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ORIGINAL ARTICLE

Characterization of two infection-induced transcription factors of *Magnaporthe oryzae* reveals their roles in regulating early infection and effector expression

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Abstract

The initial stage of rice blast fungus, *Magnaporthe oryzae*, infection, before 36 h post-inoculation, is a critical timespan for deploying pathogen effectors to overcome the host's defences and ultimately cause the disease. However, how this process is regulated at the transcription level remains largely unknown. This study functionally characterized two *M. oryzae* Early Infection-induced Transcription Factor genes (*MOEITF1* and *MOEITF2*) and analysed their roles in this process. Target gene deletion and mutant phenotype analysis showed that the mutants Δ*moeitf1* and Δ*moeitf2* were only defective for infection growth but not for vegetative growth, asexual/sexual sporulation, conidial germination, and appressoria formation. Gene expression analysis of 30 putative effectors revealed that most effector genes were down-regulated in mutants, implying a potential regulation by the transcription factors. Artificial overexpression of two severely down-regulated effectors, T1REP and T2REP, in the mutants partially restored the pathogenicity of Δ*moeitf1* and Δ*moeitf2*, respectively, indicating that these are directly regulated. Yeast one-hybrid assay and electrophoretic mobility shift assay indicated that Moeitf1 specifically bound the T1REP promoter and Moeitf2 specifically bound the T2REP promoter. Both T1REP and T2REP were predicted to be secreted during infection, and the mutants of T2REP were severely reduced in pathogenicity. Our results indicate crucial roles for the fungal-specific Moeitf1 and Moeitf2 transcription factors in regulating an essential step in *M. oryzae* early establishment after penetrating rice epidermal cells, highlighting these as possible targets for disease control.

KEYWORDS

early infection process, effectors, expression, *Magnaporthe oryzae*, transcription factors

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1 | INTRODUCTION

Rice blast caused by *Magnaporthe oryzae* is a severe rice disease attacking rice worldwide and causes high yield losses, up to 30% (Fernandez & Orth, 2018; Valent & Chumley, 1991). The infection process begins when the pathogen's conidia contact the leaf surface of the host plant (Wilson & Talbot, 2009). Within the first 2–4 h postinoculation (hpi) and under appropriate temperature and humidity conditions, conidia begin to germinate to form germ tubes that develop into appressoria at the germ tube ends after 6–8 hpi (Beckerman & Ebbole, 1996). Over time, the appressorium cell wall undergoes melanization and accumulates large amounts of glycerol intracellularly (Ryder et al., 2019). This amount of glycerol in the cytoplasm combined with the strong melanized cell wall results in the appressorium osmotically taking up surrounding water, and an immense turgor pressure develops (de Jong et al., 1997). The pressure finds an outlet through the formation of a penetration peg and then drives the penetration of the leaf epidermis 16–24 hpi (Ribot et al., 2008). Inside the host cell, hyphae develop from the penetration peg at the infection site. These hyphae enter neighbouring host cells within 36–48 hpi (Khang et al., 2010). As the infection spreads further, visible disease lesions appear on the host leaves approximately 72–96 hpi (Sakulkoo et al., 2018). At this time, new conidia form on the lesion areas and spread by wind or rain splashes to the surfaces of healthy leaves to start new infections (Wilson & Talbot, 2009). Due to the economic importance, genetic tractability, and genome sequence availability, *M. oryzae* has emerged as a model organism to study fungal pathogenesis and interaction with host plants (Ebbole, 2007).

The first 36 hpi of pathogen–host contact is named the early infection stage in this study. At this stage, the pathogen is still only in the first host cell and secretes many effectors to weaken the host immune responses (Kim et al., 2020). One of the immune responses by the host is a burst of reactive oxygen species (ROS) triggered by the innate immune system recognizing the pathogen (Jwa & Hwang, 2017; Smirnoff & Arnaud, 2019). The ROS at the penetration site can be detected by staining cells with 3,3′-diaminobenzidine (DAB) (Li et al., 2019). The pathogen–host struggle at this early biotrophic stage directly determines the outcome, whether the subsequent infection hyphae can survive and disease occurs (Vargas et al., 2012). Therefore, the processes triggered during the early infection stage are essential for *M. oryzae* survival and spread to other plants, but how these processes are regulated, especially at the transcriptional level, is still poorly understood.

Transcription factors are essential for regulating gene expression and cell development. The rice blast fungus genome encodes 495 predicted putative transcription factors in the fungal transcription factor database (Park et al., 2013). According to the InterPro classification (Zdobnov & Apweiler, 2001), these transcription factors can be divided into 44 families. The six major families are bZIP, C2H2, HMG, MADS-box, MYB, and Zn2Cys6 (Park et al., 2013). To date, dozens of transcription factors of *M. oryzae* have been functionally characterized, and the results suggest they play different roles in vegetative growth (Li et al., 2010), conidiation (Bhadauria et al., 2010; Kim et al., 2009; Matheis et al., 2017; Zhou et al., 2009), appressorium formation (Kim et al., 2009; Li et al., 2010; Odenbach et al., 2007; Tang et al., 2015), and host infection (Kim et al., 2009; Mehrabi et al., 2008; Nishimura et al., 2009; Zhou et al., 2011). However, activation of transcription factors in the early infection stage of the pathogen and their regulation of the expression of relevant effectors are rarely reported.

The rice blast fungus can express more than 800 putative effector proteins during infection (Chen et al., 2013). Therefore, it would be overwhelming to analyse the regulatory relationships between the large number of transcription factors and putative effector proteins. Because these effectors are induced during infection (Chen et al., 2013; Liu et al., 2021), this inspired us to examine our hypothesis that the transcription factors that regulate the expression of effector proteins are also expressed during infection. We found that two transcription factors, *MOEITF1* and *MOEITF2*, were specifically up-regulate during the early infection process and each specifically controls the expression of a gene for an effector protein.

2 | RESULTS

2.1 | Selection of transcription factors

We used data from our previous study (Meng et al., 2014) to analyse the expression patterns of 495 transcription factors of *M. oryzae* during all development stages. We found 30 transcription factors highly expressed during the early infection of onion epidermis. To experimentally test their regulatory roles during rice infection, we selected

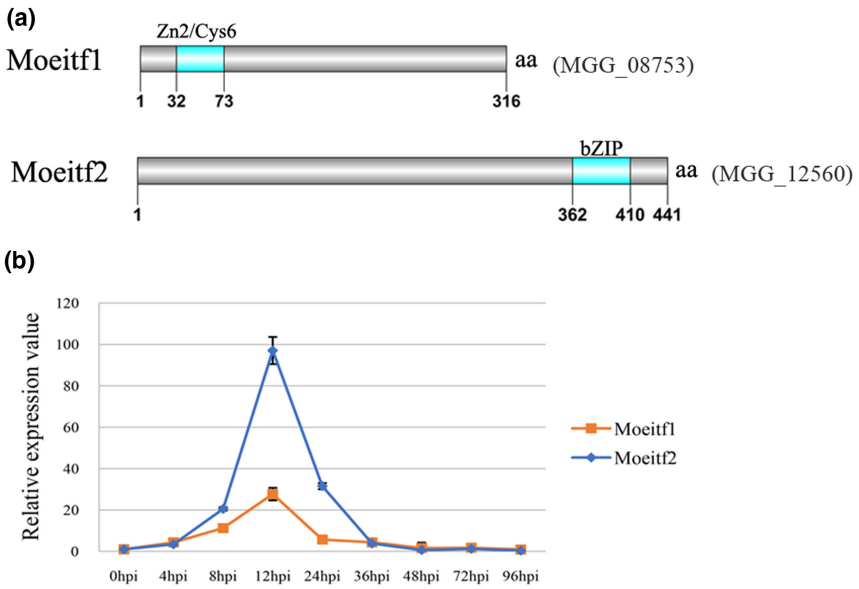

FIGURE 1 Sequence analysis and expression pattern identification of *MOEITF1* and *MOEITF2*. (a) The protein size and domain components of Moeitf1 and Moeitf2 were predicted using the InterPro website (http://www.ebi.ac.uk/interpro/). The number in parentheses indicates the locus tag number of target genes in the GenBank database. (b) The gene expression pattern of *MOEITF1* and *MOEITF2* during the whole infection process was analysed by reverse transcription-quantitative PCR (RT-qPCR). The relative expression value for each infection stage is calculated as $2^{-\Delta\Delta Ct}$ using the 0 h expression as a reference value (Livak & Schmittgen, 2001). The 0-h sample was obtained by sampling immediately after inoculation. $-\Delta\Delta C_t$ = (average C_t of the target gene − average C_t of *β-tubulin*) mutant − (average C_t of the target gene − average C_t of *β-tubulin*) 0 h postinoculation (hpi) sample. The average C_t of each gene was obtained from three RT-qPCR replicates

the top up-regulated 15 for deletion and managed to delete nine genes (authors' unpublished data). Only two of the highly up-regulated transcription factors that we managed to delete, *MOEITF1* and *MOEITF2* (ranked third and sixth in expression, respectively), showed altered infection phenotypes for their deletion mutants. These two genes were selected for further analysis. The remaining potential transcription factors are presently being investigated further in our laboratory.

