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Identification and characterization of miRNA169 family members in banana (*Musa acuminata* L.) that respond to *fusarium oxysporum f. sp. cubense* infection in banana cultivars

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MicroRNAs (miRNAs) play an important role in plant resistance to pathogen infections. However, little is known about the role of miRNAs in banana Fusarium wilt, which is the most economically devastating disease in banana production. In the present study, we identified and characterized a total of 18 miR169 family members in banana (*Musa acuminata* L.) based on small RNA sequencing. The banana miR169 family clustered into 2 groups based on miRNA evolutionary analysis. Multiple sequence alignment indicated a high degree of sequence conservation in miRNA169 family members across 28 plant species. Computational target prediction algorithms were used to identify 25 targets of miR169 family members in banana. These targets were enriched in various metabolic pathways that include the following molecules: glycine, serine, threonine, pentose, glycerolipids, nucleotide sugars, starch, and sucrose. Through miRNA transcriptomic analysis, we found that ma-miR169a and ma-miR169b displayed high expression levels, whereas the other 16 ma-miR169 members exhibited low expression in the HG and BX banana cultivars. Further experiments indicate that there were negative relationships between ma-miR169a, ma-miR169b and their targets basing on their expression levels to Foc4(*Fusarium oxysporum f. sp. cubense* tropical race 4) infection in resistant cultivars. but they were low expressed in susceptible cultivars. These results suggested that the expression levels of ma-miR169a and ma-miR169b were consistent with the resistance degree of the banana cultivars to Foc4. The analysis presented here constitutes a starting point to understand ma-miR169-mediated Fusarium wilt resistance at the transcriptional level in banana and predicts possible candidate targets for the genetic improvement of

banana resistance to Foc4.

1 **Identification and Characterization of miRNA169 Family Members in Banana (*Musa***
2 ***acuminata* L.) That Respond to *Fusarium oxysporum* f. sp. *cubense* Infection in Banana**
3 **Cultivars**

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18 **ABSTRACT**

19 MicroRNAs (miRNAs) play an important role in plant resistance to pathogen infections.
20 However, little is known about the role of miRNAs in banana Fusarium wilt, which is the most
21 economically devastating disease in banana production. In the present study, we identified and
22 characterized a total of 18 miR169 family members in banana (*Musa acuminata* L.) based on
23 small RNA sequencing. The banana miR169 family clustered into 2 groups based on miRNA
24 evolutionary analysis. Multiple sequence alignment indicated a high degree of sequence
25 conservation in miRNA169 family members across 28 plant species. Computational target
26 prediction algorithms were used to identify 25 targets of miR169 family members in banana.
27 These targets were enriched in various metabolic pathways that include the following molecules:
28 glycine, serine, threonine, pentose, glycerolipids, nucleotide sugars, starch, and sucrose. Through
29 miRNA transcriptomic analysis, we found that ma-miR169a and ma-miR169b displayed high
30 expression levels, whereas the other 16 ma-miR169 members exhibited low expression in the

31 HG and BX banana cultivars. Further experiments indicate that there were negative relationships
32 between ma-miR169a, ma-miR169b and their targets basing on their expression levels to
33 Foc4(*Fusarium oxysporum f. sp. cubense* tropical race 4) infection in resistant cultivars. but they
34 were low expressed in susceptible cultivars. These results suggested that the expression levels of
35 ma-miR169a and ma-miR169b were consistent with the resistance degree of the banana
36 cultivars to Foc4. The analysis presented here constitutes a starting point to understand ma-
37 miR169-mediated Fusarium wilt resistance at the transcriptional level in banana and predicts
38 possible candidate targets for the genetic improvement of banana resistance to Foc4.

39 INTRODUCTION

40 MicroRNAs (miRNAs) are a class of 18- to 22-nucleotide-long noncoding RNAs that play an
41 important role in gene regulation at the posttranscriptional level in plants (Slezak-Prochazka et
42 al., 2010; Bartel, 2004). Extensive research suggests that miRNAs play important roles in
43 various cellular processes, such as organ development, flowering, plant hormone signaling, and
44 plant responses to abiotic and biotic stresses (Samad et al., 2017; Zhang, 2015). Generally,
45 miRNAs silence mRNA molecules by cleaving the mRNA strand into two pieces with
46 completely complementary sequences and destabilizing the mRNA through the shortening of
47 its poly(A) tail with incompletely complementary sequences (Cuperus et al. 2011). However,
48 *Arabidopsis thaliana* miR172 was determined to be completely complementary to the open
49 reading frame (ORF) region of the target gene but was found to inhibit protein translation rather
50 than cleaving mRNA (Chen, 2004).

