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Subtle regulation of cotton resistance to Verticillium wilt mediated by MAPKK family members

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ABSTRACT

Verticillium wilt caused by soil-borne fungus of *Verticillium dahliae* Kleb. is one of the most devastating diseases of cotton. Since the hierarchically organized mitogen-activated protein kinase (MAPK) cascade plays pivotal roles in signaling plant defense against pathogen attack, and the key nodes of MAPKKs (MKKs) may serve as for the convergence and divergence of signals in MAPK cascades, the possible relations between MAPK signaling and cotton Verticillium resistance were examined in this study. A total of 24 MKK genes were identified in the *Gossypium hirsutum* L. genome and then classified based on phylogenetic analysis. Then the regulation roles of all types of cotton MKKs in activation of cotton disease resistance were tested with the virus-induced gene silencing (VIGS) method. The results showed that three types of MKKs (GhMKK4, GhMKK6 and GhMKK9) positively regulate, while GhMKK10 negatively regulate the cotton resistance to Verticillium wilt. Further, more subtle regulation of cotton resistance mediated by MKK genes were revealed. In GhMKK9, only *Gh_A12G2448* and *Gh_D12G2574* displayed positive regulation of cotton resistance; whereas only *Gh_A12G1883* and *Gh_D12G2062* displayed negative regulation of cotton resistance in GhMKK10. All these results show that MKK members in MAPK signal cascades play dual roles in subtly regulating of cotton resistance to Verticillium wilt.

1. Introduction

Cotton (*Gossypium hirsutum* L.) is the most important natural fiber crops and also an important oilseed crop in the world [1,2]. In China, more than 70% of cotton-growing area is threatened by Verticillium wilt, caused by the soil-borne fungus *Verticillium dahliae* Kleb [3,4]. Verticillium wilt leads to considerable decreases in cotton production and causes serious economic losses annually [5]. It is difficult to control *V. dahliae*, because of the long-term survival of its microsclerotia in soil even without hosts [6,7]. To date, it remains to be a great challenge to manage Verticillium wilt, despite much progress has been achieved in improving the cotton resistance through conventionally genetic breeding in the past 40 years [8,9].

The cotton defense response against to the Verticillium pathogens has been extensively studied at molecular levels for quite a long time [10,11]. Gong et al. reported that the salicylic acid signaling pathway mediated by GaRPL18 enhances the cotton resistance to Verticillium wilt [12]. Zhao et al. reported that a receptor-like kinase gene (*GbRLK*) induced by *V. dahliae* is involved in the activation of cotton disease resistance [13]. Zhang et al. also reported that RLKs, WRKY transcription factors and cytochrome p450 are involved in the cotton defense response [14]. The phenylpropanoid metabolic pathway was also reported to play a critical role in resistance to Verticillium [15,16]. Additionally, Xu et al (2011) identified the central role of lignin metabolism in cotton resistance to *V. dahliae* [17]. These findings reveal that the cotton defense response to *V. dahliae* is a complicated biological process involved by different types of signaling pathways.

Protein phosphorylation and dephosphorylation are major defense mechanisms for controlling cellular functions in response to external signals [18,19]. The mitogen-activated protein kinase (MAPK) cascades are universal modules of signal transduction involved in responses to external stimuli in eukaryotes [20,21]. The MAPK cascades comprise of the hierarchically organized MAPK kinase kinase (MKKK)-MAPK kinase (MKK)-MAPK, with each encoded by multiple genes [22–24]. Each of the three tiers of kinases in a cell contains multiple members, which contributes to the specificity of the transmitted signals [25].

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As a convergent linking point between MKKKs and MAPKs, MKKs play key roles in the MAPK cascade to regulate various stress responses in plants. Functions of some MKK genes in plant innate immunity have been studied in detail [18,26]. Gao et al reported that GhNDR1 and GhMKK2 are required for Verticillium resistance in cotton using gene silencing assays [27]. Prior research has thoroughly investigated that the constitutive activation of MKK4 and MKK5 in *Arabidopsis* leading to HR-like cell death is associated with the generation of reactive oxygen species [28]. GhMKK5 plays an important role not only in mediating innate immunity responses but also in coordinating the cold and salt signaling transduction pathways in cotton [29], and also in *Arabidopsis* [30]. Expression of active MKK9 protein in transgenic Arabidopsis plants induces the synthesis of ethylene and camalexin through the activation of the endogenous MAPK3 and MAPK6 kinases. As a consequence, transcription of multiple genes responsible for ethylene biosynthesis, ethylene responses, and camalexin biosynthesis is coordinately up-regulated [31].

Currently, the genome sequencing of land cotton has been completed [32], but roles of MKKs in cotton resistance to Verticillium wilt are still unclear. In this study, we comprehensively studied roles of MKK genes in cotton resistance to Verticillium wilt, and successfully revealed the subtle regulatory roles of cotton MKK members in resistance to Verticillium wilt.

2. Materials and methods

2.1. Collection sequence information of MKK genes in cotton

The *Gossypium hirsutum* L. MKK genes sequences were downloaded from the website http://mascotton.njau.edu.cn/. The whole Arabidopsis (*Arabidopsis thaliana*) protein sequences were acquired from the TAIR (https://www.arabidopsis.org/) genome databases.

