A Novel Maize MicroRNA Negatively Regulates the Resistance to Fusarium Verticillioides

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Abstract

Despite miRNAs regulate the defense response against multiple pathogenic fungi in diverse plant species, few efforts had been devoted to deciphering the involvement of miRNA in resistance to *Fusarium verticillioides* (*F. verticillioides*), a major pathogenic fungal in maize production. In this study, we discovered a novel *F. verticillioides*-responsive miRNA designated zma-unmiR4 in maize kernels. The expression levels of zma-unmiR4 were significantly repressed in the resistant maize line but induced in the susceptible lines upon *F. verticillioides* exposure, whereas its target gene *ZmGA2ox4* exhibited the opposite pattern of expression. Heterologous overexpression of *zma-unmiR4* in *Arabidopsis* resulted in enhanced growth and compromised resistance to *F. verticillioides*. By contrast, transgenic plants overexpressing *ZmGA2ox4* or the homolog *AtGA2ox7* showed impaired growth and enhanced resistance to *F. verticillioides*. Moreover, zma-unmiR4-mediated suppression of *AtGA2ox7* disturbed the accumulation of bioactive gibberellin (GA) in transgenic plants and perturbed a set of defense-related genes in response to *F. verticillioides*. Exogenous application of GA or GA biosynthetic inhibitor could modulate *F. verticillioides* resistance in different plants . Taken together, our results suggest that zma-unmiR4- *ZmGA2ox4* module might act as a major player in balancing growth and the resistance to *F. verticillioides* pathogen in maize.

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Running title: zma-unmiR4 confers *Fusarium Verticillioides* susceptibility

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F. verticillioides- responsive miRNA designated zma-unmiR4 in maize kernels. The expression levels of zma-unmiR4 were significantly repressed in the resistant maize line but induced in the susceptible lines upon *F. verticillioides* exposure, whereas its target gene*ZmGA2ox4* exhibited the opposite pattern of expression. Heterologous overexpression of *zma-unmiR4* in *Arabidopsis*resulted in enhanced growth and compromised resistance to *F. verticillioides* . By contrast, transgenic plants overexpressing*ZmGA2ox4* or the homolog *AtGA2ox7* showed impaired growth and enhanced resistance to *F. verticillioides* . Moreover, zma-unmiR4-mediated suppression of *AtGA2ox7* disturbed the accumulation of bioactive gibberellin (GA) in transgenic plants and perturbed a set of defense-related genes in response to *F. verticillioides* . Exogenous application of GA or GA biosynthetic inhibitor could modulate *F. verticillioides* resistance in different plants. Taken together, our results suggest that zma-unmiR4- *ZmGA2ox4* module might act as a major player in balancing growth and the resistance to *F. verticillioides*pathogen in maize.

Key words : microRNA, zma-unmiR4, *Fusarium Verticillioides* , Disease resistance, Gibberellin

Summary statement

Characterization of the roles of miRNA in *Fusarium verticillioides* resistance have a profound impact for controlling the pathogene damages. By functional analysis of the novel *F. verticillioides*- responsive miRNA zma-unmiR4, we demonstrate that zma-unmiR4-*ZmGA2ox4/AtGA2ox7* mediated bioactive GA dynamics played as a crucial regulator in *F. verticillioides* resistance and plant growth.

INTRODUCTION

MicroRNAs (miRNAs), a class of 20-24 nucleotides endogenous non-coding RNAs, have been widely found in eukaryotes and play as gene repressors by directing the cleavage or translational repression of the target transcripts (Voinnet, 2009). It has been well documented that miRNAs are involved in the control of some of the most challenging plant traits in agricultural production, such as plant development and architecture, and environmental stress and defense responses (Kumar, 2014; Rubio-Somoza and Weigel, 2011; Sunkar et al., 2012).

Accumulating evidences indicate that miRNAs regulate multiple biotic stress responses in plants, including the interactions with fungi (Chen et al., 2021; Hu et al., 2020; Zhang et al., 2016), viruses (Mengistu and Tenkegna, 2021; Yang et al., 2016; Yao et al., 2019), bacteria (Liu et al., 2019; Navarro et al., 2006; Zhang et al., 2011), as well as insects (Feng et al., 2021; Li et al., 2018). miR393, the first miRNA identified to be involved in plant immunity, could be induced by bacterial flagellin-derived peptide and contributes to restrict the growth of *Pseudomonas syringae* by repressing auxin signaling (Navarro et al., 2006). miR159a was indicated to play a positive role in rice resistance to *Magnaporthe oryzae*(Chen et al., 2021), while miR156 negatively regulates rice resistance to bacterial blight by *Xanthomonas oryzae* (Liu et al., 2019). In addition, the important roles of miR160a, miR166, miR528, miR398b, miR164, and miR168 in disease resistance had been well characterized by regulating specific target genes in various crops (Li et al., 2014). For instance, miR528 negatively regulated rice resistance to stripe virus by cleaving the transcripts of *L-ascorbate oxidase* (*AO*) gene (Wu et al., 2017). Loss function of the Osa-miR159a target genes, including *OsGAMYB* , *OsGAMYBL* , and *OsZF* , resulted in enhanced resistance to *M. oryzae* , consistent with the related phenotypes of Osa-miR159a overaccumulation plants (Chen et al., 2021). miR156 negatively regulated rice resistance against bacterial blight though decreasing its targets *IPA1* and *OsSPL7* expression levels (Liu et al., 2019). Considering the extensive regulation of miRNAs during plant immunity, further characterization of pathogen-responsive miRNAs and resultant miRNA-mediated disease defense processes will have a profound impact on the development of new strategies for controlling disease damages in crop production.

Fusarium verticillioides (*F. verticillioides*) is one of the most common pathogenic fungus that causes various prevalent diseases in crops, especially for maize, posing a great challenge to food and feed safety (Gai et al., 2018; Ju et al., 2017; Liu et al., 2020; Mu et al., 2018; Septiani et al., 2019). *F. verticillioides* infection occurs throughout the whole growth period of maize and results in seedling blight, stalk rot, ear rot, and seed rot (Machado et al., 2013; Septiani et al., 2019; Stagnati et al., 2019). Most importantly,*F. verticillioides*

-infected plants or seeds may accumulate fumonisins, a family of mycotoxins associated with several diseases in livestock and humans and are classified as probable carcinogens (Rosa Junior et al., 2019). Thus, it is of great significance to dissect the molecular mechanism of resistance to *F. verticillioides* . Although many genetic studies and omics data have identified a series of QTLs/genes associated with *F. verticillioides* resistance (Butrón et al., 2019; Chen et al., 2016; Lanubile et al., 2017; Maschietto et al., 2017; Schiwek et al., 2020; Yao et al., 2020), the molecular mechanism of plant against to *F. verticillioides* remain largely elusive, especially the role of miRNAs in this process. In our previous study (Zhou et al., 2020), a number of miRNAs including known and new predicted miRNAs were identified to be potentially associated with the resistance to *F. verticillioides* ear rot via high-throughput sequencing. Further functional analysis of these miRNAs becomes important to dissect the molecular mechanism of plant-*F. verticillioides* interaction and ultimately disease resistance improvement.

