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ghr-miR5272a-mediated regulation of GhMKK6 gene transcription contributes to the immune response in cotton

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Abstract

Fusarium wilt is a major biotic stress affecting the productivity of cotton (Gossypium hirsutum). Although mitogen-activated protein kinase (MAPK) cascades play critical roles in plant disease resistance, their intricate regulation under fungal stress remains unclear, especially with regards to microRNA-mediated regulation of MAPK gene expression. In this study, we report that the MAPK kinase gene GhMKK6 and ghr-miR5272a work together in cotton resistance to Fusarium wilt. Silencing GhMKK6 in cotton decreased resistance to F. oxysporum by repressing the expression of known disease-resistance genes. Furthermore, although GhMKK6 played a positive role in disease resistance, excessive GhMKK6 activation caused an excessive hypersensitive response. ghr-miR5272a, a major regulator, prevents this excessive response by regulating GhMKK6 expression. ghr-miR5272a targets the GhMKK6 3′-untranslated region in cotton. Overexpressing miR5272a decreased the expression of GhMKK6 and disease-resistance genes, and increased sensitivity to F. oxysporum, yielding a similar phenotype to GhMKK6-silenced cotton. Overall, these results demonstrate that the ghr-miR5272a-mediated regulation of GhMKK6 expression contributes to the immune response in cotton, and reveal a new feedback loop mechanism in plant disease response.

Key words: Cotton, feedback loop, Fusarium oxysporum, GhMKK6, ghr-miR5272a, Gossypium hirsutum, miRNA.

Introduction

Cotton (Gossypium hirsutum) is one of the most important economic crops in the world, but productivity is constrained by various biotic and abiotic stresses (Xie et al., 2015). Fungal diseases such as Fusarium and Verticillium wilt pose the largest threat to cotton (Zhang et al., 2016). These pathogens deliver effector molecules into plant cells to promote virulence and cause disease (Dangl and Jones, 2001). In particular, Fusarium wilt, which is caused by the phytopathogenic fungus Fusarium oxysporum f.sp. vasinfectum, is considered one of the most serious factors affecting yield and quality loss and is present in almost all cotton-growing regions worldwide (Gaspar et al., 2014). Two methods have traditionally been used against the pathogen: biological control measures, such as cultivar choice and crop rotation, and chemical control (Zhang et al., 2016). However, effective methods for controlling F. oxysporum infection are still lacking. The use of transgenic technology to control the cotton bollworm Helicoverpa armigera offers an alternative approach to enhance plant resistance to fungal pathogens. The mining of key resistance genes is considered the most effective method for developing pathogen-resistant varieties (Xu et al., 2016), and hence studies of cotton resistance genes and the molecular mechanism of disease resistance is very important.

Mitogen-activated protein kinase (MAPK) cascades are present in all eukaryotic organisms and function in succession to transmit a variety of cellular signals (Widmann et al., 1999).
Activation of MAPK cascades is a common mechanism for regulating innate immune responses in both animals and plants (Pedley and Martin, 2005; Rodriguez et al., 2010). A canonical MAPK cascade includes three tiered protein kinase modules: MAPK kinase kinase (MAPKKK) phosphorylates MAPK kinase (MKK), which in turn phosphorylates and activates downstream MAPKs (MAPK Group, 2002). MKKs are of particular importance (Jia et al., 2016), serving as key nodes for the convergence and divergence of signals in MAPK cascades. In Arabidopsis, MAPKKK1, MKK1/MKK2, and MAPK4 function together in a MAPK cascade to regulate innate immunity (Gao et al., 2008). mkk1/mkk2 mutant seedlings accumulate high levels of H2O2, display spontaneous cell death, constitutively express pathogenesis-related (PR) genes, and exhibit pathogen resistance (Gao et al., 2008; Pitzschke et al., 2009; Kong et al., 2012). The MKK3-mediated pathogen signalling pathway enhances resistance against Pseudomonas syringae pv. tomato DC3000 by positively regulating the expression of PR genes (Doci et al., 2007). AtMKK4/AtMKK5, which can activate the MAPKs MPK3 and MPK6, plays important roles in flagellin perception and innate immunity (Asai et al., 2002). Zhang et al. (2007) reported that AtMKK7 positively regulates plant basal and systemic acquired resistance (SAR) and that silencing MKK7 using antisense RNA not only compromises basal resistance but also blocks the induction of SAR. In contrast to model plants, our understanding of the function of MKKs under biotic stress in cotton is limited. In our previous studies, we have demonstrated that two group-C MKK genes, MKK4 and MKK5, are involved in cotton disease resistance. Overexpression of MKK4 enhanced sensitivity to bacterial and fungal pathogens (Li et al., 2014), while overexpression of MKK5 in Nicotiana benthamiana induced PR gene expression and enhanced resistance to the bacterial pathogen Ralstonia solanacearum (Zhang et al., 2012). These data collectively suggest that MKKs play an important role in plant disease resistance. However, the function of group-A MKK genes under fungal stress in cotton remains unclear.