2.2 | Sequence analysis of *MOEITF1* and *MOEITF2*

MOEITF1 is located on chromosome 6 in the *M. oryzae* genome and encodes a 316 amino acid protein with a Zn2/Cys6 DNA-binding domain at the N-terminus (Figure 1a). A similarity search of amino sequences in NCBI database showed that Moeitf1 has homologs only in ascomycete fungi (Figure S1), suggesting that Moeitf1 is conserved in ascomycetes. *MOEITF2* is located on chromosome 7 in the *M. oryzae* genome and encodes a 441 amino acid protein with a bZIP domain at the C-terminus (Figure 1a). A similarity search of amino sequences in the NCBI database showed that Moeitf2 has homologs only in the genus *Pyricularia* (Figure S2), suggesting that Moeitf2 is conserved in these fungi.

2.3 | *MOEITF1* and *MOEITF2* show a low–high–low expression pattern during infection stages

MOEITF1 and *MOEITF2* are transcription factors highly expressed at the early infection stage. To further clarify the precise expression pattern of *MOEITF1* and *MOEITF2* during all infection stages, we used reverse transcription-quantitative PCR (RT-qPCR) to examine the relative expression value in rice at 10 time points: 0, 4, 8, 12, 18, 24, 36, 48, 72, and 96 hpi. Our results showed that the expression of both *MOEITF1* and *MOEITF2* first increased and then decreased, with peak expression at 12 hpi (Figure 1b). Because 12 hpi is just the beginning of *M. oryzae* infection and penetration of host leaves, our results confirm that *MOEITF1* and *MOEITF2* have a very early infection-induced expression pattern. The result also indicates that these two transcription factors could regulate genes in the very early infection stage of *M. oryzae* infecting rice.

2.4 | Moeitf1 and Moeitf2 are typical transcription factors

Even if Moeitf1 and Moeitf2 have predicted DNA-binding domains, determining whether Moeitf1 and Moeitf2 are real and active transcription factors needs further experimental verification. In a yeast two-hybrid assay to test self-activation, yeast was transformed with pGBKT7-Moeitf1/pGADT7 or pGBKT7-Moeitf2/pGADT7 and grew on the test medium (Figure 2a) compared to positive controls (pGBKT7-53/pGADT7-T) and the empty plasmids (pGBKT7/pGADT7) used as negative controls, indicating that both Moeitf1 and Moeitf2 have transcriptional activation activities. Truncation of the putative DNA-binding domains of the Moeitfs showed these domains are needed for DNA binding; for this test positive controls were the previous negative controls and negative controls were

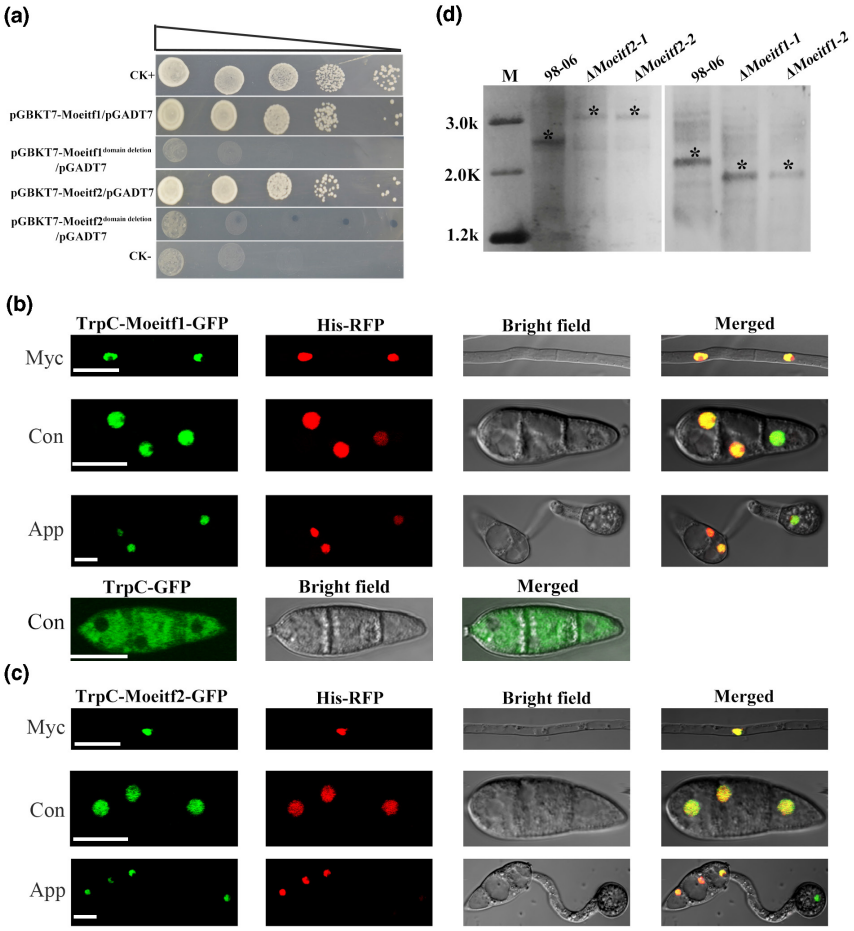

FIGURE 2 Transcription activity detection, subcellular localization analysis, and gene deletion verification of *MOEITF1* and *MOEITF2*. (a) The self-activation experiment of the yeast two-hybrid construct verified that Moeitf1 and Moeitf2 have transcriptional activity. Yeast transformed with the plasmid combination pGADT7-T/pGBKT7-53 and pGADT7/pGBKT7 was used as positive and negative controls, respectively. The DNA-binding domain deletion proteins Moeitf1 and Moeitf2 (Moeitf1$^{\text{domain deletion}}$ and Moeitf2$^{\text{domain deletion}}$) were constructed for a self-activation test. (b, c) Subcellular localization experiments show that Moeitf1 (b) and Moeitf2 (c) are localized to the nucleus. Fluorescence observation and imaging were performed using a laser confocal microscope. Myc, mycelium; Con, conidia; App, appressorium. All size bars are equal to 10 μm. (d) Southern blotting for verifying the knockout of *MOEITF1* and *MOEITF2*. The wild-type band should be approximately 2700 bp and the two *MOEITF2* mutants' bands should be approximately 3100 bp each, while for the knockout of *MOEITF1*, wild-type and mutant bands of approximately 2100 bp and 1800 bp were expected to appear, respectively. * indicates target bands

the respective transcription factors, pGBKT7-Moeitf1/pGADT7 or pGBKT7-Moeitf2/pGADT7.