51 Plant growth and development are influenced by various environmental stresses. In recent years,
52 many studies have demonstrated the involvement of miRNAs in responses to abiotic stresses
53 such as drought, low temperature, high salinity and nutrient deficiency. Ath-miR394 plays a
54 positive role in the plant response to drought stress by blocking leaf water loss (Song et al.,
55 2013; Ni et al., 2012). The stress-induced expression of miRNAs is used to activate the plant
56 stress signaling system to improve the adaptability of plants to adversity. miR169 is induced by
57 drought stress and functions to increase plant resistance to drought stress in tomato (Zhang et al.,
58 2011), but it decreases drought resistance in *Arabidopsis* (Li et al., 2008). In addition to
59 controlling targets at the posttranscriptional level in response to abiotic stresses, miRNAs also
60 regulate plant responses to biotic stresses. Tae-miR164 negatively controls the regulation of the

61 target gene *TaNAC21/22*, which is used to improve resistance to stripe rust in wheat species
62 (Feng et al., 2014). NBS-LRR proteins make up the largest class of miRNA-mediated resistance
63 proteins, the genes for which are regulated by md-miRln11. md-miRln11 affects the resistance to
64 spot leaf blight in different disease-resistant apple varieties by interacting with its target gene,
65 *NBS-LRR*, and Md-miRln11 regulates *MdNBS* gene expression to aid in adaptation to
66 pathogen infection (Ma et al., 2014). The osa-miR164-regulated NAC is repressed in response to
67 pathogenic infection (Campo et al., 2013).

68 The miR169 family is one of the largest conserved miRNA families in the plant kingdom (Sorin
69 et al., 2014). Several studies in recent years have shown that miR169 family members are
70 responsive to abiotic stresses such as salinity, cold and drought in various plant species
71 (Miaoyun et al., 2016). miR169a is substantially downregulated in both roots and shoots after
72 nitrogen-starvation treatment in Arabidopsis (Zhao et al., 2011). The zma-miR169s and their
73 target *ZmNF-YA* genes exhibit diverse changes in expression patterns after stress treatment in
74 maize leaves (Luan et al., 2015). The overexpression and cleavage of GmNFYA3 mRNA is
75 governed by miR169, which causes reduced leaf area, high water loss and enhanced drought
76 tolerance in Arabidopsis (Ni et al., 2013). miR169 regulates stress-induced flowering by
77 repressing the *AtNFYA* transcription factor (Xu et al., 2013). miR169/miR169* double mutants
78 are essential for different regulation patterns of *NFYA5* caused by miR169a and miR169l in
79 Arabidopsis (Du et al., 2017). miR169 acts as a negative regulator in rice immunity against the
80 blast fungus *Magnaporthe oryzae* by repressing the expression of nuclear factor NF-YA genes (Li
81 et al., 2017). However, how miR169 functions in banana to boost immunity remains unclear.

82 Banana (*Musa acuminata* L.) is the most traded fruit species in the world, and it is widely
83 consumed as a food commodity worldwide (Sreedharan et al., 2013). Banana Fusarium wilt (also
84 known as Panama disease) is caused by *Fusarium oxysporum* f. sp. *cubense* (Foc) and is
85 considered to be the most limiting disease for banana production worldwide. It spreads mainly
86 through the soil and attacks banana plants of all ages, resulting in wilting and yellowing of
87 banana leaves. There are four physiological races of Foc. The history and impact of Fusarium
88 wilt can be summarized with an emphasis on tropical race 4 (TR4), a 'Cavendish'-killing variant
89 of the pathogen that has spread dramatically in banana-planting countries (Ploetz, 2015). Despite
90 substantial documented progress in planting banana-resistant cultivars and implementing
91 biological, chemical, and cultural measures, management is largely restricted to excluding *F.*

92 *oxysporum f. sp. cubense*, and there is no effective prevention method or developed strategy that
93 can prevent Fusarium wilt from spreading ([Ploetz, 2015](#); [Guo et al., 2014](#)). Recently,
94 transcriptomic analysis revealed a number of important genes related to salicylic acid signaling
95 transduction([Miao et al., 2017](#); [Li et al., 2013](#)), ethylene([Wang et al., 2017](#); [Wang et al., 2015](#)),
96 and auxin biosynthesis ([Song et al., 2016](#); [Hu et al., 2015](#)) involved in the response of banana to
97 Foc infection.

98 In the present study, we identified and characterized the miR169 (ma-miR169) family members
99 from the banana genome and further investigated their evolutionary relationship, target genes,
100 and expression patterns in various banana cultivars after Foc4 infection. This systematic study
101 will increase our understanding of miRNA169-mediated immunity to *Fusarium oxysporum f. sp.*
102 *cubense* in banana and lays a foundation for the genetic improvement of banana.

103 **METHODS**

104 **Computational Identification of miR169 Family Members**

105 In our previous study, we identified miR169 family members in banana using small RNA
106 sequencing ([Song et al., 2016](#)). The mature miR169 sequences of other plant species were
107 obtained from miRbase database (Release 21.0, <http://www.mirbase.org/>). The BLAST
108 parameters were the default routine settings of the database. The Latin name of miR169
109 members of plant species were got from the miRbase though the function of online database “
110 Search by miRNA name or keyword”.

111

112 **Prediction of miR169 Target Genes and Functional Analysis**

113 The putative target genes for ma-miR169 were predicted using the plant miRNA target
114 prediction online software psRNATarget (<http://plantgrn.noble.org/psRNATarget/>) with the
115 default parameter settings. We selected the V2 Scoring Schema (2017 release), and applied
116 Musa genomics (Musa acuminata V2, Banana Genome Hub, <http://banana-genome-hub.southgreen.fr/home1>). psRNATarget is a new web server designed to integrate and analyze
117 reverse complementary matching between target transcripts and small RNAs. Another important
118 function is the evaluation of target-site accessibility through calculation of the UPE (unpaired
119 energy) utilized to unfold the secondary structure around the miRNA target site in the mRNA.
120 The following parameters were used: penalty for opening gap = 2, penalty for extending gap =

122 0.5, expectation = 10, penalty for GU pair = 1, penalty for other mismatches = 1, HSP size =19
123 and seed region = 2-7 nucleotides. KEGG pathway analysis was performed to analyze the
124 metabolic pathways and functions of unigenes (<http://www.genome.jp/kegg/>).