MAPKs generally phosphorylate their target proteins, including enzymes or transcription factors, to control the synthesis of defence hormones and signalling molecules (Bolouri Moghaddam et al., 2016). However, few studies have examined the regulation of MAPK gene expression. In recent years, many MAPK genes have been predicted as targets of microRNAs (miRNAs), which are a class of small (18–24 nt), endogenous, non-coding RNAs (Bartel, 2009). miRNAs are widely distributed in plants and animals and negatively regulate gene expression at the transcriptional, post-transcriptional, and translational levels by targeting mRNAs for degradation and/or by repressing translation (Mallory and Vaucheret, 2004; Lanet et al., 2009; Chellappan et al., 2010). The role of miRNAs as transcriptional regulators in plant disease resistance has received increasing attention (Dugas and Bartel, 2004). High-throughput sequencing has identified many plant miRNA families that participate in the response to Pseudomonas syringae infection, including miR156, miR159, miR172, and miR393 (Zhang et al., 2011). The first evidence of a role for miRNAs in plant immunity was the miR393-mediated repression of auxin signalling in bacterial resistance (Navarro et al., 2006). In cotton, Yin et al. (2012) identified 215 miRNA families by high-throughput sequencing, including 14 new miRNAs. In addition, they found that the expression of 65 of the miRNA families changed dramatically after infection by Verticillium dahlia, miR482 and its target nucleotide binding site–leucine-rich repeat (NBS-LRR) defence genes play important roles in cotton disease resistance, and miR482 expression is inhibited and the expression of NBS-LRR genes is induced after V. dahlia infection (Zhu et al., 2013). Although MKKs and miRNAs play important roles in cotton disease resistance, little is currently known about the functions and relationships between them.

In this study, a new cotton group-A MKK gene, GhMKK6, was identified and found to be indispensable for the response to F. oxysporum. However, excessive GhMKK6 activation can lead to hypersensitive response-like cell death and give rise to lesion-mimicking phenotypes. The results from a series of genomic, genetic, transgenic, and virus-induced gene-silencing (VIGS) experiments suggest that the ghr-miR5272a-mediated control of GhMKK6 gene expression contributes to the immune response. Our findings provide important information regarding the relationship between MKKs and miRNAs in the immune response in cotton.

Materials and methods

Plant material, growth conditions, and stress treatments

Seeds of cotton (Gossypium hirsutum L. cv. Lumian 22) were germinated in wet linen. Germinated seedlings were then transplanted into hydroponic cultures under greenhouse conditions at 28 ± 1 °C with a 16-h light/8-h dark cycle and 60–75% relative humidity. For salicylic acid (SA) (10 mM) and methyl jasmonate (MeJA) (100 μM) treatments, cotyledons were sprayed with each chemical. For the pathogen treatment, seedlings were inoculated with conidial Fusarium oxysporum suspensions (106 conidia ml–1) using the root-dip method. For expression pattern analyses, cotyledons from treated plants were collected, frozen in liquid nitrogen, and stored at −80 °C for RNA extraction. For F. oxysporum treatments that were performed on GhMKK6-silenced or amiR5272a-overexpressing cotton, the plants were inoculated with conidial F. oxysporum suspensions (105 conidia ml–1) using the root-dip method. Nicotiana benthamiana seeds were surface-sterilized and germinated on Murashige–Skoog (MS) medium under greenhouse conditions at 25 ± 1 °C with a 16-h light/8-h dark cycle and 60–75% relative humidity. The seedlings were then transplanted at the 2–3-leaf stage into soil and grown under greenhouse conditions. For F. oxysporum treatments that were performed on GhMKK6-overexpressing tobacco, the leaves were injected with 100 μl of conidial F. oxysporum suspensions (106 conidia ml–1) using syringes. Each treatment was repeated at least three times.