Because most transcription factors work in the cell nucleus, a subcellular localization assay was performed to examine whether Moeitf1 and Moeitf2 accumulate in *M. oryzae* nuclei. For that, we used the fungal constitutive promoter TrpC to drive the expression of Moeitf1-GFP and Moeitf2-GFP fusion proteins, and then transformed them into *M. oryzae*. The green fluorescent protein (GFP) signal of both transformants co-localized with the Histon1-RFP signal (Zhang et al., 2019), indicating high nuclear accumulation compared to the surrounding cytoplasm of both TrpC-Moeitf1-GFP and TrpC-Moeitf2-GFP in mycelia, conidia, and appressoria (Figure 2b,c). Based on these results, it can be confirmed that Moeitf1 and Moeitf2 are typical transcription factors because they both are predicted transcription factors with a DNA-binding domain, have transcriptional activation activity, and show cell nuclear accumulation.

2.5 | *MOEITF1* and *MOEITF2* are not involved in vegetative or reproductive growth

A gene deletion assay was performed to study the function of *MOEITF1* and *MOEITF2* in *M. oryzae*. For each gene, two mutants named Δ*moeitf1-1,-2* and Δ*moeitf2-1,-2* were acquired. A Southern blot assay was performed to confirm that the target genes had been successfully knocked out in the mutants (Figure 2d). Because the two replicate mutants of both genes were found to have the same phenotype, only one mutant of each, designated as Δ*moeitf1* and Δ*moeitf2*, was selected for further characterization in the following text.

We first tested the colony appearance and growth rate by growing the fungi on rice bran medium for 10 days. The results showed that Δ*moeitf1* and Δ*moeitf2* showed no difference to the wild-type strain 98-06 (Figures 3a and S3a). Further analysis of conidial production ability for each strain showed that Δ*moeitf1* and Δ*moeitf2* produced the same number of conidia compared to 98-06 (Figures 3b and S3b). After mating with TH3 (a sexually compatible strain), both Δ*moeitf1* and Δ*moeitf2* gave rise to perithecia and ascospores, similar to what was found in the wild-type strain 98-06 (Figure 3c). The absence of effects of both mutations on these phenotypes indicate that *MOEITF1* and *MOEITF2* are not critically involved in regulating the vegetative growth or reproductive growth of *M. oryzae*.

2.6 | *MOEITF1* and *MOEITF2* are not necessary for conidial germination and appressoria formation

As conidial germination and appressoria formation are prerequisite steps for *M. oryzae* infection, we tested the performance of the mutants concerning these two aspects. After incubating conidia in water for 4 h at 25°C, we analysed the germination rate of conidia and found no significant difference between the mutants Δ*moeitf1* and Δ*moeitf2* and the wild-type strain 98-06 (Figures 4a and S4a). After incubation for 8 h, the appressoria formation rate was analysed. We found that Δ*moeitf1* and Δ*moeitf2* showed a similar result to the wild-type strain 98-06 (Figures 4b and S4b). As the normal functional appressoria of *M. oryzae* develop a high turgor pressure, we also performed an appressoria collapse assay to test if the appressoria of mutants show normal turgor pressure development. As shown in Figure 4c, when treated with 2, 3, and 4 M glycerol, the Δ*moeitf1* and Δ*moeitf2* and the wild-type strain 98-06 showed a similar proportion of collapsed appressoria. These results indicate that *MOEITF1* or *MOEITF2* are not required for conidial germination, appressoria formation, or the appressorial turgor pressure generation of *M. oryzae*.

2.7 | *MOEITF1* and *MOEITF2* regulate the infection process

The above phenotype testing results showed that *MOEITF1* or *MOEITF2* were only involved in the infection stage. Therefore, we performed conidial spray inoculation of rice seedlings to determine whether these

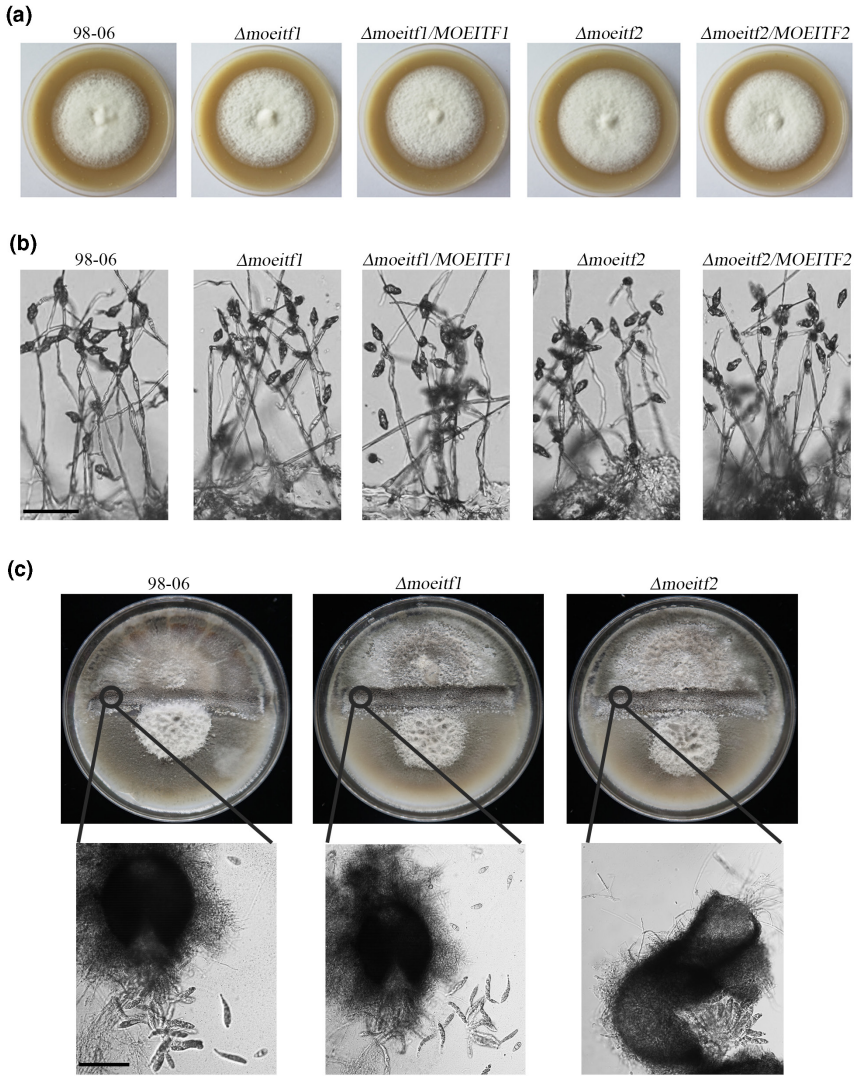

FIGURE 3 There is no alteration in the vegetative and reproductive growth of mutant Δ*moeitf1* and Δ*moeitf2*. (a) Colony morphology of each strain grown on rice bran medium for 10 days. (b) The morphology of conidia and conidiophores of each strain on the medium surface was photographed using light microscopy. The hyphal layer growing on the medium was scraped off for preparing the sample, and the medium was cut into small blocks. Then sporulation was induced by placing the blocks under continuous light for 24 h at 25°C. Size bar 50 μm. (c) Sexual reproduction-related morphology of each strain. Black perithecia appear between the two fungal colonies after 30 days of interaction between the test strain and the TH3 strain. When the perithecia are crushed, the asci and ascospores inside are visible under the microscope. Size bar 30 μm