In current study, we focused on a novel *F. verticillioides*- responsive miRNA designated zma-unmiR4, and aimed to reveal its function in plants against *F. verticillioides.* We found that the expression levels of zma-unmiR4 was significantly downregulated in the resistant maize line but upregulated in the susceptible lines after *F. verticillioides* infection, whereas the target gene *ZmGA2ox4* displayed the opposite profiles of expression. Heterologous accumulation of zma-unmiR4 resulted in impaired resistance to *F. verticillioides* infection and enhanced growth in*Arabidopsis* , however, the transgenic plants overexpressing*ZmGA2ox4* or the homolog *AtGA2ox7* showed high resistance to*F. verticillioides* as well as retarded growth. Further analyses indicated that zma-unmiR4 was able to regulate *F. verticillioides*resistance through gibberellin (GA) signaling by suppressing*AtGA2ox7* expression in*Arabidopsis* . These results provide direct evidence for the crucial role of zma-unmiR4 in regulating plant growth and disease resistance to *F. verticillioides* .

MATERIALS AND METHODS

Plant materials and growth conditions

The *F. verticillioides* -susceptible maize inbred line N6 is a Tangsipingtou lines, while the *F. verticillioides* -resistant maize line BT-1 is improved by tropical Asia material (Wang et al., 2016). Healthy maize seeds were selected and sowed, and seedlings were grown in a climate-controlled culture room at 24 ± 2 °C with a 14/10-h light/dark photoperiod.

Arabidopsis (Columbia) seeds were first sterilized with 75% ethyl alcohol for 60 s, and then soaked in 3% sodium hypochlorite for 10 min. Surface-sterilized seeds were stratified in the dark at 4°C for 2 days and sowed on MS dishes (pH 5.7) for 7 days, then seedlings were transferred to sterilized nutritional soil at 22 °C with a 16/8-h light/dark photoperiod.

The healthy seeds of *japonica* rice KY131 were soaked in 3% sodium hypochlorite for 30 min, and were accelerated germinated at 37 °C for 3 days. Then the germinating seeds were sowed into 96-well plates, and water-cultured at 28 ± 2 °C with a 14/10-h light/dark photoperiod.

Vector construction and generation of transgenic*Arabidopsis* plants

For *zma-unmiR4* overexpression (*zma-unmiR4 OE*), the 398-bp hairpin region of zma-unmiR4 was amplified from genomic DNA of N6 and ligated into the binary vector pJim19 (Bar) driven by the 35S promoter. For *ZmGA2ox4* overexpression (*ZmGA2ox4 OE*) and *AtGA2ox7* overexpression (*AtGA2ox7 OE*), the coding sequences of *ZmGA2ox4* and *AtGA2ox7* from ATG to TGA was amplified and ligated into the binary vector pCANBIA1302 (HYG), respectively, driven by the 35S promoter. After confirming the sequence,*zma-unmiR4 OE* , *ZmGA2ox4 OE* and *AtGA2ox7OE* vectors were introduced into *Agrobacterium tumefaciens*strain GV3101 and transferred into Col-0 by floral dip. For*35S::ZmGA2ox4-GUS* and *35S::AtGA2ox7-GUS* vectors, the coding sequences of *ZmGA2ox4* and *AtGA2ox7* without stop codon were amplified, respectively, and ligated into the binary vector pCAMBIA1391 (HYG) driven by the 35S promoter. For *35S:: ZmGA2ox4-YFP* vector, the coding sequences of *ZmGA2ox4* without stop codon were amplified and ligated into the binary vector pGRDR driven by the 35S promoter. For the transient expression of*zma-unmiR4* in maize protoplasts, the 398-bp hairpin region of*zma-unmiR4* was amplified from genomic DNA of N6 and ligated into

pPROTO driven by the 35S promoter. The primers used for vector construction are listed in Supplementary Table 1.

Transfection ofmaize protoplasts

Maize protoplasts were isolated from 10-days etiolated seedlings (B73) as previously described (Li et al., 2021). The *35S:: ZmGA2ox4-YFP*was co-transfected into the protoplasts with *35S:: pre-zma-unmiR4* and empty vectors, respectively, using the polyethyleneglycol (PEG)-calcium-mediated transfection. After incubation in dark for 16 h, YFP and mCherry signals were observed using laser scanning confocal microscope (Nikon, A1HD25). The relative fluorescence intensity (YFP/mCherry) was calculated by Image J software.

F. verticillioides **inoculation and phenotype investigation**

The*Fusarium verticillioides* strain was isolated from naturally infected maize kernels in Zhengzhou. A single-spore of *F. verticillioides* was isolated and propagated on sterilized maize kernels at 28degC for 7 days. The spores were then collected and diluted to the concentration of 5 x 10^6 spores mL^{-1}using sterile distilled water with 0.2 μL/mL Tween 80. The ear inoculation was performed as previously described (Wu et al., 2020; Zhou et al., 2020). The middle of the ears was injected with 2 mL *F. verticillioides* spore suspension (5×10^6 spores/mL) on the fifteenth day after pollination using a syringe. The kernels surrounding the inoculated points were sampled on 0 day and 3 days after inoculation for RNA extraction.

For *Arabidopsis* leaf inoculation, the healthy rosette leaves of four-weeks plants were inoculated with 20 μL *F. verticillioides spore* suspension with the concentration of 1×10^7spores /mL, and the control group was inoculated with sterile water. After 4-6 days culture at 22°C, the leaf was photographed and sampled for histological staining and *F. verticillioides* quantification. For spore suspension spraying, five-weeks plants were sprayed with*F. verticillioides* spore suspension (2×10^7spores/mL) or sterile water once a day for 10 days, the then the plants were photographed. For *Arabidopsis* seeds inoculation, the sterilized seeds were soaked in *F. verticillioides* spore suspension with the concentration of 1×10^7 spores /mL at 28° darkness for 48 h, and then taken out and washed with sterile water. The control group was soaked in sterile water. Then the seeds were evenly placed on a wet filter paper in petri dishes at 28° darkness for 6 days. The disease grades of the seeds rot were investigated according to the criterion showed in Figure S5.