Gene cloning and bioinformatics analysis

The GhMKK6 gene was isolated from a cotton cDNA library via PCR (primers are listed in Supplementary Table S1 at JXB online). GhMKK6-homologous protein sequences were retrieved from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned using DNAMAN 5.2.2 software (Lynnon Biosoft). The dendrogram was generated using the neighbour-joining method in MEGA 5.0 software.
RNA extraction and qRT-PCR analysis

Total RNA was isolated from cotton seedlings and tobacco leaves using the CTAB method (Wang et al., 2011) and the TRIZol reagent (Takara, Japan), respectively. First-strand cDNA was synthesized using an EasyScript First-Strand cDNA Synthesis SuperMix kit (TransGen Biotech, Beijing, China). For miRNA qRT-PCR, first-strand cDNA was synthesized using a Mir-X™ miRNA First Strand Synthesis Kit (Takara, Japan). The miRNA-specific primer was designed based on the mature miRNA sequence (see Supplementary Table S1). qRT-PCR was performed using SYBR Premix Ex Taq (Takara) in a 20-μL reaction volume on a CFX96™ Real-time Detection System (Bio-Rad). The PCR program as follows: pre-denaturation at 95 °C for 30 s; 40 cycles of 95 °C for 30 s, 55 °C for 15 s and 72 °C for 15 s; and a melt cycle from 65 to 95 °C. The 2−ΔΔCT method was used to determine the relative expression levels. The UBI and beta-actin genes from G. hirsutum and N. benthamiana, respectively, were used as standard controls. The primers used for qRT-PCR are listed in Supplementary Table S1 and S2.

Vector construction, genetic transformation, and site-directed mutagenesis

The GhMKK6 ORF was cloned and inserted into the pRI 201-AN-GUS vector (Takara, Japan). The genetic transformation was performed as described previously by Lu et al. (2013). The T3 progeny of the empty vector or transgenic plants were used for further functional studies.

A constitutively active mutant of GhMKK6, GhMKK6EE, was obtained as described previously by replacing the conserved Ser-219 and Thr-225 with Glu (Asai et al., 2008). The inactive GhMKK6 mutant, GhMKK6AA, in which the conserved Ser-219 and Thr-225 were replaced with Ala, was obtained via the same method. The activities of GhMKK6EE and GhMKK6AA have been tested (see Supplementary Fig. S7). The primers used for mutagenesis are shown in Supplementary Table S1.

ghr-miR5272a overexpression and suppression assays and virus-induced gene silencing (VIGS)

The method was performed as described by Gu et al. (2014). Two GhMKK6 fragments (nucleotides 451–934 and nucleotides 136–522), a small tandem target mimic sequence (containing two imperfect ghr-miR5272a binding sites separated by a 48-bp spacer; see Supplementary Fig. S7), were used for assays 3 weeks after inoculation. Each assay was performed with at least three independent replicates.

MAPK activation assays

Proteins were extracted from leaves using extraction buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 50 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, phosphatase and protease inhibitors (PhosStop™ and EDTA-free Complete™, Roche)]. MAPK activation assays were performed as described previously by Ovečka et al. (2014). The anti-pTEpY phospho-p44/42 MAPK antibody (Cell Signaling Technology, USA) was used to detect the activation of MAPKs.

Histochemical staining assay

For 3'-diaminobenzidine (DAB) staining, leaves were soaked in a solution containing 1 mg mL−1 DAB (pH 3.8) in the dark for 12 h. Next, chlorophyll was removed from the leaves using 95% ethanol. Trypan blue staining was performed as described previously by Zhang et al. (2012). β-Glucuronidase (GUS) staining was carried out as described by Jefferson et al. (1987).

Statistical analysis

All experiments were performed at least three times. Statistically significant differences between measurements were determined using the Tukey HSD test in IBM Statistical Product and Service Solutions (SPSS) Statistics software version 19 (IBM, USA).