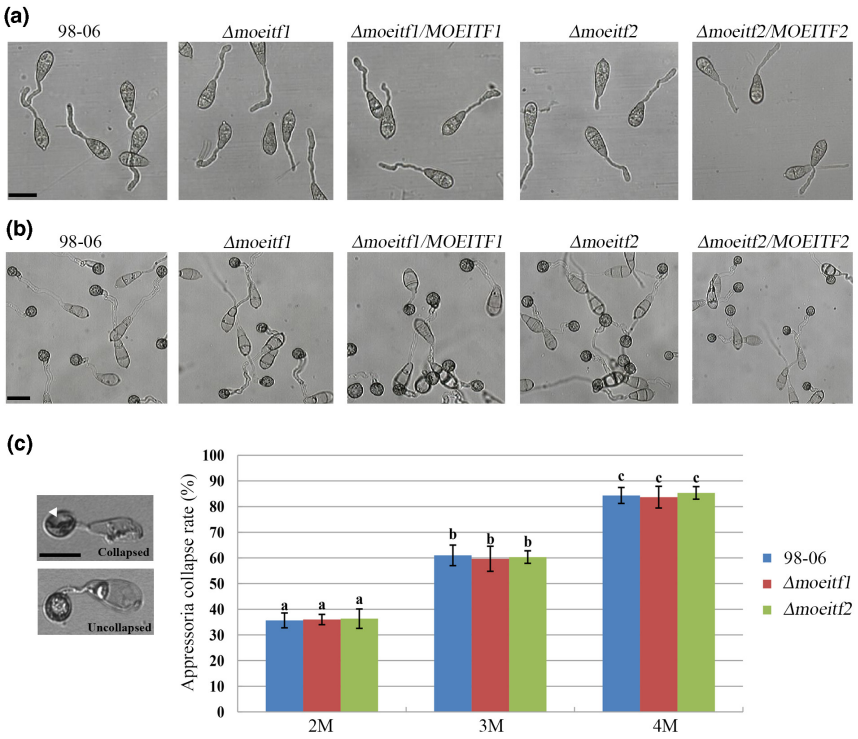

FIGURE 4 Conidial germination, appressoria formation, and turgor pressure of mutant Δ*moeitf1* and Δ*moeitf2* are normal. (a) Conidial germination was induced for 4 h on hydrophobic slide surfaces. Size bar 10 μm. (b) The development of mutant appressoria after induction on hydrophobic surfaces for 8 h. Size bar 10 μm. (c) Collapse assay tests the appressoria turgor pressure difference between the mutant and the wild-type strain. The collapse frequency of appressoria reflects this difference after treatment with different glycerol concentrations. The data come from three biological replicates, and each biological replicate was performed with three technical replicates. Size bar 10 μm. The same lowercase letters on the error bars indicate no significant differences between samples ($p > 0.05$, *t* test)

two genes contribute to *M. oryzae* infection. As shown in Figure 5a, the pathogenicity of Δ*moeitf1* and Δ*moeitf2* was significantly reduced, showing fewer and smaller lesions for the two mutants than for the wild-type strain 98-06 and the complemented strains Δ*moeitf1/MOEITF1* and Δ*moeitf2/MOEITF2*. The infection of rice sheath cells was studied to observe the mutant's infection capacities. By analysing the different infection hyphal types at 24 hpi, we observed that over 60% of mutant infection hyphae stopped developing as type 1, while fewer than 10% of type 1 was found for the wild-type strain and the complemented strains (Figure 5b). For the typical infection hyphae of types 2, 3, and 4, the mutants had lower percentages than the wild-type and complemented strains. These results confirm that *MOEITF1* and *MOEITF2* contribute to the *M. oryzae* rice infection process.

The above results also showed that the mutant could produce functional appressoria for infection, but the pathogenicity of both mutants was significantly reduced. We speculated that the reduced pathogenicity possibly resulted from not overcoming the host defences. Because the ROS burst is a common defence reaction induced by the host on infection, we used a DAB staining assay to examine whether ROS accumulated during mutant infection. As shown in Figure 5c, the infection hyphae of the mutants were surrounded by intense DAB staining, while the infection hyphae of the wild-type and complemented strains had light DAB staining. This result suggests that Δ*moeitf1* and Δ*moeitf2* are defective in overcoming host defences.

2.8 | A set of effectors were down-regulated in Δ*moeitf1* and Δ*moeitf2*

Because effector proteins of plant pathogens have essential roles in attenuating host defence reactions (Giraldo & Valent, 2013), we investigated whether the mutant inability to cope with host ROS bursts was caused by abnormal effector expression or secretion. Because the wild-type strain 98-06 has been reported to encode more than 100 predicted effectors (Dong et al., 2015), we selected 30 effectors highly expressed during 98-06 infection to test whether their expression was inhibited in the mutants Δ*moeitf1* and Δ*moeitf2*. We performed RT-qPCR assays and calculated the relative expression of these genes in mutants and 98-06. We found that the expression of 21 and 19 predicted effectors was reduced at different levels in the Δ*moeitf1* and Δ*moeitf2* strains, respectively (Tables S2 and S3). Among those, the effector *T1REP* (transcription factor 1 regulated effector protein) in Δ*moeitf1* and the effector *T2REP* (transcription factor 2 regulated effector protein) in Δ*moeitf2* were over 10-fold significantly down-regulated (Tables S2 and S3, Figure S5). These observations were corroborated in that both genes are mainly up-regulated just after penetration between 8 and 24 hpi in downloaded secondary data (Dong et al., 2015; Figure S6).

Bioinformatics analysis using SignalP showed that both T1REP and T2REP have a signal peptide (Figure S7a). DeepLoc also predicted T1REP to be located in mitochondria or plastids, and SecretomeP gave a high score for alternative secretion (Figure S7a,b). Therefore, both T1REP and T2REP are probably secreted during infection. To confirm this, we experimentally tested the T1REP and T2REP localization using red fluorescent protein (RFP) labelling. By observing the red fluorescence of 98-06 expressing T1REP-RFP or T2REP-RFP, we found that both T1REP and T2REP showed probable plant apoplast localization and a punctate accumulation at the infection hyphae forming the biotrophic interfacial complex (BIC) (Figure 6a,b), but no RFP signal was found in mycelia, conidia, or the appressorium cell (Figure S8). *T1REP-RFP* and *T2REP-RFP* were also expressed in Δ*moeitf1* and Δ*moeitf2*, respectively, and no red fluorescence was found (Figure 6a,b).