For maize leaf inoculation, the healthy second leaves were cut off and lacerated with a needle, then the 0.3-cm scratch were injected with 10 μL *Fusarium verticillioides* spore suspension with the concentration of 1×10^7 spores /mL. After incubation at 25°C for 2-5 days, the leaves were photographed or sampled for DAB and TB staining.

For rice leaf inoculation, the healthy second leaves were cut off and scratched with a needle, then the leaves were immersed in the 3 mL*Fusarium verticillioides* spore suspension with the concentration of 2×10^7 spores /mL and incubated at 25°C for 5 days. For rice seedling inoculation, healthy seedlings were sprayed with*Fusarium verticillioides* spore suspension with the concentration of 2×10^7 spores /mL once a day for 6 days. Then the leaves were photographed.

RNA analyses

For protein-coding genes, about 1 μg RNA was treated with DNase I (Promega) and reversely transcribed using the Transcriptor First Strand cDNA Synthesis Kit (TOYOBO). The qRT-PCR assay was performed using SYBR Green I Master reagent and a STEP ONE PLUS system (ThermoFisher). The expression levels of target genes were normalized to the expression levels of internal control genes using the $2^{-\Delta\Delta^{\circ}\tau}$method. For quantification of protein-coding genes and primary zma-unmiR4 in *Arabidopsis* , *Actin 2* was used as the internal control. For quantification of primary zma-unmiR4 and*ZmGA2ox4* in maize, the *elongation factor 1 alpha*(*EF1a*) of maize was used as the internal control. RNA gel blot analyses of miRNA were performed as described previously (Zhang and Li, 2013). Primer and probe sequences are listed in Supplementary Table 1.

GUS, DAB and TB staining

Transient expression assay for GUS analysis was performed as previously described (Li et al., 2021). Briefly, 5-weeks *Nicotiana benthamiana* leaves were infiltrated using *Agrobacterium tumefaciens* strains GV3101 carrying constructs together with p19 strain. After two days, the leaves were soaked in ice-cold 90% acetone under vacuum condition for 10min, then washed twice with 100 mM sodium phosphate buffer (pH 7.0) and submerged in dye solution at 37 for 12 h. Chlorophyll was removed by immersing in 95% ethanol at 95°C, then samples were photographed by a microscope (Moticam2506).

To visualize H_2O_2 accumulation, leaves were immersed in a 0.1% DAB solution in the Tris-HCl buffer (pH 6.5) and infiltrated under vacuum condition for 15 min, and then incubated at room temperature for 12 h in the dark. Then the samples were transferred into the fix solution (60% ethanol, 20% acetic acid, 20% glycerol) at 95°C for 5 min. Chlorophyll was removed by immersing in 95% ethanol at 100°C, then samples were photographed.

For cell membrane damage visualization, trypan blue staining was performed by submerging the leaves in TB solution (10 mL lactic acid, 10 mL phenol, 10 mL glycerol, 10 mL sterile water, and 10 mg trypan blue) for 30-60 min. Chlorophyll was removed by immersing in 95% ethanol at 100°C, then samples were photographed.

Hormone treatments

For maize seedlings, 7-days seedlings were sprayed with 20 μM uniconazole, 50 μM GA3, or water as the control once a day for 7 days, then the seedlings were photographed and the leaves were inoculated. For rice seedlings, 14-days seedlings were sprayed with 20 μM uniconazole, 50 μM GA3, or water once a day for 4 days, then the seedlings were sprayed with *F. verticillioides* spore suspension. For *Arabidopsis* plants, 17-days seedlings were sprayed with 20 μM uniconazole, 50 μM GA3, or water once a day for 5 days. Then the seedlings were photographed and leaves were inoculated as the methods described above.

Determination ofchlorophyll concentration

The measurement of chlorophyll concentration was performed as previously described (Arnon, 1949). Briefly, 0.2 g leaves from 5-weeks plants were sampled, well ground, and resuspended using 10 mL 80% acetone. The supernatant was then transferred to a new tube after centrifuged at 4000 rpm for 5 min and then the absorbance was measured at 663 nm, 645 nm and 652 nm, respectively, using an ELISA instrument. The chlorophyll concentration was calculated according the method as previously described (Arnon, 1949).

Gibberellin measurement

The healthy rosette leaves of four-weeks plants were harvested, immediately frozen in liquid nitrogen, and ground into powder. 50 mg of plant sample was weighed and dissolved in 500 μL HPLC-grade acetonitrile/H_2O (90:10, v/v). 10 μL internal standard mixed solution (100 ng/mL) was added into the extract as internal standards (IS) for the quantication. GAs contents were detected by MetWare (http://www.metware.cn/) based on the AB Sciex QTRAP 6500 LC-MS/MS platform. Three biological replicates were performed.

RESULTS

Zma-unmiR4 is a novel maize miRNA in response to *F. verticillioides*

Deep sequencing of small RNA libraries from maize kernels treated with or without *F. verticillioides* revealed a number of *F. verticillioides* -responsive miRNAs in our previous studies (Zhou et al., 2020), including 92 potentially novel miRNAs. These predicted miRNAs displayed various expression profiles in response to *F. verticillioides*(Figure 1A). A novel miRNA candidate designated zma-unmiR4 was characterized in more detail for its contrary expression in *F. verticillioides* susceptible maize line N6 and resistant line BT-1 (Figure 1B). Initially, successful amplification of its precursor sequence indicated that zma-unmiR4 is transcribed as an individual transcriptional unit in maize genome (Figure 1C). In addition, zma-unmiR4 was validated through RNA blotting in the maize kernels (Figure 1D). A high degree of complementarity for the precursor structure was further observed using RNAfold web server (Figure 1E). These observations support the

notion that zma-unmiR4 represents a novel recently evolved miRNA potentially regulating the resistance to *F. verticillioides* in maize. Moreover, zma-unmiR4 was found to be expressed in various maize tissues (Figure S1), implying its potential functions during various developmental stages.