Accession numbers

The GhMKK6 sequence can be found at http://cottongen.org under the Unique Name CotAD_25213_BGI-AD1_v1.0. Sequence data for the other cotton genes discussed in this paper can be found at http://cottongen.org under the following Unique Names: GhMKK5 (CotAD_01560_BGI-AD1_v1.0), GhNPR1 (CotAD_22990_BGI-AD1_v1.0), GhICS1 (CotAD_50284_BGI-AD1_v1.0), GhEDSI1 (CotAD_31858_BGI-AD1_v1.0), GhPAD4 (CotAD_57749_BGI-AD1_v1.0), GhJAZ1 (CotAD_21952_BGI-AD1_v1.0), GhJAZ3 (CotAD_67052_BGI-AD1_v1.0), GhAOS (CotAD_73823_BGI-AD1_v1.0), GhAO4 (CotAD_21497_BGI-AD1_v1.0), GhRbohB (CotAD_38218_BGI-AD1_v1.0), and GhSK11 (CotAD_53331_BGI-AD1_v1.0). Sequence data regarding the Arabidopsis genes discussed in this paper can be found in the TAIR database (http://arabidopsis.org) under the following accession numbers: AtMKK1 (AT4G26070), AtMKK2 (AT4G29810), AtMKK6
Results

**GhMKK6 is indispensable for cotton resistance to** _Fusarium oxysporum_

Previous studies have reported that the MKK6-mediated MAPK cascade pathway plays important roles in plant growth and development, production of reactive oxygen species (ROS), and disease resistance in many model plants (Asai et al., 2008; Kosetsu et al., 2010; Deng et al., 2016). MKK6 is an important component and a key regulator of this cascade. To determine the functions of the MKK6 gene in cotton, a cDNA sequence that displayed high similarity to *AtMKK6* and *NtMEK1* was identified. The full-length *GhMKK6* cDNA consisted of a 50-bp 5′-UTR, a 158-bp 3′-UTR, and a 1065-bp ORF. The ORF encodes a 354-amino-acid protein with a calculated molecular mass of 39.868 kDa and an isoelectric point of 6.73. Sequence analysis of *GhMKK6* revealed strong homology with *AtMKK6* (88.20%) and *NtMEK1* (88.42%) (Fig. 1A). The deduced amino acid sequence of *GhMKK6* was found to contain a kinase domain (S/TXXXXXS/T) from amino acids 219 to 225 (Fig. 1A). Further dendrogram analysis indicated that *GhMKK6* belongs to the group-A MKKs and is closest to *AtMKK6* and *NtMEK1* (Fig. 1B). Next, the pattern of *GhMKK6* expression was determined using qRT-PCR. *GhMKK6* was expressed in all tissues investigated in this study, particularly in the roots (Supplementary Fig. S1). As shown in Fig. 1C, *GhMKK6* expression was reduced by phytohormones such as SA and MeJA as well as by _F. oxysporum_ treatment.

To investigate the biological role of *GhMKK6*, *Agrobacterium*-mediated VIGS was used to silence *GhMKK6* in cotton. Three weeks after *Agrobacterium* infiltration, *GhMKK6* RNA and protein levels were significantly reduced (the region of *GhMKK6* used for silencing was nucleotides 451–934) (Fig. 2A). To examine the role of *GhMKK6* in the defence response to *F. oxysporum*, *CRV::00* (empty vector control) and *CRV::GhMKK6* (*CRV::01*, *CRV::02*, and *CRV::03*) were root-wounded and placed in a *F. oxysporum* spore suspension using the root-dip method. At 5 d after inoculation, *CRV::GhMKK6* leaves showed serious signs of chlorosis, whereas *CRV::00* leaves exhibited only mild disease (Fig. 2B). The pathogen disease index also showed that *CRV::GhMKK6* leaves accumulated more *F. oxysporum* than those from *CRV::00* (Fig. 2C). We then analysed the expression patterns of several genes in the SA- or JA-mediated defence pathways. As shown in Fig. 2D, the expression levels of SA-mediated
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genes (EDS1, ICS1, NPR1, and PAD4) were obviously lower in CRV::GhMKK6 leaves than in CRV::00 leaves. In contrast, the expression levels of JA-mediated genes (AOC4, AOS, JAZ1, and JAZ3) did not differ significantly between CRV::GhMKK6 and CRV::00 leaves. Because the MAPK cascade pathway can regulate both early and long-term plant stress responses, the expression of SA- and JA-mediated defence-pathway genes was also determined at 4 h and 12 h after F. oxysporum inoculation (see Supplementary Fig. S2A, B). At 4 h after F. oxysporum inoculation, the expression of SA-mediated genes exhibited no obvious changes (Fig. S2A), and only the expression of NPR1 was reduced in CRV::GhMKK6 leaves at 12 h after inoculation (Fig. S2B). To prove the validity of our data, we used VIGS to silence GhMKK6 using another fragment from nucleotides 136–522 (CRV::01-2 and CRV::02-2) (Supplementary Fig. S3B). The expression levels of SA-mediated genes were lower in CRV::01-2 and CRV::02-2 than those in CRV::00, and the expression levels of JA-mediated genes did not differ significantly (Fig. S3C). These results indicated that GhMKK6 may be associated with the SA-mediated defence pathway and play an important role in fungal defence in cotton.