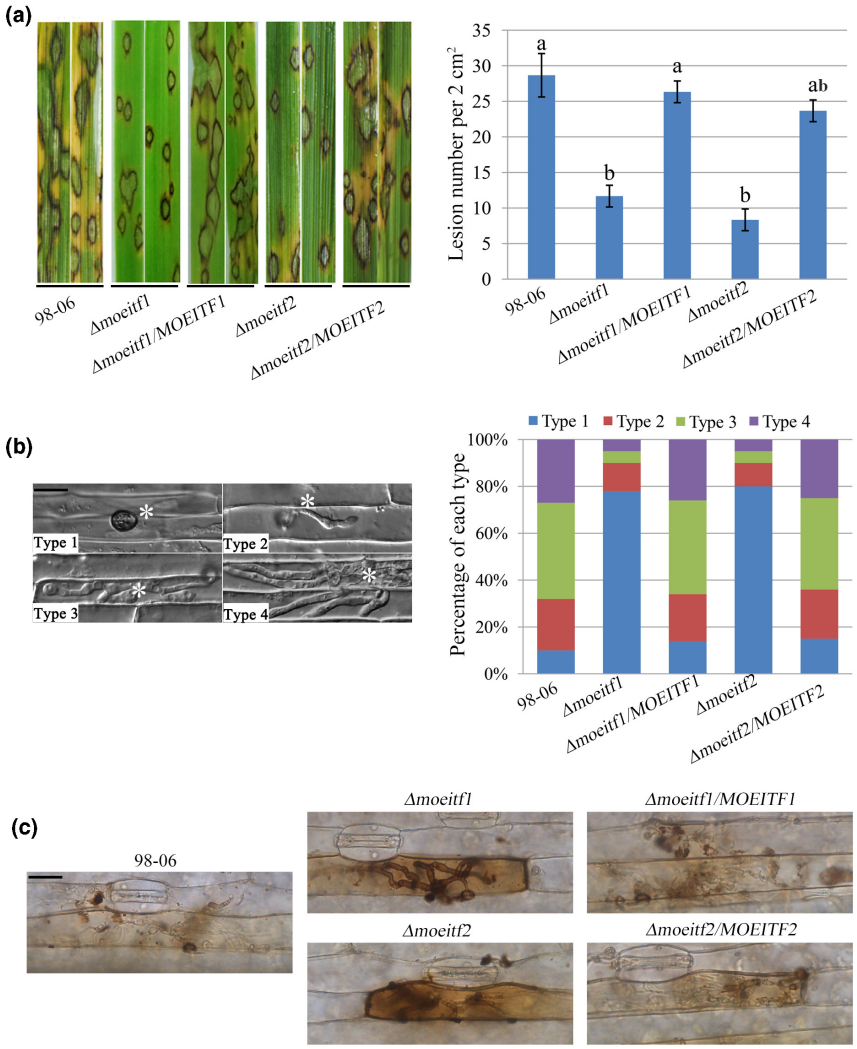

FIGURE 5 The pathogenicity of mutant Δ*moeitf1* and Δ*moeitf2* is reduced. (a) Conidial spray inoculation assay showed that the pathogenicity of Δ*moeitf1* and Δ*moeitf2* was reduced. The photographs show that the size of the disease lesions caused by the mutant were generally smaller and the number of lesions were fewer. The bar chart shows that the mutant produced fewer lesions. The data in this figure were calculated from three independent replicates. The same lowercase letters on the error bars indicate no significant differences between samples. The different lowercase letters indicate significant differences ($p < 0.05$, *t* test). (b) Conidial injection inoculation to the rice sheath showed that the early infection process of Δ*moeitf1* and Δ*moeitf2* was affected by the mutations. Most of the mutant infection hyphae remained at the type 1 stage. The percentage of different types was calculated from three biological replicates, and each of these was performed with three technical replicates. (c) 3,3′-diaminobenzidine staining assay showed that more reactive oxygen species (ROS) formed in host cells infected by the mutants, as indicated by the dark brown staining caused by the ROS. Size bar 10 μm

2.9 | The down-regulation of *T1REP* and *T2REP* could be responsible for the reduced pathogenicity of the transcription factor mutant

We used the strong promoter TrpC to drive *T1REP* and *T2REP* in Δ*moeitf1* and Δ*moeitf2*, and transformed it into both mutants to test if the down-regulation of the effectors in the respective mutants were responsible for the reduction of mutant pathogenicity. We discovered that both Δ*moeitf1/TrpC-T1REP* and Δ*moeitf2/TrpC-T2REP* could cause more disease lesions than Δ*moeitf1* and Δ*moeitf2*, respectively, although still less than found for the wild-type strain 98-06 (Figure 7a,b). Thus, overexpression of *T1REP* and *T2REP* could partially restore the pathogenicity of Δ*moeitf1* and Δ*moeitf2*, respectively, suggesting that a specific down-regulation of the effectors was mainly responsible for the reduced pathogenicity of the mutants.

To investigate whether T1REP and T2REP themselves contribute to *M. oryzae* infection, we performed a gene deletion assay and obtained two T2REP mutants, Δ*t2rep-1* and Δ*t2rep-2*, which were verified by Southern blotting (Figure 7c). Phenotype analysis showed that Δ*t2rep-1* and Δ*t2rep-2* were significantly reduced in pathogenicity (Figure 7d) but showed no alteration in vegetative growth, conidiation, conidial germination, and appressoria formation (Table S4) in comparison with the wild-type 98-06 strain. This suggested that T2REP is a virulence factor during infection. Intriguingly, we could not obtain the gene deletion mutant of T1REP even after many attempts and testing more than 400 genetic transformants, which suggests that T1REP is essential for *M. oryzae* to survive under some growth conditions and not only be active in the early infection stage.

2.10 | Moeitf1 and Moeitf2 bind with the promoter region of *T1REP* and *T2REP*, respectively

As *T1REP* and *T2REP* were significantly down-regulated in the two transcription factor mutants Δ*moeitf1* and Δ*moeitf2*, respectively, we speculated that the down-regulation of the effectors was a direct result of the deletion of the transcription factors as it would be if Moeitf1 and Moeitf2 directly controlled the *T1REP* and *T2REP* expression. We used the yeast one-hybrid assay to test whether Moeitf1 and Moeitf2 have a physical binding activity to the 1.5 kb promoter regions of *T1REP* and *T2REP*, respectively. The results showed that the yeast transformed with *Moeitf1* and the *T1REP*

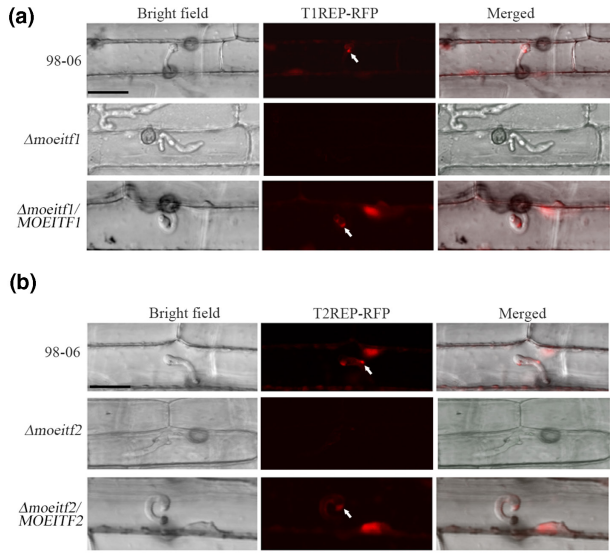

FIGURE 6 Fluorescent protein fusion and fluorescence signal observation showed that the expression of two effector proteins T1REP and T2REP is affected for mutant Δ*moeitf1* and Δ*moeitf2*, respectively. (a) Compared with the wild type, no red fluorescent protein (RFP) signal was detected in the mutant Δ*moeitf1*, indicating that the expression of T1REP is affected. Size bar 10 μm. (b) Compared with the wild type, no RFP signal was detected in the mutant Δ*moeitf2*, indicating that the expression of T2REP is affected. Size bar 10 μm. The red signals indicated by the arrow show accumulation at the biotrophic interfacial complex (BIC), the structure involved in the translocation of effectors into rice cells (Khang et al., 2010)

promoter region or transformed with *Moeitf2* and the *T2REP* promoter region could both grow normally on binding activity testing medium, while yeast that was transformed with *Moeitf1* and the *T2REP* promoter region or that transformed with *Moeitf2* and the *T1REP* promoter region could not grow on binding activity testing medium (Figure 8a,b). These results suggest that Moeitf1 and Moeitf2 can directly bind to the promoter regions of *T1REP* and *T2REP*, respectively, and regulate their expression. To further test the latter, we performed an electrophoretic mobility shift assay to check DNA binding of Moeitf1 and Moeitf2 to the 1.5 kb promoter regions of *T1REP* and *T2REP*. The results showed that Moeitf1 and Moeitf2 bound respective *T1REP* and *T2REP* promoter regions, and there were no signs of cross binding between Moeitf1 and *T2REP* or Moeitf2 and *T1REP* promoter regions (Figure 8c,d).

3 | DISCUSSION

3.1 | Transcription factors Moeitf1 and Moeitf2 specifically contribute to the early infection stage of *M. oryzae*

Our results showed that Moeitf1 and Moeitf2 are indeed typical transcription factors in that they accumulate in the nucleus (Figure 2), bind to the regulatory portions of genes (Figure 8), and regulate the genes they bind to (Figures 6 and 7). *MOEITF1* and *MOEITF2* are strongly up-regulated only during early infection, so they are not involved in appressorium formation like MoHOX7, MoLDB1, and Con7p (Kim et al., 2009; Li et al., 2010; Odenbach et al., 2007; Tang et al., 2015). They are not active during all stages of infectious growth (Mig1, Mstu1, MoHOX8, and MoMCM1) (Kim et al., 2009; Mehrabi et al., 2008; Nishimura et al., 2009; Zhou et al., 2011). Prominent stresses during the infection are light stress and oxidative stresses activating Moatf1 (Guo et al., 2010); neither Moeitf1 nor Moeitf2 seem to have roles similar to these transcription factors. Most of the mentioned transcription factors from other studies are active at several infection stages, unlike Moeitf1 and Moeitf2, which are only expressed during early infection in the biotrophic phase. The biotrophic phase appears to set the scene by regulating the genes necessary for the fungus to gain enough strength to withstand the transition to the later necrotrophic stage with plant ROS defences and lesion development, and further destruction of the plant biomass to obtain nutrients to form conidia and spread to new plants (Vargas et al., 2012).

