Protein kinase Ime2 is associated with mycelial growth, conidiation, osmoregulation, and pathogenicity in *Fusarium oxysporum*

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Abstract

*Fusarium oxysporum* f.sp. *niveum* is one of the most serious diseases impairing watermelon yield and quality. Inducer of meiosis 2 (Ime2) is the founding member of a family of serine/threonine protein kinases and plays important roles in yeasts and other filamentous fungi. In this study, we analyzed the functions of *Folme2*, the ortholog of *Saccharomyces cerevisiae* Ime2 in *F. oxysporum* f.sp. *niveum*. The *Folme2*-deleted mutants exhibited obvious morphological abnormalities, including slower vegetative growth, more branches in the edge hyphae and a reduction in conidia production. Compared to the wild type, the mutants were hypersensitive to the osmotic stressor NaCl but were more insensitive to the membrane stressor SDS. The deletion of *Folme2* also caused a reduction in pathogenicity. Transcriptional analysis revealed that *Folme2* acts downstream of *FoOpy2* which is an upstream sensor of the MAPK kinase cascade. These results indicate that *Folme2* is important in the development and pathogenicity of *F. oxysporum*, and provide new insight for the analysis of the pathogenic mechanism of *F. oxysporum*.

Keywords  *Fusarium oxysporum* · MAPKs · Protein kinase · Ime2

Abbreviations

- **Fon**  *Fusarium oxysporum* f.sp. *niveum*
- **Fon-1**  *Fusarium oxysporum* f.sp. *niveum* race1
- **Ime2**  The inducer of meiosis 2
- **MAPK**  Mitogen-activated protein kinase
- **PDA**  Potato dextrose agar medium
- **PDB**  Potato glucose liquid medium dextrose broth
- **SNA**  Synthetic low-nutrient agar medium
- **DI**  Disease index
- **WT**  Wild type strain
- **CR**  Congo red

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**Introduction**

The soil-borne, asexual fungus *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *niveum* (E.F. Sm.) W.C. Snyder & H.N. Hans (Fon), is the dominant pathogen of Fusarium wilt on watermelon (*Citrullus lanatus*) worldwide, causing adverse impacts on watermelon yield and quality (Hudson et al. 2021). To adapt to saprophytic and parasitic environments, *F. oxysporum* needs to sense and respond to numerous stimuli from their environment, including their host organisms (Kou and Naqvi 2016). Molecular perception of these stimuli is regulated by a large number of different protein kinase channels, of which mitogen-activated protein kinase (MAPK) cascades are among the most important and highly conserved members (Vandermeulen and Cullen 2020; Wu et al. 2019). Most ascomycete fungi including *F. oxysporum* possess only three MAPKs, Fmk1, Mpk1, and Hog1, which are orthologous to yeast Fus3/Kss1, Mpk1, and Hog1, respectively (Martinez-Soto and Ruiz-Herrera 2017; Pareek and Rajam 2017; Segorbe et al. 2017; Turra et al. 2014). Moreover, the inducer of meiosis 2 (Ime2) homolog pathway is believed to be a new MAPK pathway in several fungi (Xie et al. 2019). Ime2 belongs to the serine/threonine protein kinase family and is conserved in all eukaryotes. A major characteristic of these kinases is their similarity in the N-terminal
region, which includes a TXY motif, typically found in the activation loop of MAPKs (Irniger 2011). Ime2 was first identified as a gene expressed exclusively during meiosis in *S. cerevisiae* (Smith and Mitchell 1989; Schindler and Winter 2006). Subsequent studies demonstrated that it also plays key roles in sporulation and pseudohyphal growth in yeast (Sari et al. 2008; Strudwick et al. 2010). During the last few years, Ime2-related protein kinases from various fungal species have been studied. Surprisingly, these protein kinases exhibited striking diversity in their cellular functions. In *Aspergillus nidulans*, the Ime2 homologous protein IMEB does not participate in meiosis, but functions as an inhibitor of sexual development in the presence of light. IMEB also promotes asexual reproduction, and regulates the production of secondary metabolites in *A. nidulans* (Bayram et al. 2009). For *Neurospora crassa*, Ime2 is needed for the inhibition of protoperithecia formation in response to the availability of sufficient nitrogen (Hutchison and Glass 2010). Ime2 is also reported to be a negative regulator of the cell death pathway to specifically regulate nonself recognition and cell death in *N. crassa* (Hutchison et al. 2012). The analysis of the Ime2 homolog Crk1 in the phylum of the basidiomycete suggests that this protein kinase is involved in the regulation of mating. However, it negatively affects mating in *Cryptococcus neoformans* but promotes mating by regulating the expression of the *prf1* gene in *Ustilago maydis* (Garrido et al. 2004; Liu and Shen 2011). In *Trichoderma reesei*, Ime2 is involved in the degradation process of the plant cell wall: deletion of the Ime2 homologous genes results in significantly upregulated expression of cellulase *cbh1*, *cbh2*, and *eg1* (Chen et al. 2015). In the nematode predatory fungus *Arthrobotrys oligospora*, the absence of Ime2 results in slow growth, reduced sporulation, loose cell wall structure, reduced trapping structure, and lower nematode capture quantity than that of the wild type (Xie et al. 2019). Thus, Ime2-related kinases exhibit an amazing variety of functions in controlling cellular processes in fungi.