Excessive GhMKK6 activation leads to lesion-mimicking phenotypes

In recent studies, silencing MEK1 (the gene homologous to GhMKK6) in tobacco was found to decrease resistance to pathogens (Asai et al., 2008; Liu et al., 2004), and we noticed that silencing GhMKK6 in cotton produced similar phenotypes to those observed after pathogen infection. To determine the function of GhMKK6 in plant disease resistance, we overexpressed GhMKK6 in N. benthamiana. Three independent lines were selected for further functional analysis (see Supplementary Fig. S4).

To confirm the resistance of GhMKK6-overexpressing (OE) plants to F. oxysporum, we examined 6-week-old empty vector (Vec) and transgenic lines. At 3 d after F. oxysporum inoculation, the MAPK phosphorylation levels in transgenic plants were much higher than those in Vec plants (see Supplementary Fig. S5). At 4 h and at 3 d after F. oxysporum inoculation, changes were detected in the expression patterns of several genes in the SA- and JA-mediated defence pathways. The expression levels of the selected genes in the OE lines were higher than those in the Vec plants (shown at 4 h...
in Supplementary Fig. S6 and at 3 d in Fig. 3A). Although pathogen growth assays showed that the F. oxysporum disease indices in leaves from OE lines and Vec plants were not obviously different (Fig. 3B), the OE lines showed serious signs of chlorosis (Fig. 3D). Histochemical staining with Trypan blue (depicting cell death) and DAB (depicting ROS accumulation) showed that OE leaves accumulated more of the stains than the Vec leaves (Fig. 3D).

Based on the above results and previous studies, we speculated that excessive GhMKK6 activation causes lesion-mimicking phenotypes in GhMKK6-overexpressing lines after F. oxysporum inoculation. To confirm our hypothesis, we constructed a constitutively active GhMKK6 mutant, GhMKK6EE, to simulate activated GhMKK6, and an inactive version of GhMKK6, GhMKK6AA, and expressed each under the control of the CaMV 35S promoter (see Supplementary Fig. S7A). Then, GhMKK6, GhMKK6EE, and GhMKK6AA were expressed transiently by Agrobacterium-infiltration in N. benthamiana (Fig. 4B). At 5 d after infiltration (=120 h post-infiltration, hpi), leaves expressing GhMKK6EE showed the lesion-mimicking phenotype (Fig. 4A). The expression patterns of SA- and JA-mediated defence-pathway genes were also examined. As shown in Fig. 4D, while the expression levels of those genes in GhMKK6 plants were normal, they were remarkably higher in GhMKK6EE leaves than in GhMKK6 or GhMKK6AA leaves. The respiratory burst oxidase homolog protein B (RbohB) plays important roles in ROS production, and RbohB expression levels in OE- and GhMKK6EE-expressing leaves were increased (Fig. 3C and 4C). At 3 d after N. benthamiana leaves were infected with GhMKK6, GhMKK6EE, and GhMKK6AA, detached leaves were placed in an F. oxysporum spore suspension (10^6 conidia ml^-1). Although all of the leaves exhibited signs of chlorosis, the pathogen growth assay showed that leaves expressing GhMKK6EE accumulated less F. oxysporum than those expressing GhMKK6AA (Supplementary Fig. S7B, C). Thus, we confirmed that excessive GhMKK6 activation engendered a lesion-mimicking phenotype.

GhMKK6 is a target of ghr-miR5272a

Based on these results, we deduced that although GhMKK6 played a positive role in cotton disease resistance, excessive GhMKK6 activation could be harmful. From the expression pattern assays, we speculated that the transcriptional and/or post-transcriptional regulation of GhMKK6 may be used to avoid excessive GhMKK6 activation. miRNAs are ubiquitous transcriptional and/or post-transcriptional regulators in plants, and previous studies have shown that they play important roles in cotton disease resistance (He et al., 2014). By analysing high-throughput sequencing data from previous studies, we found that ghr-miR5272a matched the GhMKK6 3´-UTR (Xie et al., 2015). In nature, a functional miRNA and its target can contain up to five mismatches (Schwab et al., 2005); our analysis revealed 3.5 mismatches within the predicted complementary region between ghr-miR5272a and GhMKK6.
and GhMKK6, with G:U pairs counting as 0.5 mismatches (Fig. 5A). This result indicated that ghr-miR5272a might cleave the GhMKK6 transcript.