ZmGA2ox4 and its homolog *AtGA2ox7* are the targets of zma-unmiR4

Based on target gene prediction (http://rna.informatik.uni-freiburg.de), zma-unmiR4 showed extensive sequence complementarity with the gene*Zm00001d017294* encoding for gibberellin 2-oxidase 4 (*ZmGA2ox4* ; Figure 2A). Notably, the accumulation of*ZmGA2ox4* transcripts was drastically increased in BT-1 but decreased in N6 after *F. verticillioides* inoculation (Figure 2B), contrary to zma-unmiR4 expressions (Figure 1A-C). To confirm that*ZmGA2ox4* is regulated by zma-unmiR4 *in planta* , the constructs respectively expressing zma-unmiR4 (*35S:pre-unmiR4*) and *ZmGA2ox4-YFP* (*35S: ZmGA2ox4-YFP*) were co-transformed into maize protoplasts (Figure 2C), and YFP signals were significantly decreased in the protoplasts (Figure 2D). We further expressed*35S: ZmGA2ox4-GUS* and *35S:pre-unmiR4* transgenes in tobacco leaves, and found that the GUS signals were nearly undetectable compared with the strong GUS staining when expressing *35S:ZmGA2ox4-GUS*alone (Figure 2E). RT-qPCR also showed that *ZmGA2ox4* transcripts were significantly decreased when co-expressing both transgenes (Figure 2F). Together, these results demonstrate that *ZmGA2ox4* is a direct target of zma-unmiR4.

Arabidopsis AtGA2ox7 and *AtGA2ox8* encoding for the homologous proteins of ZmGA2ox4 were predicted to be the putative heterologous targets of zma-unmiR4 (Figure 3A). We then compared the expression changes of *AtGA2ox7* or *AtGA2ox8* between wild type and zma-unmiR4 overexpressing (*zma-unmiR4 OE* ; Figure 3B) plants. As shown in Figure 3C, *AtGA2ox7* was remarkedly downregulated while *AtGA2ox8* displayed no obvious changes in both zma-unmiR4 overexpressors, suggesting that *AtGA2ox7* may be targeted by zma-unmiR4. To verify this regulation *in planta* , *AtGA2ox7* was fused with *GUS* gene and transiently co-expressed with *35S:pre-unmiR4* in tobacco. *GUS*activities and transcripts were dramatically decreased compared with the vector control (Figure 3D and 3E), manisfested by the greatly reduction of *AtGA2ox7* transcripts (Figure 3F). These data demonstrate that zma-unmiR4 negatively regulates *AtGA2ox7* in *Arabidopsis* .

Overexpression of zma-unmiR4 confers *Arabidopsis* growth and *F. verticillioides* susceptibility

To investigate the biological functions of zma-unmiR4, we developed homozygous transgenic *Arabidopsis* lines overexpressing zma-unmiR4 (*zma-unmiR4 OE* , Figure 3A),*ZmGA2ox4* (*ZmGA2ox4 OE* ; Figure S2A*)* , and *AtGA2ox7* (*AtGA2ox7 OE* ; Figure S2B), respectively. Interestingly, we found that *zma-unmiR4 OE*plants displayed increased plant height, early flowering, and large leaf size compared with WT plants, which were similar with the phenotypes of*atga2ox7* mutants (SALK_055721C; Figure 4A; Figure S3). On the contrary, ectopic expression of *ZmGA2ox4* or*AtGA2ox7* greatly reduced plant height, delayed flowering time, and shorten leaf radius, and the leaves of *AtGA2ox7* OE appeared dark green with higher chlorophyll content (Figure 4A and 4B; Figure S3), as observed previously (Porri et al., 2012; Shu et al., 2016). In addition, exogenous application of bioactive GA could partially rescue the dwarf phenotype of *ZmGA2ox4 OE* and *AtGA2ox7 OE*plants (Figure S4), implying the conserved functions of *ZmGA2ox4*and *AtGA2ox7* in GA-mediated plant growth.

We then determined the *F. verticillioides* resistance of various genotypic plants by inoculating fungal spore suspension. On the 5th day post inoculation, young leaves of*zma-unmiR4 OE* and*atga2ox7* mutant plants displayed obvious disease symptoms, and the size of yellow necrotic lesions were much larger than that of WT (Figure 4B). By contrast, the leaves of *AtGA2ox7 OE* and*ZmGA2ox4 OE* plants exhibited slight yellowish necrosis (Figure 4B). In addition, the rosette leaves of *zma-unmiR4* OE and *atga2ox7* adult plants displayed severer blight or death phenotypes after spraying with*F. verticillioides* spore suspension, however, the transgenic plants of *ZmGA2ox4OE* or *AtGA2ox7 OE* were almost unaffected (Figure 4C).

We further tested whether there existed differences in *F. verticillioides* seed rot among WT, *atga2ox7* mutant, and the transgenic plants indicated above. To this end, the seeds from various genotypes were incubated with *F. verticillioides* spore suspension, and the phenotypes of fungal mycelia growth on seeds surface were

recorded on 6th day. Compared to the water-treatment control, the growth and invasion areas of fungal mycelia showed significant difference among various genotypes after *F. verticillioides*inoculation. The seeds from *zma-unmiR4 OE* or *atga2ox7*mutant plants were more sensitive to *F. verticillioides* but resistant from *ZmGA2ox4 OE* or *AtGA2ox7 OE*transgenic plants (Figure 4D). In details, more than half of the seeds from *zma-unmiR4 OE* and *atga2ox7* mutant plants exhibited disease grades II and III, however, most of seeds from *ZmGA2ox4OE* and *AtGA2ox7 OE* plants belonged to grade I according to the three grades of disease resistance (Figure S5). These data suggested that zma-unmiR4 could regulated plant growth positively and *F. verticillioides* resistance negatively by manipulating*AtGA2ox7* or *ZmGA2ox4*expression.

Altered resistance to *F. verticillioides* by zma-unmiR4 is associated with the production of H_2O_2

As one kind of necrotrophic fungal pathogens, *F. verticillioides* might ultimately kill and benefit from the infected host cells (Rivas-San Vicente et al., 2013). Leaves of various genotypic plants were incubated with 3,3'-diaminobenzidine (DAB) to detect H_2O_2 or stained with trypan blue (TB) to reveal dead cells. We first compared the H_2O_2 level and cell death between the leaves of *F. verticillioides* susceptible maize line N6 and resistant line BT-1 after *F. verticillioides* infection. Higher levels of H_2O_2 production and clusters of dead cells were observed in N6 leaves but less in BT-1 leaves (Figure 5A and 5B), implying that *F. verticillioides* hypersensitivity led to H_2O_2 burst and cell death in maize. Consistent with the differences of *F. verticillioides* resistance, we also noted higher H_2O_2levels (Figure 5C) and more cell death (Figure 5D) in *zma-unmiR4 OE* leaves compared with that of WT. On the contrary, the H_2O_2 accumulation and cell death were nearly undetectable in *ZmGA2ox4 OE* and *AtGA2ox7OE* leaves (Figure 5 C and 5D).