To date, the functions of the protein kinase Ime2 in *F. oxysporum* have not been reported. In this study, we identified homologs of *S. cerevisiae* Ime2 in *F. oxysporum*. The functionalities of *FoIme2* in *F. oxysporum* were investigated by constructing deletion mutants. Compared to the wild type, the *FoIme2*-deleted mutants exhibited obviously decreased mycelial growth and conidiation production, and more branches in the edge hyphae. The mutants were hypersensitive to the osmotic stressor NaCl but more insensitive to the membrane stressor SDS. The deletion of *FoIme2* also caused a reduction in pathogenicity. Our results suggest that *FoIme2* is a general regulator of morphogenesis and virulence in *F. oxysporum*.

### Materials and methods

#### Fungal strains and culture conditions

The wild type strain *F. oxysporum* f.sp. *niveum* races 1 (Fon-1) was isolated from the roots of symptomatic field-grown plants in Changsha, China. Race determination was performed on watermelon differentials Black Diamond, Charleston Gray, and Calhoun Gray (Fulton et al. 2021). Fon-1 was used as the parental strain for the transformation experiments and was maintained as a stock culture in our lab. Potato dextrose agar (PDA) was used as the routine medium for wild type cultures. PDA supplemented with 200 μg/ml neomycin (Solarbio, Beijing, China) was used for subculturing deletion mutants. Potato dextrose broth (PDB) was used for fungal mycelia and conidia production or DNA and RNA extraction. All cultures were grown at 28 °C. All fungal isolates were purified as single spore cultures and stored at −80 °C in a 30% (v/v) glycerol solution.

#### Sequence analysis of *FoIme2* in *F. oxysporum*

The *FoIme2* gene of *F. oxysporum* was originally identified through the homology searches of the *F. oxysporum* genome sequence, using the BLASTX algorithm and the sequences *S. cerevisiae* Ime2 protein sequence as the query.

#### Construction of *FoIme2*-deleted mutants

Target gene deletion was carried out by replacing the *FoIme2* gene (GenBank number: MAMH01000468.) with a neomycin resistance marker cassette using the split-marker methodology. The vector pKN- *FoIme2*-KO was created to generate two overlapping gene deletion constructs. All primers are listed in Table 1. A 1597 bp upstream fragment and a 1365 bp downstream fragment of the target gene were amplified from the genomic DNA of the WT strain with the primer pairs IU*-SacI-F*/IU*-NotI-R* and ID*-SpeI-F*/ID*-ApaI-R*, respectively. The upstream fragment was digested using the restriction enzymes *SacI*/NotI and inserted into the restriction sites *SacI*/NotI of pKN vector containing *neo* (encoding neomycin phosphotransferase) to produce the vector pKN-*FoIme2*-FS. Next, the downstream fragment was digested using the restriction enzymes *SpeI*/ApaI and inserted into the restriction sites *SpeI*/ApaI of pKN to produce vector pKN-*FoIme2*-KO. The first fragment contained the upstream sequence of *FoIme2*, and the 3’ end (approximately 75%) of the neomycin cassette, was obtained by amplification with the specific primer pair IU*-SacI-F*/Neo-R*. The second fragment contained the terminator region of
the target gene and the 5′ end (approximately 75%) of the neomycin cassette was obtained by amplification with the specific primer pair Neo-F/ID-Apal-R.