To investigate whether ghr-miR5272a cleaves GhMKK6 mRNAs, we expressed ghr-miR5272a using the precursor of ath-miR319a as a backbone (Gu et al., 2014). We exchanged the native ath-miR319a sequence with ghr-miR5272a, yielding amiR5272, which was then inserted into the pRI 201-AN vector (the GUS gene was removed). Agrobacterium-mediated transient expression was used to analyse the cleavage of GhMKK6 mRNA by amiR5272. Leaves transformed with pRI 201-AN-GUS (harbouring the GUS gene) were used as a control. As shown in Fig. 5B, leaves transiently expressing 35S::GUS, 35S::GUS-GhMKK6 + 3 (containing the GhMKK6 ORF and 3′-UTR), and 35S::GUS-GhMKK6 (only containing the GhMKK6 ORF) showed similar GUS levels. In contrast, leaves inoculated with 35S::amiR5272 showed no GUS signal. When 35S::GUS-GhMKK6 + 3 was co-transformed into leaves with 35S::amiR5272, only very weak GUS staining was detected compared with leaves that only expressed 35S::GUS-GhMKK6 + 3. Meanwhile, leaves inoculated with both 35S::GUS-GhMKK6 and 35S::amiR5272 showed no obvious change in GUS staining compared with leaves that only expressed 35S::GUS-GhMKK6. The levels of GhMKK6 expression in the transient leaves were also analysed. As shown in Fig. 5C, GhMKK6 mRNA levels were reduced in leaves co-transformed with 35S::GUS-GhMKK6 + 3 and 35S::amiR5272. The protein level of GhMKK6 was also obviously reduced when ghr-miR5272a was co-expressed with GUS::GhMKK6 + 3 but not with GUS::GhMKK6 (see Supplementary Fig. S8). In N. benthamiana, NbMEK1 cannot be targeted by ghr-miR5272a due to the limited complementarity of the sequence (Supplementary Fig. S9A). Ectopic expression of ghr-miR5272a in N. benthamiana leaves did not obviously alter NbMEK1 expression (Fig. S9B). This result shows that ghr-miR5272a targets GhMKK6.

ghr-miR5272a characterization and expression patterns in cotton

We determined ghr-miR5272a expression after SA, MeJA, or F. oxysporum treatment to see if a response could be detected. As shown in Fig. 6A, ghr-miR5272a expression peaked at 1 h and decreased after 6 h following MeJA treatment. Treatment with F. oxysporum also obviously induced ghr-miR5272a expression (Fig. 6A). However, no significant changes in ghr-miR5272a expression were detected following SA treatment (see Supplementary Fig. S10). Thus, ghr-miR5272a expression behaved reciprocally to that of GhMKK6 upon treatment with MeJA or F. oxysporum (Fig. 6A). These results indicated that GhMKK6 is transcriptionally and/or post-transcriptionally regulated by ghr-miR5272a in vivo.

ghr-miR5272a affects the cotton immune response to Fusarium oxysporum

Many studies have reported that short tandem target mimics (STTMs) can be used to inhibit miRNA function (Yan et al.,...
To investigate the function of ghr-miR5272a, we constructed the pCLCrVA-STTM vector, which contains two imperfect ghr-miR5272a binding sites separated by a 48-bp spacer described by Yan et al. (2012) (see Supplementary Fig. S11A). At 3 weeks after Agrobacterium infiltration, the expression of ghr-miR5272a was reduced (Fig. S11B). Leaves from CRV::00 and CRV::STTM were then placed in F. oxysporum spore suspensions. As shown in Fig. S11C, GhMKK6 expression was not significantly altered in CRV::STTM cotton. At 5 d after inoculation, the expression of several SA- and JA-mediated defence pathway genes was detected and, as shown in Fig. S11D, the accumulation of these genes was increased.