Development of *F. verticillioides* resistance by zma-unmiR4 correlates with the expression of defense-related genes

We measured the relative levels of a set of defense-related genes*PR1* , *PR4* , *PR5* ,*PDF1.2* , *NPR1* , *WRKY70* , *ORA59* , and*HCHIB/PR3* mRNAs in the transgenic plants indicated above. Notably, the expression levels of these defense-related genes were significantly induced in response to*F. verticillioides* infection in WT and *AtGA2ox7 OE*plants (Figure 6). In addition, all these genes except for *PR5*were greatly upregulated in *AtGA2ox7 OE* plants compared to the WT upon *F. verticillioides* infection (Figure 6). However, for*zma-unmiR4 OE* plants, the expression levels of *PR1* ,*PR4* , *PR5* , and *ORA59* displayed no obvious changes after *F. verticillioides* inoculation, and *PDF1* ,*NPR1* , *WRKY70* , and *HCHIB* were even downregulated after *F. verticillioides* inoculation (Figure 6). These data suggested that zma-unmiR4-mediated suppression of *AtGA2ox7* might disturb the induction of defense genes by *F. verticillioides* , thus resulting in resistance variations.

GA accumulation is associated with *F. verticillioides* resistance

AtGA2ox7, a member of gibberellin 2-oxidase family, is a class of 2-oxoglutarate-dependent dioxygenases that regulate the deactivation of bioactive GAs (Li et al., 2019). We then analyzed the endogenous contents of bioactive GAs including GA1, GA3, GA4, and GA7 in the rosette leaves of four-week-old plants. The levels of GA3 and GA4 were too low to detect in WT, *zma-unmiR4 OE* , and *AtGA2ox7OE* samples tested, but GA1 were accumulated to higher levels in*zma-unmiR4 OE* plants than both WT and *AtGA2ox7 OE*plants (Figure 7A). In addition, compared with WT, *zma-unmiR4 OE*transgenic plants accumulated higher levels of GA7 while the contents of GA7 were significantly decreased in *AtGA2ox7 OE* (Figure 7B). These results suggested that zma-unmiR4-*AtGA2ox7* module mediated plant growth and *F. verticillioides* resistance were more likely through regulating endogenous bioactive GAs accumulation.

To further investigate the effects of GA on plant disease resistance and growth, 17-day-old seedlings of WT, *zma-unmiR4 OE* and*AtGA2ox7 OE* transgenes were sprayed with GA (50 μM) or GA synthesis inhibitor uniconazole (20 μM). As expected, the growth of WT and *AtGA2ox7 OE* seedlings was enhanced by GA treatment, but growth inhibition was clearly observed for both the *zma-unmiR4 OE* and WT plants when treated with uniconazole (Figure 8A). We then inoculated the leaves with *F. verticillioides* spore suspension. Compared to the water control treatment, the leaves of WT and*AtGA2ox7 OE* plants treated with GA

displayed larger yellow necrotic lesions, and more H_2O_2 production as well as cell death (Figure 8B). By contrast, both WT and *zma-unmiR4 OE*treated with uniconazole exhibited slight necrotic lesions, and the extents of H_2O_2 production and cell death were much lower than the non-treated control (Figure 8B).

Moreover, we applied GA or uniconazole on the susceptible maize line N6 to test the resistance changes to *F. verticillioides* . Compared with the application of water, the *F. verticillioides*susceptibility of N6 seedlings was greatly promoted by GA, consistent with increased *F. verticillioides* -caused cell death and H_2O_2 accumulation (Figure 8C-E). By contrast, maize seedling treated with uniconazole displayed smaller necrotic lesions (Figure 8D), and decreased cell death and H_2O_2 production (Figure 8E). Furthermore, the similar results were also observed in rice seedlings treated with GA and GA inhibitors (Figure S6). When the rice seedlings were sprayed directly with *F. verticillioides* spore suspension, the disease symptom of the seedlings treated with GA was obvious enhanced, while seedlings treated with uniconazole was the opposite. Collectively, these results demonstrate that GA plays a negative role in plants resistance to *F. verticillioides.*

DISCUSSION

Fusarium verticillioides (*F. verticillioides*) is one of the most common pathogenic fungus that can cause many prevalent diseases in crops, especially for maize, such as seedling blight, root rot, stalk rot, ear rot, and seed rot, leading to poor grain yields and quality, thus posing a great challenge to food and feed safety (Gai et al., 2018; Ju et al., 2017; Mu et al., 2018; Septiani et al., 2019; Zhou et al., 2018). Identification of genes related to *F. verticillioides*resistance and subsequent development of*F. verticillioides* -resistance crops are considered to be the most economical and environment-friendly strategy.

Given that miRNAs provide quantitative regulation of target gene expression rather than switching regulation, the dynamic accumulation of pathogen-responsive miRNAs can provide fine-tuning of target gene expression during pathogen infection, thus in turn enhancing the plant's disease-resist ability (Campo et al., 2013). High-throughput sequencing of small RNA is an effective method to discover pathogen-responsive miRNAs, including conserved and novel miRNAs. Although false-positive prediction of novel miRNAs cannot be ruled out during sequencing and data processing, the function of the young evolved miRNAs in pathogen resistance should be fully considered. For instance, the Md-miRln20 (Zhang et al., 2019), osa-miR7695 (Campo et al., 2013), and Md-miRLn11 (Ma et al., 2014) were characterized by small RNA sequencing and experimentally validated for their function in disease resistance. In the previous study, multiple *F. verticillioides* -responsive miRNAs were identified using small RNA deep sequencing (Zhou et al., 2020), and one of novel miRNAs zma-unmiR4 displayed entirely different expression patterns between the susceptible and resistant maize line after *F. verticillioides* infection (Figures 1A-C), and RNA blotting provided evidence for the existence of zma-unmiR4 in maize (Figure 1D). The significantly reduction of zma-unmiR4 in BT-1 upon *F. verticillioides* exposure indicated that it may function as a negative regulator of maize immunity against *F. verticillioides* (Figures 1A-C), manifested by the compromised resistance of transgenic plants ectopically expressing zma-unmiR4 in *Arabidopsis* (Figure 4).