3.2 | Effectors T1REP and T2REP are regulated explicitly by the early infection-stage transcription factors Moeitf1 and Moeitf2

During *M. oryzae* infection more than 6000 expressed genes can be detected, of which more than 800 are putative effectors (Chen et al., 2013). Given the vital role of effectors in attenuating host immunity (Jaswal et al., 2020), we speculated that the reduced infection ability of transcription factor mutants in this study might be due to the abnormal expression of pathogenicity-related effectors needed to hide the fungus from the plant innate immunity or turn off plant defences (Vargas et al., 2012). The former is likely during the early biotrophic infection phase. As we expected, the RT-qPCR results found that most of the 30 highly expressed effectors in the wild-type strain 98-06 were down-regulated in the mutants, and two of them, T1REP and T2REP, were down-regulated by more than 10-fold. The functions of T1REP and T2REP are unknown, but both are relatively small, secreted proteins, as would be expected for effectors in this infection phase. Neither of the two effectors have any known enzyme-like domains. T2REP is predicted to have a positive charge with an even number of cysteines at the C-terminus (https://aps.unmc.edu/prediction), like many antimicrobial peptides and peptide effectors (Ku et al., 2020; Lazzaro et al., 2020). Thus, T2REP could potentially interfere with the host membranes. T1REP is, on the other hand, predicted to be cationic (https://aps.unmc.edu/prediction) but also predicted to localize to mitochondria and plastids as well as potentially to become alternatively secreted (Figure S7b). Therefore, T1REP might be needed in the fungal mitochondria and also be secreted as an effector during host invasion. This would explain why we have not succeeded in deleting it. Additional evidence that both proteins are indeed effectors when regulated by Moeitf1 or Moeitf2 comes from overexpressing them in the corresponding transcription factor mutants, when the pathogenicity of the mutants was partially restored (Figure 6). Moeitf1 and Moeitf2 bound the

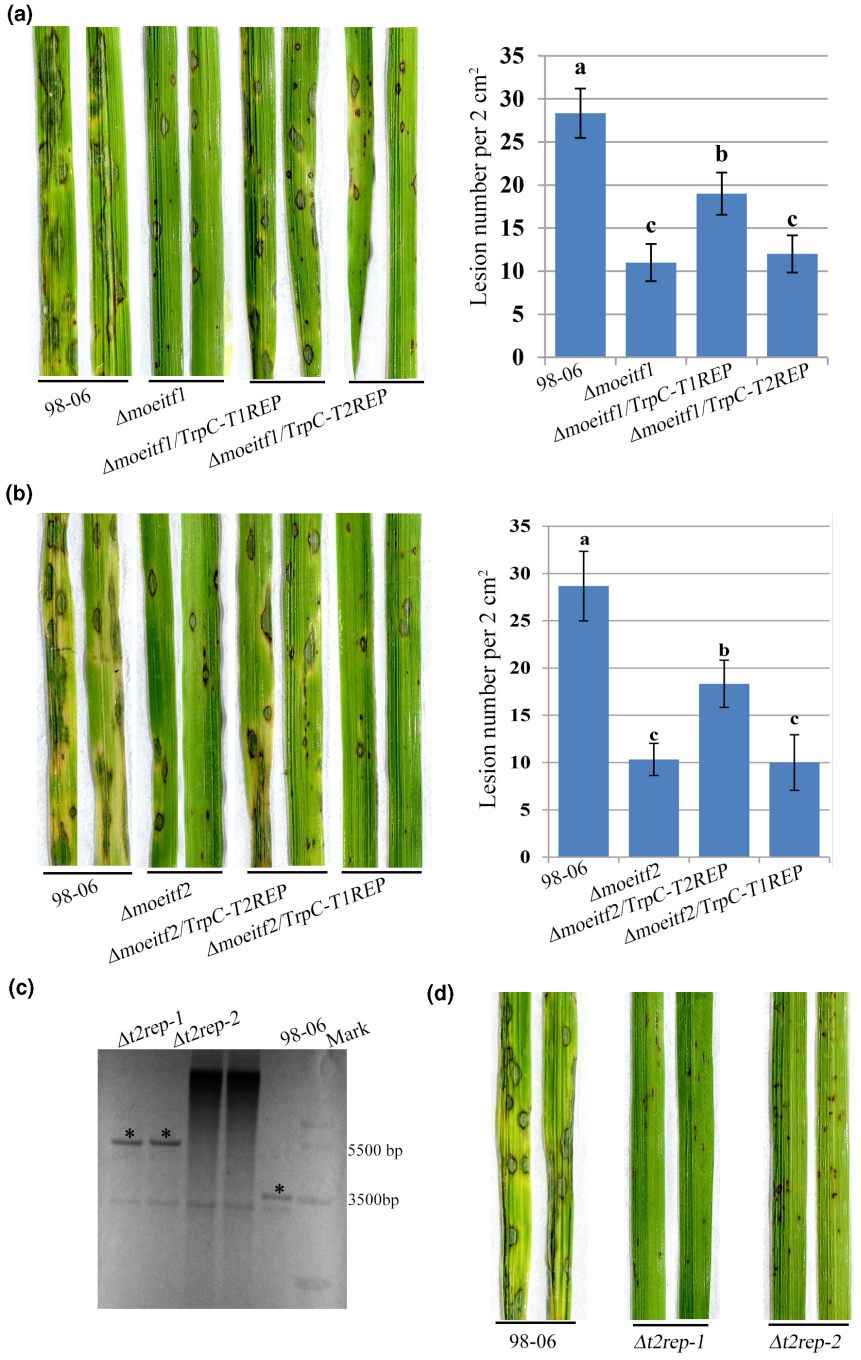

FIGURE 7 Overexpression of *T1REP* and *T2REP* partially restored the pathogenicity of Δ*moeitf1* and Δ*moeitf2*, respectively. (a) Conidial spray inoculation assay showed that the strain Δ*moeitf1*/*TrpC-T1REP* caused more lesions than Δ*moeitf1*, but still less than the wild type. (b) Conidial spray inoculation assay showed that the strain Δ*moeitf2*/*TrpC-T2REP* caused more lesions than Δ*moeitf2*, but still less than the wild type. The average lesion number on 2 cm^2 rice leaf was calculated from three biological replicates, and three technical replicates were performed for each biological replicates. The same lowercase letters on the error bars indicate no significant differences between samples. Different lowercase letters indicate significant differences ($p < 0.05$, *t* test). (c) Gene deletion verification of the T2REP mutant Δ*t2rep-1* and Δ*t2rep-2* by Southern blotting. The 780 bp segment before the target gene coding region was amplified and labelled as the hybridization probe. The genomic DNA was digested using *Sma*I, and after blotting, two bands of approximately 3500 and 5700 bp were expected to appear in the wild-type 98-06 and mutants, respectively. * indicates the target bands. (d) Conidial spray inoculation assay showed that the pathogenicity of Δ*t2rep-1* and Δ*t2rep-2* was significantly reduced

promoter regions of *T2REP* and *T1REP*, respectively, and the binding was specific for each transcription factor and effector (Figure 8).To our knowledge, this is the first discovery in *M. oryzae* that individual transcription factors specifically regulate the expression of proteins that act as effectors.