To further investigate the function of ghr-miR5272a, we inserted the amiR5272 precursor into the pCLCrVA vector to produce pCLCrVA-amiR5272. At 3 weeks after Agrobacterium infiltration, qPCR and western blotting were performed to detect GhMKK6 and ghr-miR5272a expression. As shown in Fig. 6B, miR5272a transcript abundance was significantly increased in CRV::amiR cotton compared with CRV::00. As a target of ghr-miR5272a, GhMKK6 transcription and translation were reduced in the CRV::amiR lines (Fig. 6E).

To examine the resistance of CRV::amiR cotton to infection, CRV::00 and CRV::amiR were root-wounded and inoculated with F. oxysporum spore suspensions using the root-dip method. At 5 d after inoculation, the CRV::amiR leaves showed more serious signs of chlorosis than the CRV::00 leaves, and they also accumulated more F. oxysporum (Fig. 6C, D). The expression patterns of several SA- and JA-mediated defence pathway genes were also examined, and the expression levels of SA-mediated genes (EDS1, ICS1, NPR1, and PAD4) were clearly lower in CRV::amiR leaves than in CRV::00 leaves (Fig. 6F). In contrast, the expression levels of JA-mediated genes (AOC4, AOS, JAZ1, and JAZ3) were unaffected. These results showed that GhMKK6-silenced and ghr-miR5272a-overexpressing cotton have the same expression profiles under F. oxysporum treatment.

**Discussion**

The role of MKK6 has been widely studied in model plants. In Arabidopsis, ANPs, MKK6/ANQ (homologous to MKK6 in Arabidopsis), and MPK4 function together in a MAPK cascade that is required for cytokinesis (Krysan et al., 2002; Kosetsu et al., 2010), and the mkk6/anq mutant exhibits severe cytokinesis defects (Takahashi et al., 2010). However, in tobacco, the MKK1/MKK2-mediated MAPK pathway plays a different role in plant immune responses. MKK1 (which is homologous to MKK6 in tobacco) -NTF6 participates in the regulation by INF1 elicitin of bursts of ROS, and constitutively expressing MEK1DD induces RbohB-dependent oxidative bursts (Asai et al., 2008). Liu et al. (2004) also reported that the MEK1-NTF6 pathway was involved in virus immunity in N. benthamiana; silencing MEK1 using VIGS decreased resistance to Tobacco mosaic virus. These data collectively suggest that the function of MKK6 is very complex, and hence studying it in other plants is very important to understand the molecular mechanisms of plant disease resistance. In the present study, we confirmed that GhMKK6 also plays an important role in cotton disease resistance, and silencing GhMKK6 decreased tolerance to Fusarium wilt and...
Fig. 6. ghr-miR5272a overexpression sensitizes cotton to *F. oxysporum*. (A) ghr-miR5272a expression patterns in cotton under MeJA and *F. oxysporum* treatments. (B) miR5272 expression levels in cotton transfected with pCLCrVA-amiR5272. (C) Pathogen disease index in amiR5272-overexpressing plants at 5 d after *F. oxysporum* infection. (D) Representative phenotypes of amiR5272-overexpressing plants infected with *F. oxysporum* (5 d after infection). (E) GhMKK6 mRNA relative expression and protein levels in cotton transfected with pCLCrVA-amiR5272. (F) qRT-PCR analysis of expression of SA- and JA-mediated defence pathway genes in amiR5272-overexpressing plants at 5 d after *F. oxysporum* infection. CRV::00 served as the empty vector control. Data in (A–C, E, F) are means ± SE of three independent experiments (*n* = 6). Different letters indicate significant differences (*P* < 0.05) based on Tukey’s HSD test. (This figure is available in colour at *JXB* online.)
decreased the expression of several resistance genes, including *EDS1*, *PAD4*, and *ICS1* (Fig. 2). However, gain-of-function analyses showed that excessive *GhMKK6* activation over-balanced SA-mediated defence responses and ROS production, and resulted in lesion-mimicking phenotypes (Fig. 3). Although the use of overexpression of *GhMKK6* in tobacco to determine its function in cotton has some limitations, our data do indicate the potential function of *GhMKK6* in cotton. Interestingly, we noticed that many genes involved in JA biosynthesis, such as *AOS* and *AOC4*, and in JA signalling, such as *JAZ1* and *JAZ3*, were up-regulated in *GhMKK6*-overexpressing lines, whereas the expression of these genes showed no significant changes in *GhMKK6*-silenced cotton (Figs. 2 and 3). These results imply that *GhMKK6* may also participate in the JA-mediated defence pathway, and other genes may play the same role in JA-mediated defence responses when *F. oxysporum* infects cotton. Interestingly, we noticed that many genes involved in JA biosynthesis, such as *AOS* and *AOC4*, and in JA signalling, such as *JAZ1* and *JAZ3*, were up-regulated in *GhMKK6*-overexpressing lines, whereas the expression of these genes showed no significant changes in *GhMKK6*-silenced cotton (Figs. 2 and 3). These results imply that *GhMKK6* may also participate in the JA-mediated defence pathway, and other genes may play the same role in JA-mediated defence responses when *F. oxysporum* infects cotton. They found that ghr-miR5272a expression was higher in plants infected with *V. dahlia* D07038 (an intermediately aggressive strain) than in plants infected with *V. dahlia* V991 (a highly toxic strain). In our study, we characterized the expression patterns and biological functions of ghr-miR5272a and provided evidence of ghr-miR5272a-mediated *GhMKK6* expression regulation as a new regulatory mechanism to prevent excessive autoimmunity in cotton under *F. oxysporum* infection. Due to the limitations of experimental conditions, transient transfections in cotton are very difficult. Tobacco was therefore used instead, and our results showed that ghr-miR5272a could cleave *GhMKK6* mRNA in vivo (Fig. 5). However, the cleavage site may not exist in *N. benthamiana* MKK1, as determined by a bioinformatics analysis, and transient expression of ghr-miR5272a in *N. benthamiana* showed no obvious effects on MKK1 expression. Therefore, this miR5272a-mediated gene expression regulation mechanism may be limited to certain species. In plants, feedback loops are important regulatory mechanisms during development and stress responses. In Arabidopsis, the bHLH transcription factor *HBII* functions as a major node in a complex feedback loop that mediates the trade-off between growth and immunity (Fan et al., 2014). Another bHLH transcript factor, *MYC2*, is a component of the MKK3–MPK6–MYC2 feedback loop, which is involved in blue-light-mediated seedling development (Sethi et al., 2014). As with many transcription factors, miRNAs also play important roles in feedback loop mechanisms. Arabidopsis miR171 and its target scarecrow-like proteins constitute a feedback loop that is critical for mediating gibberellin–DELLA signalling in light (Ma et al., 2014). In the current study, we report a new feedback loop that includes miR5272a and the MAPK cascade (Fig. 7). When *F. oxysporum* infects cotton, *GhMKK6*-mediated signal transduction is
activated. This signalling pathway induces the expression of downstream resistance genes, ROS production, and SA- and/or JA-mediated defence responses (see Supplementary Fig. S12). Similar to SA-mediated defence responses, pathogen-activated respiratory bursts have also been implicated in controlling HR (Yun et al., 2011). Uncontrolled HR harms plants, and in cotton ghr-miR5272a expression acts to prevent an excessive HR by regulating GhMKK6 expression after F. oxysporum infection. Taken together, our data suggest that GhMKK6 is critical for cotton disease resistance and that the regulatory function of ghr-miR5272a in the immune response is based on its GhMKK6 mRNA-cleavage activity.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Relative expression of GhMKK6 in different tissues.