According to the different life styles, plant pathogens can be divided into biotrophs (prefer living cells) and necrotrophs (prefer dead cells) (Barna et al., 2012). In the case of the necrotrophic pathogen such as*Botrytis cinerea* , ROS-overaccumulation-caused cell death and tissue necrosis during pathogen infection were reported to benefit the pathogen invasion by offering a growth substrate, thus increasing host susceptibility (Hanif et al., 2018; Tian et al., 2019; Wang et al., 2018). *F. verticillioides* was reported to be one kind of necrotrophic fungal pathogens (Rivas-San Vicente et al., 2013). In line with this, more cell death and H_2O_2production were detected in the leaves of susceptible maize line N6 compared with the resistant maize line BT-1 after *F. verticillioides* infection (Figure 5). Similarly, the susceptibility to*F. verticillioides* infection was also correlated with the levels of cell death and H_2O_2 production in WT,*zma-unmiR4 OE* , *AtGA2ox7* OE and *ZmGA2ox4* OE plant (Figure 4 and Figure 5). Therefore, cell death and H_2O_2 accumulation can be used as the indicators of *F. verticillioides* susceptibility in maize cultivars.

Gibberellins are widely recognized as phytohormones that play multiple roles in plant development and stress responses (Rizza and Jones, 2019; Schomburg et al., 2003). Endogenous levels of bioactive GAs are

maintained through a balance of biosynthesis and inactivation. AtGA2ox7 is a class of 2-oxoglutarate-dependent dioxygenases that regulate the deactivation of bioactive GAs (Li et al., 2019). Consistently, the transgenic plants overexpressing*AtGA2ox7* showed significant reduction of bioactive GAs compared to the WT, thus exhibiting GA-deficient phenotypes, such as dwarf plant, delayed flowering, and small dark green leaves (Porri et al., 2012; Schomburg et al., 2003; Shu et al., 2016) (Figure 4A and 4B; Figure 7; Figure S3). By contrast, dysfunction of *AtGA2ox7* resulted in GA-induced phenotypes, including enhanced growth, large leaf size, and early flowering(Magome et al., 2008; Rieu et al., 2008; Shu et al., 2016), which was consistent with the phenotypes of *zma-unmiR4 OE*plants and higher levels of GA contents (Figure 4A and 4B; Figure 7; Figure S3). Therefore, we have reason to believe that the high level of bioactive GAs by zma-unmiR4-mediated repression of *AtGA2ox7* is responsible for the phenotypic changes of *zma-unmiR4 OE* plants.

Although the function of bioactive GA in plant growth and development is well reported, the role of GA in plant resistance to *F. verticillioides* remains unclear. In fact, GA was first identified from*Gibberella fujikuroi* (*Fusarium moniliforme*), one kind of necrotrophic fungus that causes rice bakanae disease (Yabuta and Sumiki, 1938). Overexpression of the GA deactivating enzyme Eui could increase the resistance to bacterial blight and rice blast respectively caused by*Xanthomonas oryzae* and *Magnaporthe oryzae* , however, transgenic rice overexpressing *OsGA20ox3* (encoding for a GA biosynthetic enzyme) displayed hypersensitivity to both diseases (Yang et al., 2008; Qin et al., 2013). Similarly, our current results demonstrated that GAs also exhibited a negative effect on the resistance to *F. verticillioides* through genetic and physiological analysis in *Arabidopsis* or maize plants (Figures 4 and 8). Engineering the expression level of GA-deactivating enzyme *AtGA2ox7* or*ZmGA2ox4* could modify the *F. verticillioides* resistance (Figure 4). Despite of the enhanced resistance to *F. verticillioides* in *Arabidopsis* with overaccumulation of*AtGA2ox7* , many adverse effects were also occurred on development, such as dwarf plants and delayed flowering (Figure 4A), which would be likely expected in maize. In addition, it has been reported that application of GA inhibitor uniconazole significantly reduced lodging rate and enhanced yield in maize (Ahmad et al., 2021). Our data also showed the positive effects of uniconazole on *F. verticillioides*resistance and dwarf traits in maize and rice (Figure 8C-E and Figure S6). Therefore, fine-tuning zma-unmiR4-ZmGA2ox4 regulatory module could theoretically be an alternative way to generate desirable *F. verticillioides* and lodging resistance without growth or yield penalty in maize breeding.

Given the crucial role of GA in plant innate immunity (De Vleesschauwer et al., 2016; Qin et al., 2013; Yang et al., 2008), it is not surprising to find the divergent resistance to *F. verticillioides* between the GA-deficient (*AtGA2ox7* OE) and GA-sufficient (*zma-unmiR4* OE) plants (Figure 4, 5, 8). On the one hand, GA may give rise to an indirect attenuation of pathogenesis-related (PR) genes, thus facilitating pathogen prevalence. Indeed, the expression levels of*PR1* , *PR3* , *PR4* , and *PR5* were significantly induced in response to *F. verticillioides* infection in*AtGA2ox7* OE plants, however, they displayed no obvious changes after *F. verticillioides* inoculation in *zma-unmiR4 OE*(Figure 6). On the other hand, GA may hinder disease defense responses via modulating the homeostasis of the archetypal immunity hormones (Verma et al., 2016; Wild and Achard, 2013), such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). In rice, overexpression or dysfunction of GA deactivating enzyme Eui results in disturbed homeostasis of SA and JA, thus leading to the altered disease susceptibility (Yang et al., 2008). In addition, the SA receptors non-expresser of pathogenesis-related genes 1 (*NPR1*) is a master regulator of systemic acquired resistance in plants, overaccumulation of NPR1 leads to enhanced disease resistance to diverse pathogens (Ding et al., 2018). In support of this notion, we found that *NPR1* was significantly induced after *F. verticillioides* infection in *AtGA2ox7OE* plants but repressed in *zma-unmiR4 OE* plants (Figure 6), implying divergent SA signaling dynamics in those transgenic plants upon pathogen attack. Moreover, NPR1 might activate several WRKY transcription factors, such as WRKY70, subsequently leading to massive induction of antimicrobial genes (Saleh et al., 2015). As expected, *F. verticillioides* -induced expressions of *WRKY70*in the WT, *zma-unmiR4 OE* and *AtGA2ox7* OE plants was exactly similar with that of *NPR1* (Figure 6). Additionally, the divergent expression profiles of *ORA59* and the JA- and ET-responsive plant defensin gene *PDF1.2*(Zarei et al., 2011) further revealed the different dynamics of JA- and ET-associated resistant

responses in the transgenic plants upon *F. verticillioides*exposure. Further genome-wide transcriptome analysis with the*zma-unmiR4 OE* and *AtGA2ox7* OE plants should provide much-needed insights into to the interactions between GA and other phytohormones signaling pathways that underpin plant resistance in response to *F. verticillioides* challenging.