3.3 | Effectors T1REP and T2REP appear to localize to the BIC structure

During infection by rice blast fungus, multiple effectors are secreted and translocated into rice cells (Li et al., 2009; Mosquera et al., 2009; Wu et al., 2015; Yoshida et al., 2009; Zhang & Xu, 2014). Two different secretion systems have been identified in *M. oryzae* (Giraldo et al., 2013). One system uses the conserved endoplasmic reticulum to Golgi secretory pathway to secrete effectors into the extracellular space between the fungal cell wall and the extra-invasive hyphal membrane produced by the plant cells (Kankanala et al., 2007). As the effectors stay in the extracellular space, effectors secreted by this system are called apoplastic effectors (Giraldo et al., 2013). The other system is an *M. oryzae*-specific plant-derived structure, called the BIC; these effectors accumulate for later delivery into the rice cells (Giraldo et al., 2013). The effectors secreted by this system mainly go inside host cells, so they have been named cytoplasmic

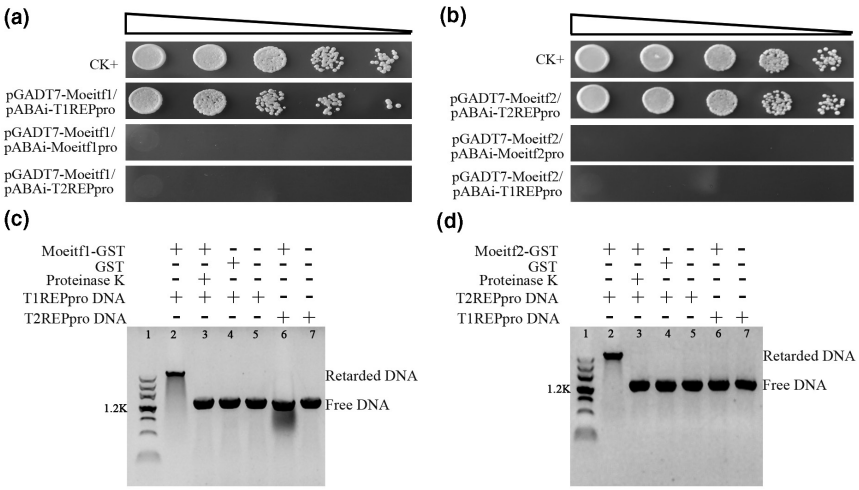

FIGURE 8 Yeast one-hybrid assay (a, b) and electrophoretic mobility shift assay (c, d) showed that Moeitf1 and Moeitf2 can bind the promoter region of *T1REP* and *T2REP*, respectively. (a) Yeast transformed with pGADT7-Moeitf1 and pABAi-T1REPpro could grow on the medium with 100 ng/ml abscisic acid (ABA). Yeast transformed with the plasmid combination pGADT7-Moeitf1/pABAi-Moeitf1pro and pGADT7-Moeitf1/pABAi-T2REPpro was used as two types of controls. (b) Yeast transformed with pGADT7-Moeitf2 and pABAi-T2REPpro could grow on the medium with 100 ng/ml ABA. Yeasts transformed with the plasmid combination pGADT7-Moeitf2/pABAi-Moeitf2pro and pGADT7-Moeitf2/pABAi-T1REPpro were used as two type controls. The positive controls in (a) and (b) are yeast transformed with p53-AbAi and pGADT7-Rec-53. (c) The purified Moeitf1-GST protein retarded the *T1REP* promoter DNA T1REPpro (lane 2), but not T2REPpro (lane 6), indicating a binding event between Moeitf1 and the *T1REP* promoter. The addition of proteinase K (lane 3) and glutathione S-transferase (GST) protein (lane 4) were used as negative controls. The samples were loaded on 1% agarose gel for electrophoresis. (d) The purified Moeitf2-GST protein retarded the *T2REP* promoter DNA T2REPpro (lane 2), but not T1REPpro (lane 6), indicating a binding event between Moeitf2 and *T2REP* promoter. The addition of proteinase K (lane 3) and GST (lane 4) was used as negative controls. The samples were loaded on 1% agarose gel for electrophoresis

effectors (Giraldo et al., 2013). Imaging a fungus expressing the fluorescently labelled cytoplasmic effector Pwl2 showed that the BICs are located at concentrated regions of infection hyphae (Giraldo et al., 2013). In our study, T1REP and T2REP also showed a similar BIC accumulation in addition to what seems to be a general localization in the plant apoplast (Figure 6a,b), suggesting that they are possibly two new cytoplasmic effectors. Because both effectors are regulated during early infection, the effect of these effectors is likely to pave the way for other effectors needed later in pathogenicity. This could explain why regulating either of these effectors by the two transcription factors Moeitf1 and Moeitf2 substantially affects overall pathogenicity. However, further experimental verification is needed to show if the proteins enter the plant cytoplasm.

3.4 | Moeitf1 and Moeitf2 as possible targets for disease control

The most economical and effective method for controlling the rice blast disease currently is to use disease-resistant rice varieties (Li et al., 2021). However, the pathogen mutates quickly under field conditions. Thus, new disease-resistant rice cultivars might lose their disease resistance within 3–5 years of planting (Zhou et al., 2007). Chemical fungicides have been widely applied to control pathogenic fungi but may cause serious adverse effects, including environmental pollution and pathogenic resistance (Cools & Hammond-Kosack, 2013; Ma & Uddin, 2009; Zhang et al., 2006); therefore, there is an urgent need to develop new disease control methods. Pathogen-specific transcription factors like Moeitf1 and Moeitf2, which regulate the effector proteins needed for infection, provide potential unique targets for developing control chemicals with limited adverse side effects. These two transcription factors are only found in ascomycetes and have no orthologs in plants and animals. Thus, chemicals specifically interfering with Moeitf1 and Moeitf2 will probably be safe for rice and people who eat rice. If chemicals could be used to interfere with the transcription factor expression and systematically reduce the pathogen's infection ability, instead of killing it, it could lead to low-level asymptomatic infections. The use of such chemicals would be expected to lead to reduced crop damage and a lower rate of evolution of new *M. oryzae* strains with an improved potential to overcome plant defences.

3.5 | Conclusion

We conclude that two early infection-induced transcription factors, Moeitf1 and Moeitf2 of *M. oryzae*, are involved in regulating infection growth but do not regulate vegetative growth, asexual/sexual sporulation, conidial germination, and appressoria formation. Mutants of Moeitf1 and Moeitf2 were defective in coping with host ROS stress development and down-regulated a set of putative effectors. Overexpression of two strongly down-regulated effectors, T1REP and T2REP, in the mutants Δ*moeitf1* and Δ*moeitf2* could partially restore the infection ability. Deletion of *T2REP* severely

weakened the fungal pathogenicity. We failed to knock out *T1REP*, possibly because it is essential for the fungus to survive. Our findings support that both T1REP and T2REP are critical effectors needed by *M. oryzae* for the infection of rice. Our binding data indicate that Moeitf1 specifically regulates T1REP and Moeitf2 specifically regulates T2REP.

4 | EXPERIMENTAL PROCEDURES

4.1 | Strains, rice plants, media, and culture conditions

The *M. oryzae* wild-type strain 98-06 (Dong et al., 2015) was used as the background for gene deletion. The susceptible indica rice cv. CO-39 was grown for 2 weeks for the spray inoculation assay. A rice bran medium, made from crushed rice seed coats and 15 g/L agar, was used to grow *M. oryzae* and induce conidial production. Oat medium (50 g/L oatmeal, 15 g/L agar) was used to perform a sexual reproduction assay (Li et al., 2015). Vegetative growth was tested by measuring the colony diameter after 10 days of growth on rice bran medium in 9-cm Petri dishes incubated at 25°C under 12 h/12 h light/dark periods. Conidial production was evaluated by flooding the 12-day-old colony with double distilled water, filtering out the mycelia by gauze, and then counting the conidia using a haemocytometer.

4.2 | RT-qPCR assay

Total RNA was extracted using Eastep Super Total RNA Extraction Kit (Promega), and 5 mg of RNA was reverse-transcribed to cDNA using the Evo M-MLV RT kit with gDNA Clean for qPCR (Accurate Biotechnology) according to the manufacturer's instructions. The resulting cDNA was then diluted 10-fold and used as the template of qPCR. qPCRs were performed using an Applied Biosystems 7500 Real-Time PCR System. Each reaction contained 25 μl of SuperRealPreMix Plus SYBR Green (Tiangen Biotechnology), 1 μl of cDNA, and 1.5 μl of each primer. The thermal cycling conditions were 15 min at 95°C followed by 40 cycles of 10 s at 95°C and 20 s at 60°C. The threshold cycle (C_t) values were obtained by analysing amplification curves with a normalized reporter threshold of 0.1. The primers used in this study are listed in Table S1.