125 **Phylogenetic Analysis of miR169 Family Members**

126 Molecular evolutionary analyses were conducted using the MEGA 7.0 program. To evaluate the
127 reliability of the different phylogenetic groups among miR169 family members, a phylogenetic
128 tree was constructed using the maximum likelihood (ML) algorithm in the MEGA 7.0 program.
129 Editing was performed in FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). The
130 sequences were aligned using ClustalW. Based on the neighbor-joining method, the molecular
131 evolutionary history was inferred using 1000 bootstrap replicates to assess the robustness of the
132 tree branches.

133 **Plant Material and Growth Conditions**

134 Young banana seedlings of four cultivars, named Baxi banana (abbreviated BX), HongYan
135 banana (abbreviated HY), FenJiao banana (abbreviated FJ), and BaoDaojiao banana (abbreviated
136 BDJ), were obtained. BX is a triploid (AAA) cultivar with high yield, high quality, and the
137 capacity for long-term storage. FJ is also a triploid cultivar, but with a different genotype (AAB),
138 and it is characterized by good flavor, rapid ripening, and tolerance to abiotic stresses. BX and FJ
139 are Fusarium wilt-susceptible cultivars that withers and die after infection. Other cultivars
140 exhibit some resistance to Fusarium wilt, and the infection rate can be controlled at 5–8%. Both
141 are widely cultivated in banana-planting areas. The seedlings used for RT-PCR were tissue
142 culture seedlings that were not exposed to the environment, and the seedling height was
143 approximately 6-8 cm. qRT-PCR seedlings needed to be transplanted, and the seedling height
144 was approximately 25-30 cm.

145 **Pathogen Infection and Microscopy Analysis**

146 All banana species were cultured and propagated separately. Foc4 was confirmed to efficiently
147 infect banana plants and induce disease symptoms. At the seedling stage, the hydroponic
148 suspension was replaced with a Foc4 spore suspension (1.5×10^6 conidia/mL). The pseudostem
149 and roots of the banana were sampled and stored at -70°C , and part of the postinfection banana
150 was transverse and longitudinally cut for microscopic examination. Macroelements and
151 microelements were supplied throughout the growing stage to maintain growth. The seedlings
152 were grown under a long photoperiod (16 h:8 h/light: dark).

153 **Primer design, cDNA synthesis and qRT-PCR Analysis**

154 In our previous study, ma-miR169a and ma-miR169b displayed high expression according to the
155 sequencing results. We used the reads per kilobases million values (Additional file 8) to build a
156 heatmap (Becker et al., 1998). First, we identified the expression of ma-miR169a ,ma-miR169b
157 and their targets. For this purpose, RT-PCR and qRT-PCR primers were designed according to
158 the predicted miRNA and target sequences (Additional file 6). The small RNA samples were
159 converted to miR-cDNA using an RT primer pool with reverse transcriptase. A specific primer
160 pair was designed for each miRNA, after which PCR amplification using a SYBR Premix Ex
161 Taq™ Kit (Takara, Dalian, China) was carried out with a Rotor-Gene 6000 machine (Corbett
162 Robotics, Australia). Quantification of the relative expression of miRNAs was performed using
163 the $\Delta\Delta$ CT method, and the U6 gene was used as a control. (Song et al., 2018). Quantification of
164 the target was also carried out through qRT-PCR-using the actin gene as a control. The primers
165 of the U6 and actin were listed in the additional file 6. The forward and reverse primer sequences
166 flanked the binding region in which the miRNA interacted with its target mRNA.

167 **Statistical Analysis**

168 The quantitative expression data were analyzed statistically using one-way ANOVA followed by
169 post hoc Tukey's HSD (honest significant difference) tests. The p value <0.01 was considered
170 statistically significant.

171 **RESULTS**

172 **Identification and Genomic Distribution of ma-miR169 Family Members in Banana**

173 Based on our previous miRNA transcriptomic data (Song et al., 2016), a total of 18 ma-miR169
174 members were identified from the banana genome. The sequences of the ma-miR169 family
175 were 18-22 nucleotides in length, and the sequence similarity reached 73.67% (Figure 1A).
176 Evolutionary analysis clustered the ma-miR169 family members into two groups (Figure 1B). To
177 identify other various plant ma-miR169 members, a genome-wide search was carried out using
178 the microRNA database, miRbase (<http://www.mirbase.org/cgi-bin/browse.pl>). A total of 209
179 mature miR169 sequences were predicted in 28 plant species (Additional file 1). The numbers of
180 miR169 family members differed greatly among the various species. Compared with the number
181 of miR169 members in other plant species, including 11 in *Arabidopsis thaliana*, 3 in *Oryza*
182 *sativa*, 8 in *Zea mays* and 17 in *Glycine max*, the number in banana, 18 was the highest (Figure
183 2). The sequences of the miR169 family members were 18-23 nucleotides in length, and

184 the sequence similarity reached 68.85% (Additional file 2), suggesting that the mature
185 sequences of these miR169 members were highly conserved.