PEG-mediated transformation was performed as described previously (Di Pietro and Roncero 1998). The transformants were cultured in PDA plates supplemented with 200 μg/ml neomycin and grown at 28 °C. To identify the gene-deleted mutants, the resulting transformants were screened by PCR with the primer pairs Neo-check-F/Neo-check-R, and Ime2-check-F/Ime2-check-R (Table 1) which were used to identify the neomycin resistance and FoIme2 gene, respectively. The deletion mutants were further confirmed by Southern blot analysis of EagI-digested genomic DNA using a PrF/PrR (Table 1) PCR-amplified 523 bp fragment as the probe.

### Table 1 All primers used in the study

| Primers       | Sequence(5′–3′)                                                                 | PCR purpose                                                                 |
|---------------|--------------------------------------------------------------------------------|----------------------------------------------------------------------------|
| IU- SacI-F    | CGAGCTCTTTTGTGCTCTACGGAGT        | Amplify the left homologous arm of foime2                                   |
| IU- Nol-R     | ATGAATGCGAGCGCGATGTTGCG         | Amplify the right homologous arm of foime2                                  |
| ID-SpeI-F     | GACGAGGTCGTCGTCCGGAGGG          | Amplify the neo-cassettes and downstream                                      |
| ID-ApaI-R     | AGCCAAACCTATGTGATACGCCGCCTCG   | Amplify the upstream and neo-cassettes                                       |
| Neo-F         | ACATATTACGGTGGTTGCTGGGTCTGG    | Amplify the 1665 bp fragment of Neo                                          |
| Neo-R         | TGATCGGGGAGAATATGAGGCCGATG     | Amplify the 1116 bp fragment of foime2                                       |
| Neo-check-F   | AGCCAACTCTGTCGACGAGT            | Amplify the 523 bp probe for Southern blot                                   |
| Neo-check-R   | TAATAGCGAGCAGAGAGAGGG          | Detecting the expression of FoOpy2                                           |
| Ime2-check-F  | GACGAGGTCGTCGTCCGGAGGG          | Detecting the expression of FoSho1                                           |
| Ime2-check-R  | AGCCAACTCTGTCGACGAGT            | Detecting the expression of FoSte50                                          |
| PrF           | ATGCTACGGTGGGAGGAGGGCatG       | Detecting the expression of FoSte12                                          |
| PrR           | ATGCTACGGTGGGAGGAGGGCatG       | Detecting the expression of FOXG_12855                                      |
| C1-F          | CCCCTGCTTCTCCAGGAGG           | Detecting the expression of FOXG_13111                                      |
| C1-R          | CTTCCTCACCTCCTCCGAGG          | Detecting the expression of FOXG_16880                                      |
| C2-F          | CGACACGGCAAGTAGAGCATG         | Detecting the expression of FOXG_14504                                      |
| C2-R          | TGACTCCAGGACGAGGACAGG         | Detecting the expression of Actin                                            |
| C3-F          | GACTCTGACGGTGGTCTGGG          | Detecting the expression of EF2                                               |
| C3-R          | CGCGATCCGACGAGGACAGG         | Detecting the expression of FOXG_14504                                      |
| C4-F          | GATCTCAGGACGAGGACAGG         | Detecting the expression of FOXG_14504                                      |
| C4-R          | AGAGCGTTGCGAAGCAGGAGG         | Detecting the expression of FOXG_12855                                      |
| Actin-F       | ATGTGACCCACTTCACCTCACC        | Detecting the expression of FOXG_12855                                      |
| Actin-R       | CTCTCGTCGTACTCTCGT           | Detecting the expression of Actin                                            |
| EF-F          | CATCGCCACGGCTGAACACTCT        | Detecting the expression of FOXG_12855                                      |
| EF-R          | AGAACCAGGGCCTACTTGGAAAA       | Detecting the expression of FOXG_12855                                      |

“—” Represents the restriction site or guard base

### Mycelial growth and conidiation

To determine the mycelium growth rate, mycelial plugs of wild-type or mutant strain (5 mm diameter) from the edge of a 5-day-old colony were transferred to potato PDA containing the corresponding antibiotics, and grown in the dark at 28 °C. Each strain was represented by three replicate plates. After 3 days, the colony diameter in each plate was measured and the average diameter was calculated in each group. These experiments were repeated four times.