Under stress conditions, plants are believed to actively repress their growth for survival, but accompanied with undesirable crop productivity (Zhang et al., 2020). As a master regulator of plant growth and development, however, GA confers susceptibility to multiple plant diseases (Qin et al., 2013; Yang et al., 2008). Overall, our results revealed the novel miRNA zma-unmiR4 played as a crucial regulator in*F. verticillioides* resistance and plant growth by suppressing bioactive GA accumulation (Figure 9). Thus, engineering the zma-unmiR4-*ZmGA2ox4* module may thereby represent an alternative strategy that warrants a better balance between *F. verticillioides* disease resistance and growth in corn breeding.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Figure Legends

Figure 1. Identification and validation of zma-unmiR4

(**A**) Expression heatmap of the 92 predicted novel miRNAs candidates in BT-1 and N6 kernels after *F. verticillioides*inoculation by small RNA sequencing (Zhou et al., 2020). Maize kernels of BT-1 and N6 at 0 d or 3 d post inoculation were sampled for small RNA libraries construction. (**B**) Distinct expression of zma-unmiR4 in BT-1 and N6 maize lines after *F. verticillioides* inoculation by RT-qPCR. Maize *Ef1a* was used as the internal control, and the expression level of N6 on day 0 was set to 1. Data are means +- SD from three biological replicates. ** $P < 0.01$ by student's*t* -test. (**C** and **D**) Verification of zma-unmiR4 through RT-PCR amplifying its precursor (C) and RNA blotting (D). (**E**) Predicted hairpin structure of zma-unmiR4 by RNAfold software.

Figure 2. Maize *ZmGA2ox4* is targeted by zma-unmiR4.

(**A**) Schematic diagram of *ZmGA2ox4* showing the target site of zma-unmiR4. (**B**) *ZmGA2ox4* was differentially regulated in BT-1 and N6 kernels after *F. verticillioides*inoculation. Maize *Ef1a* was used as the internal control, and the expression level of N6 on day 0 was set to 1. Data are means +- SD from three biological replicates. (**C**) Structure of various constructs used in the transient transformation assay in maize protoplast and tobacco plants. (**D**) Maize protoplasts were transfected with the reporter plasmid with empty effector or*35S:pre-unmiR4* effector, then the protoplasts were kept in darkness for 16 h. The fluorescence intensity of YFP was normalized to the mCherry. Data are represented as mean +- SD (n [?] 200 cells) from three biological replicates. (**E** and **F**) Tobacco leaves were transfected with *35S: ZmGA2ox4-GUS* reporter plasmid with empty effector or *35S:pre-unmiR4* effector. After two days, the transfected leaves were used for GUS staining (E) and isolation of total RNA for detecting *ZmGA2ox4* expression by RT-qPCR (F). Data are means +- SD from three biological replicates. ** $P < 0.01$ by student's t -test.

Figure 3. *Arabidopsis AtGA2ox7* is a heterologous target of zma-unmiR4.

(**A**) Phylogenetic tree of *ZmGA2ox4* homologs in maize and*Arabidopsis* . The sequences of *ZmGA2ox4* homologs from maize and *Arabidopsis* were obtained from NCBI database searches (https://blast.ncbi.nlm.nih.gov), and phylogenetic analysis was performed using MEGA7.0. The neighbor-joining method was used with 1,000 bootstrap replications. (**B**) Measurement of zma-unmiR4 precursor

enrichment in two independent transgenic homozygous lines (*zma-unmiR4 OE*) by RT-PCR. (**C**) Expression levels of *AtGA2ox7* or *AtGA2ox8*were quantified in WT and *zma-unmiR4 OE* plants by RT-qPCR. (**D**) GUS staining in tobacco leaves co-transformed with*35S:AtGA2ox7-GUS* construct and empty vector or the*35S:pre-unmiR4* effector as mentioned in panel C of Figure 2. (**E** and **F**) Quantification of GUS (E) and*AtGA2ox7* (F) transcripts in the samples described in panel D. Data are means +- SD of three biological replicates. ** $P< 0.01$ by Student's t -test; ns, no significant difference.

Figure 4. Zma-unmiR4 regulates *F. verticillioides*resistance negatively and growth positively through the target gene*AtGA2ox7* or *ZmGA2ox4*.

(**A**) Growth phenotypes of WT, *atga2ox7* mutant,*zma-unmiR4 OE* , *AtGA2ox7 OE* , and *ZmGA2ox4 OE* plants. Four-week-old seedlings grown in soil were photographed. (**B**) The disease symptoms on the representative leaves of WT, *AtGA2ox7*mutant, *zma-unmiR4* OE, *AtGA2ox7* OE, and *ZmGA2ox4* OE plants at 5 days post inoculation. Healthy rosette leaves of 4-week-old plants were inoculated with 20 µl *F. verticillioides* spore suspension (*F. V*) or sterile water (Mock). (**C**) The disease symptoms of WT, *atga2ox7* mutant, *zma-unmiR4 OE* , *AtGA2ox7 OE* , and *ZmGA2ox4 OE* plants after*F. verticillioides* spraying. Four-week-old plants grown in soil were sprayed with *F. verticillioides* spore suspension or sterile water. (**D**) The seed rot symptoms of WT, *atga2ox7* mutant, *zma-unmiR4 OE* ,*AtGA2ox7 OE* , and *ZmGA2ox4 OE* plants. Healthy dry seeds were sterilized, then immersed in*F. verticillioides* spore suspension for 48 h and placed in sterile filter paper for 6 days.

Figure 5. *Fusarium verticillioides* susceptibility is proceeded by H_2O_2 accumulation.

(**A** and **B**) H_2O_2production and cell death in maize leaves were revealed by diaminobenzidine (DAB) straining (A) and trypan blue (TB) straining (B), respectively. Healthy second leaves of BT-1 and N6 maize lines were injected with *F. verticillioides* spore suspension (*F. V*) or sterile water (Mock), and the leaves were sampled for staining 2 days after inoculation. (**C** and **D**) DAB staining (C) and TB staining (D) analysis in *Arabidopsis* leaves of indicated genotypic plants. Healthy rosette leaves of 4-week-old plants were injected with *F. verticillioides* spore suspension or sterile water, and were sampled for straining 4 days after inoculation. The amplification and spread of H_2O_2 production and cell death in the whole leaf were shown in a large section of a leaf at ×5 magnification.