186 **Prediction of ma-miR169 Targets**

187 Based on the 18 mature ma-miR169 sequences in banana, we used all the banana gene coding
188 sequences to predict the ma-miR169 targets with the psRNATarget online software(Li et al.,
189 2017). A total of 25 targets were obtained, and the number of targets differed greatly for the
190 different miR169 members. Compared with other miR169 members, ma-miR169h, ma-miR169f,
191 ma-miR169c, ma-miR169d, ma-miR169i, and ma-miR169k had more targets, with 3, 3, 2, 2, and
192 2 targets, respectively (Additional file 3). In addition, we found a complicated relationship
193 between miRNAs and their targets. ma-miR169b, ma-miR169c, ma-miR169e, ma-miR169g, ma-
194 miR169i, ma-miR169j, ma-miR169k, ma-miR169l, ma-miR169m, ma-miR169n, ma-miR169p,
195 and ma-miR169q had the same target (GSMUA_Achr8T16690_001); ma-miR169c and ma-
196 miR169k had the same target (GSMUA_Achr11T16520_001); and ma-miR169h and ma-
197 miR169f shared 3 targets (GSMUA_Achr10T02910_001, GSMUA_Achr4T09610_001, and
198 GSMUA_Achr8T20870_001). The targets of ma-miR169a (GSMUA_Achr4T12800_001) and
199 ma-miR169o (GSMUA_Achr2T19800_001) were unique and were not common to other ma-
200 miR169 members; a similar situation was observed for ma-miR169d
201 (GSMUA_Achr8T24960_001 and Achr9T12370_001) and ma-miR169i
202 (GSMUA_Achr1T03730_001 and Achr8T16690_001), which each had two targets.

203 **Functional Annotation of Target Genes**

204 Within the molecular function category, a large number of target genes were assigned to
205 transcription factors, cellular processes, binding, metabolic processes, and organelles. To
206 understand the biological function of these target genes, we got the metabolic pathway of those
207 targets using the KEGG database (KEGG PATHWAY Database, 2017;
208 <http://www.kegg.jp/kegg/>). Five target genes (GSMUA_Achr1T03730_001,
209 GSMUA_Achr2T19800_001, GSMUA_Achr4T12800_001, GSMUA_Achr8T20870_001, and
210 GSMUA_Achr11T16520_001) were marked in five metabolic pathways. These pathways
211 included glycine, serine and threonine metabolism; pentose and glucuronate interconversion;
212 glycerolipid metabolism; amino sugar and nucleotide sugar metabolism; and starch and sucrose
213 metabolism. (Additional file 4, 5).

214 **Expression Profiles of ma-miR169 members in Two Banana Varieties**

215 To investigate the expression patterns of the ma-miR169 family members, roots of HG and BX
216 were collected for transcriptome sequencing (Figure 3). Generally, when the value of FPKM
217 (Reads per Kilo bases per Million reads) is more than 20, it is considered to be high expression
218 level (Song et al., 2016; Chen et al, 2014), the FPKM of ma-miR169a and ma-miR169b were
219 68.41 and 36.17 respectively in HG, showing a high expression levels, In contrast, the other 16
220 ma-miR169 members FPKM values were between 0 to 12.32, exhibited low expression levels .
221 Thus, ma-miR169a and ma-miR169b may be important candidates for the next research.

222 **The involvement of ma-miR169a and ma-miR169b in the banana response to Foc4**

223 The most typical symptoms of the banana infected by Foc4 were brown spots or stains on the
224 roots and protocorms. . At 30 days after Foc4 inoculation, no or slight brown staining in the roots
225 and protocorms of HY (Figure 4A and 4J) and BDJ (Figure 4B,4C, 4K, 4L) were observed,
226 whereas there were obvious brown stains in the roots and protocorms of FJ and BX (Figure 4D
227 and 4G). These results indicate that HY and BDJ are more resistant to Foc4 than FJ and BX.

228 Because of the obvious phenotypic differences, this set of samples was also used for the
229 expression analysis of ma-miR169a and ma-miR169b. we synthesized reverse transcription
230 primers and qRT-pcr primers (Additional file 7), and cloned those miRNAs into HG and BX,
231 and confirmed the success of the ma-miR169a and ma-miR169b cloning through sequencing
232 (Figure 5). The expression levels of ma-miR169a and ma-miR169b were significantly increased
233 in the roots of HY and BDJ by Foc4 treatment comparing with controls and their increased fold
234 were approximately 3 to 43, in contrast, ma-miR169a and ma-miR169b showed low expression
235 or repression in the roots of FJ and BX after Foc4 treatment. Further experiments indicate that
236 there were negative relationships between ma-miR169a, ma-miR169b and their targets basing on
237 their expression levels to Foc4 infection in the roots of HY and BDJ, but they were low
238 expressed in FJ and BX(Figure 6; Additional file 8). Together, it suggested that the expression
239 characteristics of ma-miR169a and ma-miR169b were consistent with the resistance to Foc4 in
240 banana cultivars.