4.3 | Transcription activity tested by yeast two-hybrid assay

Using *Eco*RI and *Pst*I, the full-length of *MOEITF1* and *MOEITF2* without intron regions were cloned into pGBKT7. The resulting plasmids were transformed with empty pGADT7 into the yeast strain AH109. Growth of yeast transformants on the test medium (SD/−Trp/−Leu/−His/−Ade) for reporter gene activation indicated that Moeitf1 or Moeitf2 activated the transcription of the yeast reporter gene. Yeast transformed with the combination of pGADT7-T/pGBKT7-53 and pGADT7/pGBKT7 served as positive and negative controls, respectively.

4.4 | Sexual reproduction assay

Strains tested were crossed with the sexually compatible strain TH3 on oatmeal medium for at least 30 days (Li et al., 2015). If the tested strains have sexual reproduction activity, black perithecia develop at the intersection of the two strains, visible to the naked eye on the agar surface. Crushing perithecia releases clavate asci and ascospores visible by microscopy (BX51; Olympus).

4.5 | Molecular manipulation

The target genes' 1 kb upstream and downstream fragments were amplified with a 15 bp adapter sequence of *HPH* (hygromycin phosphotransferase) gene to construct *MOEITF1* and *MOEITF2* gene deletion cassettes. Then, the fragments were fused with the N-terminus or C-terminus of the *HPH* gene by overlapping PCR. *MOEITF1* and *MOEITF2* gene complemented vectors were constructed using the full length of the target genes. The upstream 1.5 kb native promoter was cloned into pCB1532 between *Xba*I and *Bam*HI sites using a seamless cloning method (ClonExpress II One Step Cloning Kit). Moeitf1 and Moeitf2 localization vectors were constructed as follows. The TrpC promoter and *GFP* sequences were fused with the target gene's N-terminus and C-terminus, and then inserted into the plasmid pCB1532 between the *Xba*I and the *Bam*HI sites.

4.6 | Fungal transformation

The fungal transformation was performed using the polyethylene glycol-mediated protoplast transformation method (Li et al., 2016). The protoplast cells were prepared as described previously (Li et al., 2019), then the DNA was introduced to the protoplasts. For gene deletion assay, at least 2 μg of gene deletion cassette DNA was transformed into the wild-type strain 98-06, and the transformants were screened on TB3 medium (6 g/L casamino acids, 6 g/L yeast extract, 200 g/L sucrose, 15 g/L agar) with 250 μg/ml hygromycin. Southern blotting was conducted to verify which transformants had successfully replaced the target genes with the *HPH* deletion construct using a digoxigenin high prime DNA labelling and detection starter kit I (Roche). Southern blotting was used for verifying the knockout of *MOEITF1* and *MOEITF2*. The 800 bp segment before the target gene coding region was amplified and labelled as the hybridization probe. To verify *MOEITF2* knockout, *Nhe*I and *Spl*I were used to digest the genomic DNA, and after blotting two bands of approximately 2700 bp and 3100 bp were expected to appear in the wild type and mutants, respectively. To verify the *MOEITF1* knockout,

*Pst*I and *Dra*I were used to digest the genomic DNA, and after blotting two bands of approximately 2100 bp and 1800 bp were expected to appear in the wild type and mutants, respectively.

At least 5–10 μg of complementation vector DNA was transformed into target gene mutants for gene complementation. The transformants were screened on basal medium (Yang & Naqvi, 2014) supplemented with 50 μg/ml chlorimuron-ethyl (Sigma Aldrich). The gene complementation transformants were verified by showing phenotypes that resemble the wild-type strain 98-06. Using the same plasmid for constructing vectors, the method for effector overexpression transformation was similar to that of the gene complementation transformations.

4.7 | Conidial germination, appressoria formation, and pathogenicity assay

Conidial germination assay and appressoria formation assay were performed by incubating conidial suspensions of 5×10^4 spores/ml on a hydrophobic surface in a sealed humid environment at 25°C for 4 and 8 h, respectively (Li et al., 2014). Conidial germination rate and appressoria formation rate were calculated by counting the percentage of germinated conidia and appressoria-forming conidia.

A sprayer pump bottle was used for conidial inoculation of 10 2-week-old rice seedlings with 5 ml of conidial suspension adjusted to 5×10^4 spores/ml. The conidial suspension was evenly sprayed onto the seedlings. The inoculated plants were incubated at 25°C for 24 h in a controlled environment chamber with 90% humidity and then moved to a standard rice-growing environment for another 4–5 days until disease lesions appeared. The pathogenicity of different strains was evaluated by counting the number of lesions and comparing their sizes. Injection inoculation was performed by injecting the prepared conidial suspension into rice sheath cavum taken from 21-day-old plants. The injected sheaths were then incubated for 24 h at 80% humidity. After that, the inner sheath surfaces were peeled and made into slide samples to observe infection hyphal growth by microscopy. The infection hyphae were grouped into four types to evaluate the infection ability: type 1, a small infection peg formed; type 2, the small infection peg begins hyphae-like growth; type 3, the infection hyphae fill the first infected host cell; type 4, the infection hyphae spread to the neighbouring host cell.

4.8 | Appressoria collapse assay

The appressoria collapse assay was performed as described previously (Li et al., 2016) to test whether the appressoria turgor pressure was normal. As a high glycerol concentration generates the appressoria turgor pressure, they were treated with exogenous glycerol to observe if they collapsed. Conidial suspension drops, 10 μl each, were placed on hydrophobic slides and incubated, as described above, for 24 h at 25°C to allow appressoria maturation. Then the covering water was carefully removed and replaced with an equal volume of 2, 3, or 4 M glycerol solution. After incubation for another 15 min, the ratio of collapsed to normal-looking appressoria was determined using microscopy. A high ratio of collapsed appressoria at a low glycerol concentration indicates low appressorial turgor pressure.

4.9 | DAB staining

The DAB staining to indicate host ROS formed during *M. oryzae* infection was performed as described previously (Li et al., 2019). A conidial suspension of 5×10^4 spores/ml was sprayed onto 2-week-old barley and incubated for 24 h. The inoculated leaves were plucked and placed in 1 mg/ml DAB solution for 8 h at room temperature. Then the samples were soaked in a washing solution (ethanol:acetic acid 94:4, vol/vol) for 2–3 h. The ROS are detected as dark brown precipitates visible in the infected host cells when observed under a microscope.

4.10 | Yeast one-hybrid assay

The yeast one-hybrid assay (Zhang & Xu, 2014) was used to check whether the target transcription factor can bind to the promoter region of the tested effector genes. First, we amplified the full-length coding sequence of the target transcription factor and cloned it into the pGADT7 vector using *Eco*RI and *Bam*HI restriction enzymes. Subsequently, an approximately 1.5 kb sequence of the promoter region of the effector was amplified and cloned into the pAbAi plasmid using the seamless ligation kit as mentioned above. Then, the obtained plasmids above were cotransformed into the yeast strain Y1H Gold. After obtaining the transformants, we checked whether the transformants could grow on a medium containing 100 ng/ml abscisic acid. The transformed yeast containing the combination of the two plasmids, p53-AbAi and pGADT7-Rec-53, served as a positive control. The crossover combination of two target transcription factors and two tested effectors was used as a negative control.

4.11 | Electrophoretic mobility shift assay

The *MOEITF1* and *MOEITF2* cDNA sequences were amplified and cloned into prokaryotic expression vector pGEX-KG, respectively, containing a C-terminal glutathione S-transferase (GST) tag. The resulting Moeitf1-GST and Moeitf2-GST proteins were expressed by *Escherchia coli* BL21 and purified using glutathione magarose beads (Smart Lifesciences). The 1.5 kb putative promoter region DNA (0.1 μg) of *T1REP* and *T2REP* was amplified and incubated with the purified Moeitf1-GST and Moeitf2-GST (0.1 μg), respectively, for 20 min at 25°C. Then 1% agarose gel electrophoresis was performed to test whether the promoter DNA could be retarded due to binding the corresponding protein. The addition of GST and proteinase K worked as negative controls.

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CONFLICT OF INTEREST

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available within the paper and its supplements.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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