH 7.0), 0.5% polyvinylpolypyrrolidone, 5 mM Na2VO4, 10 mM NaF, 50 mM β-glycerophosphate, 10% glycerol, 2 mM DTT, 1 mM PMSF, and complete EDTA Free protease inhibitor cocktail (Roche, China)]. The protein concentration of the extract was quantified. The extract was precleared with a protein A and G agarose mixture (Roche, China) for 3 h at 4°C. Then, the agarose was removed and the cleaned protein was incubated with anti-MPKK10.2 antibody overnight at 4°C. Then, the protein A and G agarose mixture was added for further incubation for 4 h at 4°C. The immunocomplex was collected by centrifugation and washed three times with wash buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 5 mM Na2VO4, 50 mM β-glycerophosphate, 0.1% Tween 20, 1 mM PMSF, and complete EDTA Free protease inhibitor cocktail]. Then, 5 × SDS-PAGE loading buffer containing 250 mM Tris-HCl (pH 6.8), 10% SDS (W/V), 0.5% bromphenol blue (W/V), 50% glycerol and 50 mM DTT was added, and the samples were boiled for 3–5 min. Western blot analyses were performed. Each co-immunoprecipitation analysis was biologically repeated twice with similar results, and only one replicate was presented.

Immunoprecipitated protein kinase activity assay

For immunoprecipitated MPKK10.2 kinase activity assay, total proteins were extracted from 100 mg rice leaves of WT before and after Xoc infection and drought stress in extraction buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8.0), 5 mM EGTA (pH 7.0), 5 mM Na2VO4, 10 mM NaF, 50 mM β-glycerophosphate, 10% glycerol, 1 mM PMSF, and complete EDTA Free protease inhibitor cocktail]. MPKK10.2 was immunoprecipitated using anti-MPKK10.2 antibody, and protein A and 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), once with wash buffer 3 (extraction buffer containing 0.1% Tween 20), Kinase buffer [25 mM Tris-HCl (pH 7.5), 1 mM EGTA, 12 mM MgCl2, 0.1 mM Na2VO4, 0.1 mM ATP] containing His-MPK6 (His-MPK6) or His-MPK6 (His-MPK6) was added for reaction at room temperature for 40 min. Then, 5 × non-reducing buffer containing 300 mM Tris-HCl (pH 6.8), 5% SDS (W/V), 0.5% bromphenol blue (W/V) and 50% glycerol was added to minimize the denaturation of co-eluting antibody. The samples were heated for 10 min at 75°C. Western blot analyses were performed. Phosphorylated MAPK was analyzed using anti-pTeP-Y antibody, and immunoprecipitated MPKK10.2 was analyzed using anti-MPKK10.2 antibody.
For immunoprecipitated MPK3 kinase activity assay, MPK3 was immunoprecipitated using anti-MPK3 antibody from total protein extracted from rice leaves. The phosphorylation of immunoprecipitated MPK3 was detected using anti-pT3EY antibody. Each kinase activity assay was biologically repeated twice with similar results and only one replicate was presented.

Statistical analysis

The significant differences between control and treatment of the samples were analyzed by the pair-wise t-test installed in the Microsoft Office Excel program. The correlation analysis between disease and gene expression levels was performed using the CORREL analysis installed in the Microsoft Office Excel program.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Gene expression analysis in MPKK10.2 transgenic plants.

Figure S2. Analysis of lesion length and MPKK10.2 expression level of MPKK10.2-RNAi transgenic plants in three T1 families.

Figure S3. Analysis of lesion length and MPKK10.2 expression level of MPK10.2-oe transgenic plants in three T1 families.

Figure S4. MPK3 expression in MPK3suppressing (RNAi) and MPK3overexpressing (OE) transgenic plants.

Figure S5. Effects of modulating MPK3 expression on responses to drought stress in rice.

Figure S6. Effect of suppressing MPK3 on responses to Xoc infection in rice.

Figure S7. Effects of overexpressing MPK3 on responses to Xoc infection in rice.

Figure S8. Effect of MPK3 knockdown (MPK3-R1; original name MPK5-R1) rice plants (provided by Dr Yinong Yang of Pennsylvania State University) on response to Xoc infection.

Figure S9. Analysis of MPK6 protein expression level.

Figure S10. MPK6 expression in MPK6overexpressing (OE) transgenic plants.

Figure S11. Analysis of lesion length and MPK6 expression level of MPK6oe transgenic plants.

Figure S12. Analysis of lesion length and MPK6 expression level of MPK6-RNAi transgenic plants.

Figure S13. Analysis of lesion length and MPK6 expression level of MPKK10.2-oe/MPK6-RNAi transgenic plants.

Figure S14. Co-immunoprecipitation assays between MPKK10.2 and MPK6 or MPK3 in WT, mpk6 and MPK3-RNAi plants.

Figure S15. The S/T-X-S/T motif of rice MPKks.

Table S1. Primers used for vector construction.

Table S2. PCR primers used for gene expression analysis.

Table S3. PCR primers used for PCR-mediated site mutagenesis.

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