241 **DISCUSSION**

242

243 The miR169 family is one of the largest and most conserved miRNA families in plants (Miaoyun
244 et al., 2016). The miR169 family plays an important role in plant responses to abiotic and biotic
245 stresses (Zhang, 2015). The miR169 members information of plant species can be obtained in the

246 miRbase database, including miRNA precursors, target genes, however, due to the lack of a
247 miR169 reference sequence in the banana genome, the miR169 family of banana is not available
248 in miRbase currently, and little information is known. In this study, 18 miR169 family members
249 were identified in banana, a greater number than that in many plant species (Samad et al., 2017;
250 Zhang and Wang, 2015). These miRNA sequences from the ma-miR169 family are 18-22
251 nucleotides in length and are comparable to the miR169 family members in other plants (Noman
252 et al., 2017; Li et al., 2016). The nucleotide sequence homology of the ma-miR169 family was
253 observed to be 73.67%, indicating their conserved nature (Ni et al., 2013). Evolutionary analysis
254 divided the banana miR169 family members into two groups, which is consistent with the
255 findings of previous studies classifying miR169 members in poplar (Noman et al., 2017; Liu et
256 al., 2013). The miRNA target gene prediction algorithm provides a basis for the verification of
257 miRNA function. Several studies have proven that NY-FA family members, as the main targets
258 of miR169 family members, are involved in the development of plant root architecture(Sorin et
259 al., 2014), nodule formation(Zhao et al., 2011), disease resistance(Li et al., 2017), and abiotic
260 stress responses (; Ni et al., 2013). Notably, NY-FA family members participate in regulating
261 plant resistance to abiotic and biotic stresses mainly through the ABA pathway (Ding et al., 2016;
262 Luan et al., 2015; Zhao et al., 2009).

263 Overexpression of *HAP2* improves plant resistance to exogenous ABA in aspen and poplar.
264 Overexpression of *GmNFYA3* increases plant tolerance to drought and sensitivity to exogenous
265 ABA in soybeans (Ni et al., 2013). The ma-miR169 family has 25 target genes, mainly CK1_
266 casein kinase, single myb histone, plant neutral invertase, 60S ribosomal protein L12, UDP-
267 glucuronic acid decarboxylase, probable mono-galactosyl diacylglycerol synthase, and
268 erythronate-4-phosphate dehydrogenase. We have noted that CK1_ casein kinase was predicted
269 to be the target of 12 miR169 family members, suggesting their crucial function. CK1_ casein
270 kinases have also been found to be involved in plant responses to stress through the ABA
271 pathway. EL1-like casein kinases function in regulating the stability and function of ABA
272 receptors through phosphorylation (Chen et al., 2018). Casein kinase II mutants (*ckb1*) exhibit
273 reduced sensitivity to ABA and increased stomatal aperture, leaf water loss and proline
274 accumulation (Yuan et al., 2017). Plastid casein kinase 2 mutants show reduced ABA sensitivity
275 and thermotolerance (Wang et al., 2014). Together, these findings suggest that CK1_ casein
276 kinase may be the target of the ma-miR169 family and may play an important role in the

277 regulation of the ABA pathway. Previous reports have found that cca-miR169a, cca-miR169c
278 and cca-miR169h are expressed in female flowers of hickory (Sun et al., 2017). vvi-miR169e,
279 vvi-miR169f, and vvi-miR169g are expressed in the leaf, callus, and stem of grapevine,
280 respectively (Mica et al., 2009). Moreover, miR169 is widely involved in plant resistance to
281 abiotic and biotic stresses. Arabidopsis miR169 can promote leaf dehydration (Ni et al., 2012; Li
282 et al., 2008). miR169-overexpressing tomato plants show improved resistance to drought stress
283 (Zhang et al., 2011). Overexpression of miR169a results in hypersusceptibility to different
284 *Magnaporthe oryzae* strains by inhibiting its targets, NF-YA family members. Overexpression of
285 miR169o leads to susceptibility to bacterial blight in rice (Yu et al., 2018). Tomato miR169
286 negatively regulates NF-YA5 to enhance tomato resistance to gray mold (Li et al., 2016; Gu et
287 al., 2010).

288 In this study, we conducted a comprehensive expression analysis of ma-miR169a and ma-
289 miR169b, which exhibited high expression levels in HG and BX banana cultivars. Expression
290 was observed in the roots of four banana cultivars. These cultivars were HY, FJ, BX and BDJ.
291 However, the function of ma-miR169 in banana responses to *Fusarium wilt* remains to be
292 explored. In this study, we provided evidence that ma-miR169a and ma-miR169b were induced
293 in the roots of HY and BDJ (two cultivars resistant to Foc4) but repressed in FJ and BX (two
294 cultivars sensitive to Foc4) after Foc4 treatment. There was a negative relationship between ma-
295 miR169a,ma-miR169b and their targets basing on their expression levels to Foc4 infection.
296 Thus, the expression levels of ma-miR169a and ma-miR169b are positively correlated with the
297 resistance of banana cultivars to Foc4, implying a positive role of these miR169 family members
298 in immunity to Foc4 in banana.

299 CONCLUSIONS

300 This study identified 18 miR169 family members in banana. Their evolutionary relationships,
301 target genes, and expression patterns in various cultivars after Foc4 infection were systematically
302 analyzed. The ma-miR169a and ma-miR169b can be induced to upregulate by Foc4 and have
303 high expression level in banana resistant varieties. Our study will give a novel sight on miR169's
304 function which associated with *Fusarium wilt*.

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Figure 1

Figure 1. Multiple sequence alignment analysis of ma-miR169 family members.

A, Sequence similarity analysis; B, Evolutionary analysis clustered the ma-miR169 family.