Figure 6. Expression of the defense-related genes in transgenic*Arabidopsis*.

Healthy leaves of 4-week-old WT, *zma-unmiR4 OE* , and*AtGA2ox7 OE* plants were inoculated with *F. verticillioides*spore suspension, and were sampled at indicated time point for total RNA extraction. Gene expression levels were quantified by RT-qPCR.*Actin 2* was used as an internal control. Expression level in WT at 0 h was set to 1. Data are means ± SD of three biological replicates.

Figure 7. Quantification of endogenous bioactive GAs in WT,*zma-unmiR4 OE*, and *AtGA2ox7 OE* plants.

Shoots of 4-week-old various genotypic plants were sampled for quantifying GA contents. Data are means ± SD of three biological replicates. ** $P < 0.01$ by Student's t -test.

Figure 8.Exogenous application of GA or GA inhibitors alters plant resistance to *F. verticillioides*

(**A**) Growth phenotypes of WT, *zma-unmiR4 OE* and *AtGA2ox7 OE* plants. 17-day-old indicated plants were sprayed with H_2O, GA (50 µM) or uniconazole (20 µM) once a day for 5 days, then photographed. (**B**) The disease symptoms (top), DAB staining (middle), and TB staining (bottom) of the representative leaves from WT, *zma-unmiR4* OE and *AtGA2ox7*OE plants. Healthy leaves of 17-day-old seedlings treated as above were inoculated with *F. verticillioides* spore suspension, then photographed or stained 4 days after inoculation. (**C**) Growth phenotypes of susceptible maize line N6 after exogenous spraying of H_2O, GA (50 µM) or uniconazole (20 µM). (**D** and**E**) The disease symptoms (D), TB straining (E, left), and DAB straining (E, right) of N6 leaves. 7-days seedlings were sprayed with H_2O, GA (50 µM) or uniconazole (20

μM) once a day for 7 days, then inoculated with *F. verticillioides* (*F. V*) spore suspension or sterile water (Mock), and photographed or stained at 4 days after inoculation.

Figure 9. A hypothetical model of zma-unmiR4 in mediating plant resistance to *F. verticillioides* and growth.

zma-unmiR4 suppresses *ZmGA2ox4* or *AtGA2ox7* expression by cleaving their transcripts, leading to bioactive gibberellin (GA) accumulation. On the one hand, increased GA perturbs JA/SA-mediated defense signaling, thereby resulting in compromised *F. verticillioides* resistance. On the other hand, increased bioactive GA confers plant growth and development. Thus, manipulating the zma-unmiR4- ZmGA2ox4 module may represent an alternative strategy that warrants a better balance between *F. verticillioides* disease resistance and growth in maize.

Supplemental Information

Figure S1. Tissue-specific expression of zma-unmiR4.

YR, YS and YL represented roots, stem and leaves from 8-day-old B73 seedlings, respectively, and stem and leaf were taken from the plants at the flowering stage. Total RNA was extracted from indicated tissues and treated with DNase I, and reverse-transcribed into cDNA for PCR amplification. *Ef1a* was used as a control.

Figure S2. Molecular identification of *ZmGA2ox4 OE* and *AtGA2ox7 OE* transgenic lines.

Leaves of 4-week-old *ZmGA2ox4 OE* and *AtGA2ox7 OE* transgenic plants were sampled for total RNA extraction, and RT-qPCR assays were performed to measure the transcript levels of *ZmGA2ox4* (A) and *AtGA2ox7* (B) genes. *Actin2* was used as an internal control. Data are means ± SD of three biological replicates. ** $P< 0.01$ by Student's t -test.

Figure S3. Quantitative comparison of flowering time, plant height, leaf size andchlorophyll contents among WT, *atga2ox7* mutant, and transgenic plants.

The timing of the first opened flower (A), rosette leaf number at time of the first open flower (B), plant height (C), leaf size (D), leaf area (E), and chlorophyll concentration (F) of WT, *atga2ox7* mutant,*zma-unmiR4 OE* , *AtGA2ox7 OE* , and *ZmGA2ox4 OE* plants. Four-week-old seedlings grown in soil were used for analysis. Data are means ± SD. Panel A and B, n = 24 plants; Panel C, n = 20 plants; Panel E, the largest rosette leaf was used for measuring leaf area by Image J software, n = 18 plants. Panel F, n = three biological replicates. ns, no significant difference; * $P < 0.05$, ** $P< 0.01$ by Student's t -test.

Figure S4. Exogenous GA partially rescued the dwarf phenotype by *AtGA2ox7* or *ZmGA2ox4* overaccumulation in *Arabidopsis*.

Four-week-old *AtGA2ox7* OE and *ZmGA2ox4* OE transgenic plants grown in soil were sprayed with GA (50 μM) once a day for 10 days, and then photographed.

Figure S5. Investigation of *F. verticillioides* seed rot resistance.

(**A**) Three disease grades were divided according to the infected area (IA, the area covered by mycelia on one seed / the total area of same seed). Grade I, 0<IA[?]25%; Grade II, 25<IA[?]50%; Grade III, 50<IA[?]100%. (**B**) The disease grades of seed rot were varied among indicated genotypes. Data are from a total of 150 seeds for each genotype.

Figure S6. Exogenous application of GA alters rice resistance to *F. verticillioides*.

(**A**) Growth phenotype of KY131 rice seedlings. 14-d-old seedlings were sprayed with H_2O, GA (50 μM) or uniconazole (20 μM) once a day for 4 days, and then photographed. (**B, C**) The disease symptoms of KY131 leaves upon *F. verticillioides* exposure. For B, 14-d-old seedlings were treated as panel (A), then sprayed with *F. verticillioides* spore suspension (*F. V*) and sterile water (Mock) once a day for 6 days and

photographed. For (C), 14-d-old seedlings were treated as panel (A), then the leaves were immersed in *F. V* spore suspension for 5 days, then photographed.

Table S1. Primer sequences used in this study.

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`Figures.pdf` available at https://authorea.com/users/456409/articles/553457-a-novel-maize-microrna-negatively-regulates-the-resistance-to-fusarium-verticillioides