Fig. S2. Expression levels of SA- and JA-mediated defence pathway genes in GhMKK6-silenced cotton after F. oxysporum infection.

Fig. S3. Silencing of GhMKK6 by the fragment from nucleotides 136 to 522 in cotton reduces resistance to F. oxysporum.

Fig. S4. Expression levels of GhMKK6 in transgenic lines.

Fig. S5. The activation of MAPKs in empty vector and transgenic tobacco lines with or without F. oxysporum infection.

Fig. S6. Expression levels of SA- and JA-mediated defence pathway genes in GhMKK6-overexpressing tobacco 4 h after F. oxysporum infection.

Fig. S7. The activity of GhMKK6EE and GhMKK6AA.

Fig. S8. Expression levels of GhMKK6 in different cotransformed tobacco leaves.

Fig. S9. Prediction of miR5272a targets in tobacco.

Fig. S10. The expression pattern of ghr-miR5272a in cotton under SA treatment.

Fig. S11. Overexpression of a ghr-miR5272a mimic with imperfect binding sites inhibits the function of ghr-miR5272a.

Fig. S12. The expression levels of SA- and JA-mediated defence pathway genes in wild-type cotton.

Table S1. Oligonucleotide primers used in gene cloning, vector construction, and qPCR.

Table S2. Oligonucleotide primers used in qRT-PCR.

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