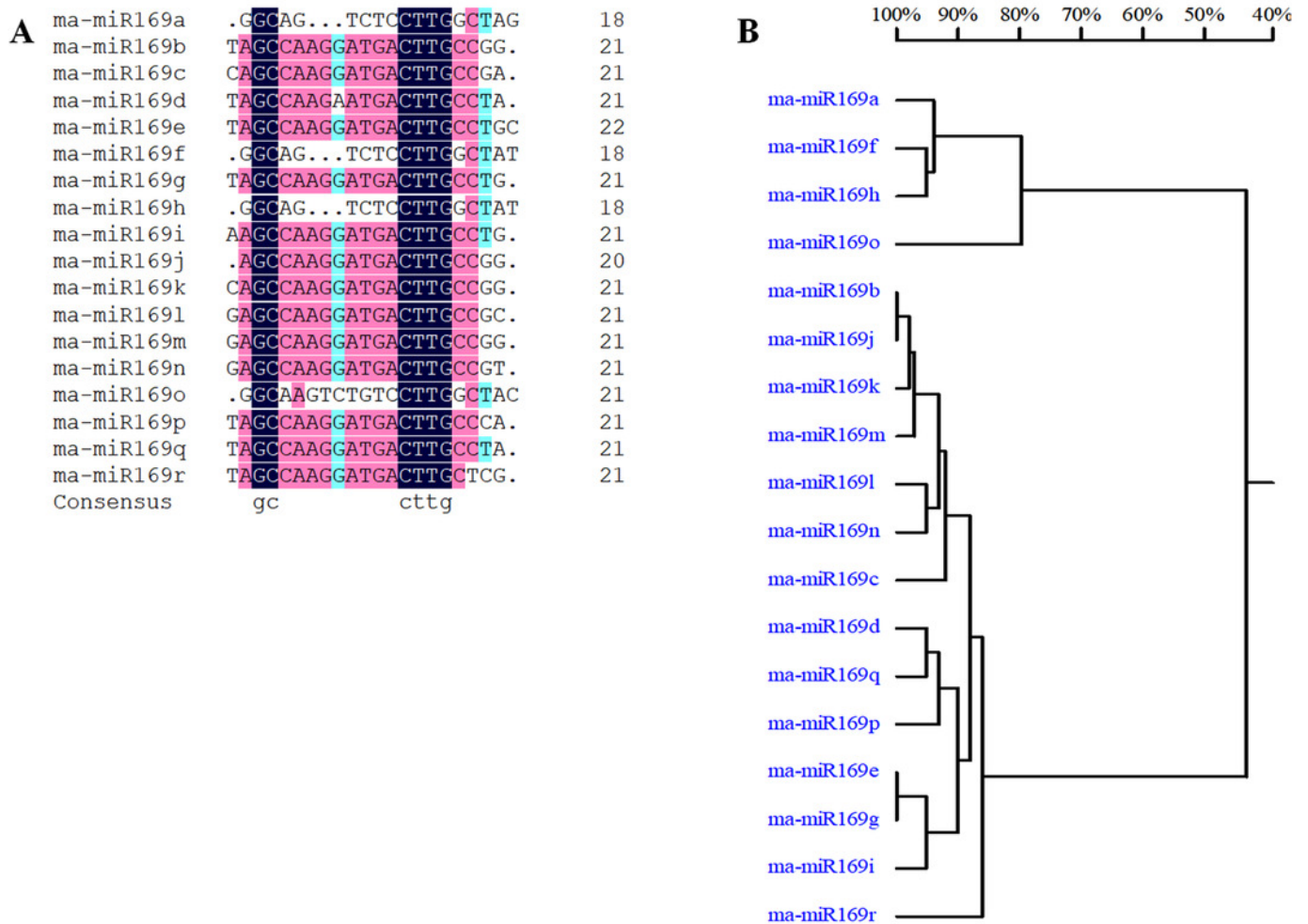


Figure 2(on next page)

Figure 2. The number of mature sequences from miR169 family members in different species.

aly, *Arabidopsis lyrata*; aqc, *Aquilegia caerulea*; ata, *Aegilops tauschii*; ath, *Arabidopsis thaliana*; bdi, *Sorghum bicolor*; bna, *Brassica napus*; cme, *Cucumis melo*. cpa (*Carica papaya*), csi (*Citrus sinensis*), ghr (*Gossypium hirsutum*), gma (*Glycine max*), lus (*Linum usitatissimum*), ma (*Musa acuminata* L.), mdm (*Malus domestica*), mes (*Manihot esculenta*), mtr (*Medicago truncatula*), nta (*Nicotiana tabacum*), osa (*Oryza sativa*), pde (*Pinus densata*), ppe (*Prunus persica*), ptc (*Populus trichocarpa*), rco (*Ricinus communis*), sbi (*Sorghum bicolor*), sly (*Solanum lycopersicum*), tcc (*Theobroma cacao*), vun (*Vigna unguiculata*), vvi (*Vitis vinifera*), zma (*Zea mays*).