For the conidiation assay, three mycelial plugs (5 mm diameter) from the edge of a 5-day-old colony were transferred to a flask containing 50 ml of PDB (150 rpm, 28 °C). After 4 days, microconidia were counted with a hemocytometer. Each strain was represented by three
replicate flasks. The spores were imaged through a microscope, to detect changes in the conidium morphology. The experiment was repeated three times.

Synthetic low-nutrient agar medium (SNA), containing (in w/v) 0.1% KH₂PO₄, 0.1% KNO₃, 0.05% MgSO₄·7H₂O, 0.05% KCl, 0.02% glucose, 0.02% sucrose, and 2% agar, was used to induce macroconidia and chlamydo-spores. Agar blocks (5 mm in diameter) carrying mycelia of wild-type or mutant strains were inoculated onto SNA and incubated at 28 °C for 7 days under continuous black–blue light. The conidiation was observed with a light microscope (Nguyen et al. 2019).

Sensitivity to compounds causing osmotic, cell wall, cytoplasm membrane stress or oxidative stress

Mycelial plugs (5 mm diameter) taken from the edge of a 5-day-old colony were grown in the dark at 28 °C in 5-cm-diameter Petri plates (each plate contains one Fon-1 wild type plug, one ΔFolme2-2 plug and one ΔFolme2-19 plug) containing PDA amended with different concentrations of NaCl, KCl, SDS, Congo red (CR), and H₂O₂ for 3 days. The diameter of the colonies were measured every 24 h. The percentage of mycelial radial growth inhibition (RGI) was calculated using the formula RGI = [(C−N) / (C−5)] × 100%, where C is the colony diameter of the control and N is that of a treatment. Each combination of strain and stressor was represented by three replicate plates, and the experiment was repeated three times.

RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from the mycelia of each sample using RNA-easy™ Isolation Reagent (Vazyme Biotech, Nanjing, China). One microgram of each RNA sample was used for reverse transcription with a HiScript® II 1st Strand cDNA Synthesis Kit (+ gDNAwiper) (Vazyme Biotech, Nanjing, China). The expression levels of FoOpy2, FoSho1, FoSte50, FoSte12 and four cellulase genes (FOXG_12855; FOXG_13111; FOXG_16880; FOXG_14504) under induction in PDB medium were determined by quantitative real-time reverse transcriptase PCR (qRT–PCR) with the primers listed in Table 1. For each sample, the F. oxysporum Actin and EF2 genes were used as reference sequences. The analysis of relative gene expression levels using the qRT–PCR data was performed according to the 2^−ΔΔCT method described previously (Livak and Schmittgen 2001). The experiment was repeated three times.

Plant material and inoculation

“Zaojia 8424” [Citrullus lanatus (Thunb.) Matsum. and Nakai., Xinjiang Academy of Agricultural Sciences, China], a commercial watermelon variety that is susceptible to race 1 of FON, was used for this study. Watermelon germinating seeds were planted in soils that were sterilized and inoculated with conidial suspensions (conidial content of 1 × 10⁴ conidia/g) and maintained in a growth chamber (14 h light at 28 °C/10 h dark at 24 °C). The disease severity of each plant was evaluated based on external yellowing and wilting scores as follows: 0, no symptoms; 1, slight yellowing of cotyledons; 2, yellowing and wilting of cotyledons; 3, yellowing of cotyledons and true leaves; 4, yellowing and wilting of all leaves except the heart; and 5, wilting of all leaves or plant death. The disease index (DI) was calculated using the formula DI = Σ(Xi*n)/5*N×100%, where N is the number of investigative watermelon seedlings for each strain; Xi is the disease score of strain and stressor was represented by three replicate flasks, and the experiment was repeated 3 times.

Results

Identification of ime2 from F. oxysporum

Folme2 (GenBank number: MAMH01000468.) was originally predicted by BLAST analysis of the model fungi S. cerevisiae and N. crassa. Ime2 proteins against the F. oxysporum f. sp. niveum genome sequence (available at https://www.ncbi.nlm.nih.gov/genome/707?genome_assembly_id=280512). The full Folme2 gene in F. oxysporum is 2412 bp long, contains two 57 bp introns and encodes a protein with 765 amino acids. The search for conserved domains against the NCBI Conserved Domain program revealed that Folme2 contains the conserved catalytic domain of S_TKc associated serine/threonine kinases (residues 24–352).