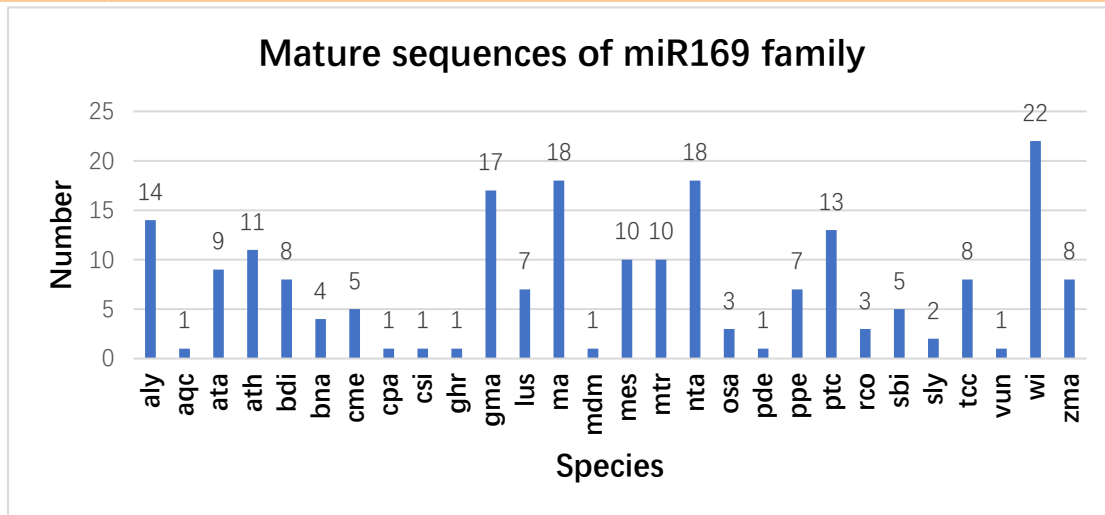


Figure 3 (on next page)

Figure 3. Expression of ma-miR169 family members.

The Y-axis represents ma-miR169 family members; the X-axis represents HG (Hainan Gong banana) and BX (Baxi banana).

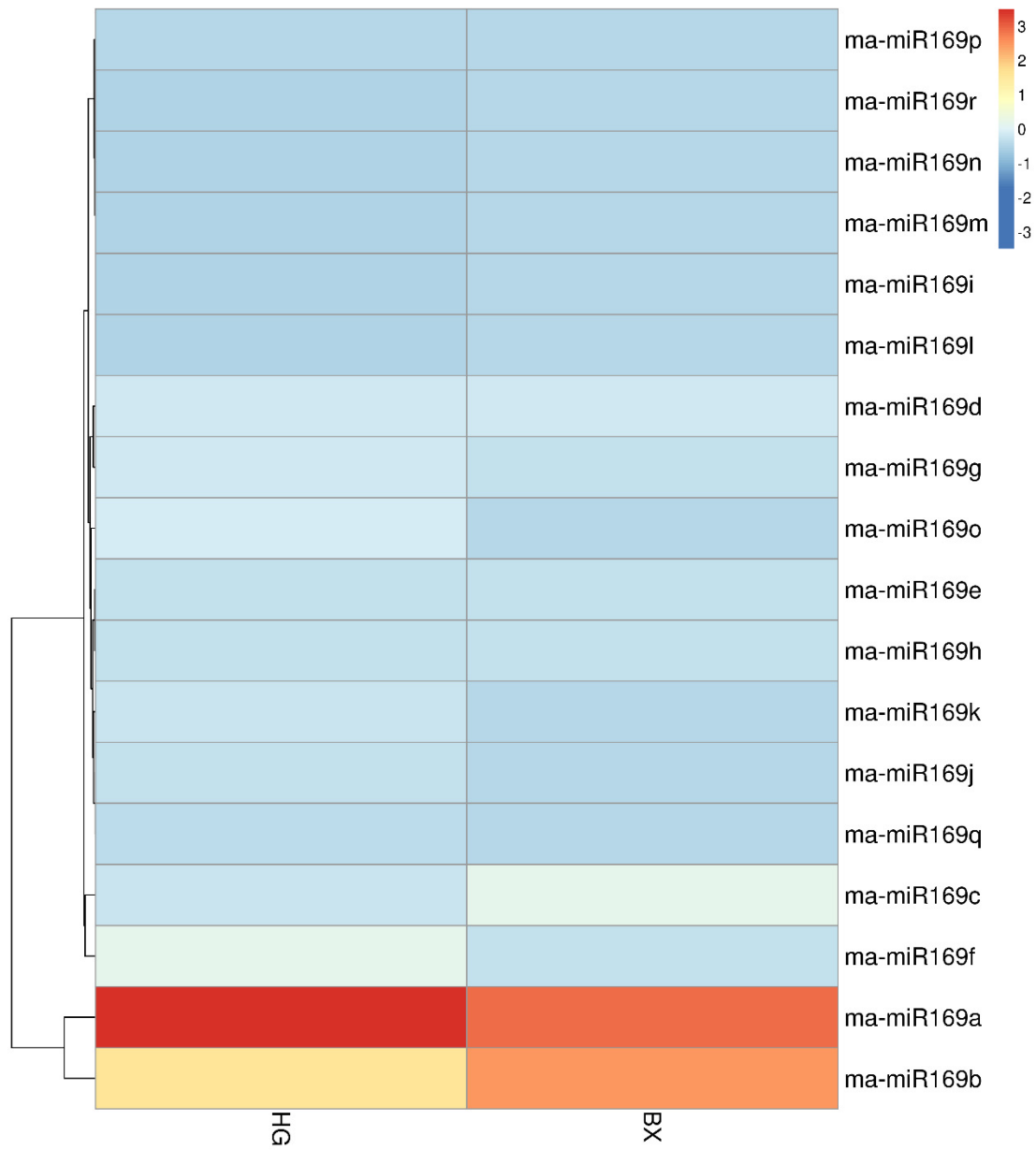


Figure 4

Figure 4. Disease symptoms from four banana cultivars. The roots and pseudostems after treatment with Foc4 are shown.

The symbols A, B and C represent HY; D, E and F represent FJ; G, H and I represent BX; J, K, and L represent BDJ. The A, D, G and J represent root crosscutting; B, E, H and K represent protocorm longitudinal cutting; C, F, I and L represent pseudostem crosscutting. The red arrow indicates the brown stain. All banana cultivars were examined 30 days after Foc4 inoculation.



Figure 5

Figure 5. RT-PCR detection of ma-miR169a and ma-miRNA169b expression.

A, Detection of ma-miR169a; B, Detection of ma-miR169b; M: DL2000; 1: The whole seedling of Hongyan banana; 2: The whole seedling of Fenjiao banana; 3: The whole seedling of Baxi banana; 4: The whole seedling of Baodaojiao banana.

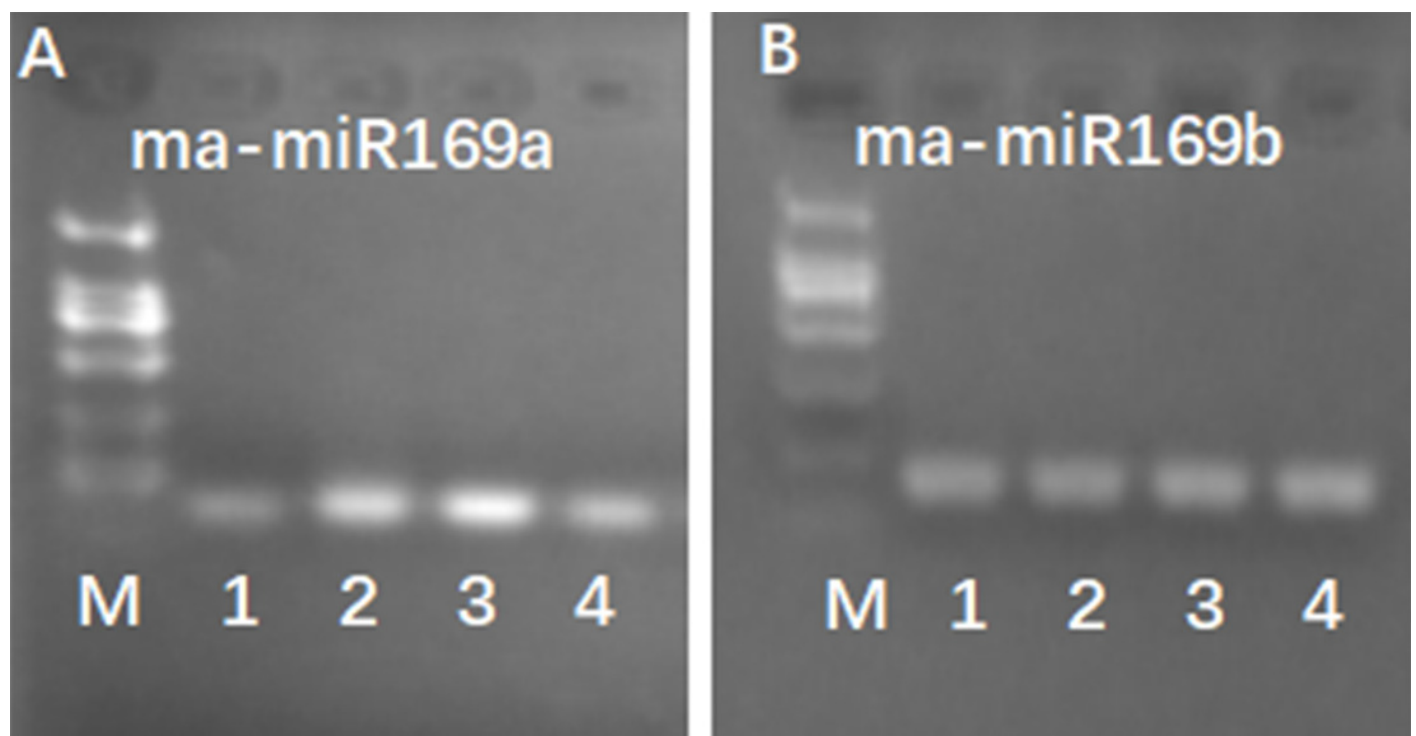


Figure 6

Figure 6. The Expression of ma-miR169a, ma-miR169b and their targets in root in response to Foc4

A-D, The expression of ma-miR169a and the target gene in different varieties of root; E-H, The expression of ma-miR169b and the target gene in different varieties of root. A and E represent HY(Hongyan banana); B and F represent FJ(Fenjiao banana), C and G represent BX(Baxi banana), D and H represent BDJ(Baodao jiao banana). The Y-main axis represents the percentage of U6 expression, the Y-sub-axis represents the percentage of actin gene expression; the X-axis represents control(CK, without Foc4 infection), and treatment(Foc4 infection). Expression was analyzed 30 days after Foc4 infection. The values represent the means, and the error bars represent the standard errors for independent experiments conducted in triplicate.