Deletion of Folme2 in F. oxysporum

To understand the biological functions of Folme2 in F. oxysporum, we generated gene-deletion mutants using a fusion PCR-based deletion strategy (Fig. 1a). After PCR screening (Fig. 1b), two stable mutants were used for Southern blot analysis.

Southern blot analysis used a 523 bp fragment located upstream of Folme2 as the probe. When probed, the deletion mutants produced an anticipated 2669 bp band, whereas the wild-type strain produced an anticipated 5649 bp band.
The genetically stable gene deletion transformants for *F. oxysporum* wild-type strains were named ΔFoIme2-2 and ΔFoIme2-19, respectively. Deletion of *FoIme2* affects mycelium growth and conidial yield.

Significant reductions were observed in the growth of two independent mutants (ΔFoIme2-2 and ΔFoIme2-19) in comparison to the wild-type (Fon-1) colonies. Furthermore, when incubated on PDA at 28 °C for a long time (more than 10 days), the edge hyphae of the mutants colonies were more likely to produce many more branches than those of WT colonies (Fig. 2a). The macroconidia produced by the mutants were not different in morphology from those produced by Fon-1. However, a significantly reduced number of microconidia was produced by mutants in PDB (Fig. 2c).

**FoIme2 contributes to the stress response in *F. oxysporum***

The *FoIme2* mutants displayed decreased resistance to extracellular osmotic (NaCl) stress and increased resistance to membrane (SDS) stress compared to the wild-type strain. However, no differences in the tolerance to intracellular osmotic (KCl), cell wall (CR), and oxidative (0.08%H₂O₂) stress were identified between the mutants and the wild-type strain (Fig. 3a, b).

**FoIme2 is essential for the full virulence of *F. oxysporum***

The virulence of the *FoIme2*-deleted mutants was determined by plant infection assays. Watermelon planted in soils inoculated with wild type or mutant strains all showed characteristic wilt symptoms. However, the mutants exhibited impaired pathogenicity due to a significant delays in initial symptom appearance (Fig. 4a, b). The results demonstrated that FoIme2 is essential for the potency of pathogenicity in *F. oxysporum*. 
Discussion

In *S. cerevisiae*, Ime2 was first identified as a gene expressed exclusively during meiosis which was initiated by the transcriptional activator Ime1 (Smith and Mitchell 1989). The sexual reproduction period of *F. oxysporum* is currently unknown and the homologous genes of *ime1* are absent in the *F. oxysporum* genome. In this study, we identified the function of the protein kinase Ime2 in *F. oxysporum*, which regulates mycelium growth, microconidia development, extracellular osmotic and membrane integrity. These results suggested that Ime2 may play more roles in addition to meiosis in fungi whose sexual reproduction is not important for their life-cycle stage.

Significant reductions in the growth of two independent *FoIme2-deleted* mutants was observed. The hyphal morphology of the mutants was different from that of the wild type. This result agrees with what has been observed with the ime2 mutants of several filamentous fungi. In *Nomuraea rileyi*, *A. oligospora*, and *U. maydis*, the deletion of the *ime2 homologous gene severely affected the growth rate of the strain and the filamentous growth (Garrido et al. 2004; Li 2018; Xie et al. 2019). The phenotype of *imeB* mutants in *A. nidulans* also showed a reduction in vegetative growth (Bayram et al. 2009).

A previous study showed that the filamentation of *F. oxysporum* was regulated by Fmk1 MAPK cascades (Di Pietro et al. 2001; Segorbe et al. 2017). *FoOpy2*, *FoSho1*, *FoSte50*, and *FoSte12* are participants in Fmk1 MAPK cascades. The transmembrane proteins Opy2 and Sho1 are upstream sensors of the Ste50-Ste11-Ste7-Fmk1-Ste12 kinase cascade in *S. cerevisiae* and other fungi (Gu et al. 2015; Guo et al. 2017; Herrero de Dios et al. 2013; Rispail and Di Pietro 2010; Sharmeen et al. 2019; Takayama et al. 2019; Yamamoto et al. 2010). Here we compared the transcript levels of genes whose levels of transcription were reported to be regulated by the transcription factor *FoSte12* by quantitative real-time reverse transcriptase PCR (Gu et al. 2015). The results showed that the transcript levels of *FoOpy2* and three cellulase
genes (FOXG_14504; FOXG_13111; FOXG_16880) were increased dramatically, but the transcript levels of other genes did not change significantly, in ΔFolme2 (Fig. 5). These results indicate that Folme2 may act downstream of FoOpy2 and function in parallel of FoSte12. Deletion with Folme2 induced overexpress of FoOpy2 through negative feedback regulation, and the overexpression FoOpy2 increased the phosphorylation level of the downstream kinase Ste12, which eventually resulted in increased expression of two cellulase genes. The analysis agrees with what was reported in U. maydis, where the Ime2 homolog Crk1 is regulated by phosphorylation of both Fuz7 (the Ste7 homolog) and Kpp2 (the Fus3/Kss1 homolog), and it acts in the signal transduction pathway both in parallel to Kpp2 and as a substrate of this MAPK (Garrido et al. 2004). The process reported above is similar to what was shown in T. reesei, where the deletion of Ime2 homologous genes results in significantly upregulated cellulose expression (Chen et al. 2015).

We also recorded a notable decrease (relative to WT) in the number of conidia in the Folme2-mutants, however the mutation did not affect the conidial morphology. Our results are similar to the Nrime2 mutant in N. rileyi: deletion of Nrime2 delayed the sporulation time but did not affect conidial morphology (Li 2018). However, different results were reported in the case of the deletion of Aolme2...
in *A. ligoospora*, the deletion of Scime2 in *S. cerevisiae* and the deletion of Sppt1 and spnde3, the two Ime2 homolog in *Schizosaccharomyces pombe*, which suggested that Ime2 plays an important role in conidial production and conidial morphology (Abe and Shimoda 2000; Schindler and Winter 2006; Xie et al. 2019).

The *FoIme2*-mutants displayed decreased resistance to extracellular osmotic (NaCl) stress and increased resistance to membrane (SDS) stress compared to the wild-type strain. However, no differences in tolerance to intracellular osmotic (KCl), cell wall (CR) and oxidative (H2O2) stress was identified. This is similar to the deletion of *AoIme2* in *A. ligoospora*: *AoIme2* mutants were inhibited under hyperosmotic stresses but were unaffected by oxidative and cell-wall-perturbing stresses. In the case of *N. rileyi*, the *Nrime2* gene was not affected by oxidative and cell-wall-perturbing stresses. In the case of *N. rileyi*, the *Nrime2* homolog also plays a crucial role in environmental adaptation in *U. maydis* (Garrido and Pérez-Martín 2004). These results indicate that *Ime2* is involved in the adaptation to high osmolarity stress in *F. oxysporum* and other fungi. In *S. cerevisiae*, the interaction between the transmembrane protein OpY2 and Sho1 enhances the signalling efficiency of the Hog1 MAP kinase cascade, which regulates the high osmosality stress response (Takayama et al. 2019; Yamamoto et al. 2010). The different appearances of the *FoIme2*-mutants and the wild type in the presence of osmotic pressure may be caused by the *FoOPY2* overexpression in the mutants.

Despite the high expression levels of some cellulase genes, *FoIme2* mutants displayed decreased virulence partly due to mycelial vegetative growth defects and decreased conidial yield. In the nematode-trapping fungi *A. ligoospora*, *AoIme2* mutants displayed defects in capturing and infecting nematodes due to fewer traps, a fungal part that is essential to capture and infect nematodes. In *N. rileyi*, *Nrime2* deletion was also reported to severely affect virality (Li 2018), and crk1 in *U. maydis* was required for pathogenicity (Garrido et al., 2004). These results indicate that *Ime2* plays a key role in fungal pathogenicity.

**Conclusions**

In summary, our data indicate that the protein kinase *FoIme2* plays a significant role in the hyphal growth, hyphal branching, conidiation, stress response and pathogenicity of *F. oxysporum*. However, how *FoIme2* exerts these functions, and the roles of *FoIme2* in the MAPK system, require further study.

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**Declarations**

**Conflict of interest** The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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