2.2. Multiple-sequence alignment and construction of phylogenetic tree

The *Gossypium hirsutum* L. and Arabidopsis MKK family proteins were aligned with the software of MEGA 6.06, and the parameters were the default. A phylogenetic tree was constructed by employing the neighbor-joining method wrapped in the PHYLIP software suite (http://evolution.genetics.washington.edu/phylip.html). The tree was viewed with MEGA6.06, which bootstrap values for 1000 replicates, respectively [33,34].

2.3. Plant materials, growth conditions, and pathogen inoculation

Verticillium wilt-resistant cultivar Zhongzhimian KV3 and susceptible cultivar Xinluzao7 (XLZ7) were used in this study. The cotton seeds were surface-sterilized for 5 min in 75% (v/v) ethanol and 2 h in 3% (v/v) hydrogen peroxide solution, then thoroughly washed with sterilized water and transferred to 10-cm diameter plastic pots filled with a mixture of nutritive soil: commercial vermiculite (2:1, v/v). All cotton plants were grown in a 28 °C/22 °C (day/night) green house at approximately 70% relative humidity until Virus-induced gene silencing (VIGS) was performed by *Agroinfiltration* method on two fully expanded cotyledons of 10-day-old upland cotton seedlings as previously described [35].

The VIGS silenced cotton seedlings were transferred to grown at 25°C/20°C (day/night) conditions. When the second true leave appeared after more 15 days of growth, these silenced cotton plants were stem inoculated with toothpick method [36]. Each plant was inoculated with 10 μL of highly toxic and defoliant strain of *V. dahliae* V991 at a concentration of $1.0 \times \mathbf{10^6 conidia}$/mL.

2.4. Construction of silencing constructs corresponding to targeted cotton MKK genes

Each silencing vector was designed to silencing certain targeted MKK genes according to the highly similarity in sequence. SGN VIGS (http://vigs.solgenomics.net/) tool was used to select appropriate gene target sequence regions for designing the silencing vectors, which were used for silencing certain target gene [37]. According to the design approach, there was only a corresponding silencing vector for silencing of GhMKK3 (*Gh_A11G0616*, *Gh_D11G0703*), GhMKK4 (*Gh_D06G1960*), and GhMKK5 (*Gh_A05G0965*, *Gh_D05G1074*) respectively. While six GhMKK2 genes were divided into three silencing groups, GhMKK2-1 (*Gh_A05G0237*, *Gh_D05G0323*), GhMKK2-2 (*Gh_A06G0660*, *Gh_D06G0748*) and GhMKK2-3 (*Gh_A07G0124*, *Gh_D07G2384*). And each GhMKK2 silencing group was designed to be only silenced by its corresponding silencing vector.

Similarly, four GhMKK6 genes were classified into two silencing groups, GhMKK6-1 (*Gh_A13G1734*, *Gh_D13G2082*), GhMKK6-2 (*Gh_A07G0085*, *Gh_D07G0094*). It should be pointed out the frustration in the construction of the vectors for silencing GhMKK6-2 genes in our experiments. Three GhMKK9 genes were divided two silencing groups, GhMKK9-1 (*Gh_A12G2448*, *Gh_D12G2574*), GhMKK9-2 (*Gh_D12G2575*). And four GhMKK10 genes were fallen into two silencing categories of GhMKK10-1 (*Gh_A03G1976*, *Gh_D03G1645*) and GhMKK10-2 (*Gh_A12G1883*, *Gh_D12G2062*). All these GhMKK silencing groups were designed to be target silenced by its corresponding silencing vectors.

The length of gene target sequence regions should be between 200 and 400 nucleotides, longer fragments may increase the opportunities of target-off silencing [37,38]. Primer 5.0 was used to design the primers [39] (Supplemental Table 1). The construction of silencing constructs was briefly described as follows: *pCLCrVA* and *pCLCrVB*, the cotton leaf crumple virus silencing vectors [40], were used for the construction GhMKK genes silencing vectors. First total cotton RNA was isolated from resistant variety of Zhongzhimian KV-3 using a cetyltrimethy lammonium bromide (CTAB) method [41,42]. Then the cDNA was synthesized using the SMARTer™ cDNA Synthesis Kit (Clontech, San Jose, CA, US) for amplification of MKK gene target sequence. The verified sequence amplicons were inserted into *pCLCrVA* vector. Then, *pCLCrVA*, *pCLCrVB* and derivatives of *pCLCrVA* were respectively transferred into *Agrobacterium tumefaciens* EHA105 via freeze-thawing method [43,44].

2.5. GhMKK genes silenced by single or merged vectors

VIGS was performed by Agroinfiltration method on two fully expanded cotyledons of 10-day-old upland cotton seedlings (resistant variety of Zhongzhimian KV-3 and susceptible variety of Xinluzao 7) as previously described [45]. Briefly, for single vector silencing, *Agrobacterium* harboring the empty vector of *pCLCrVA* or one of its derivatives was mixed with an equal volume of Agrobacterium harboring *pCLCrVB*. The mixed *Agrobacterium* solutions were infiltrated into the back of the cotton seedling using a syringe without needle. Inoculating seedlings were grown at 25°C/20°C (day/night) conditions. The magnesium chelatase subunit I gene (*GhChlI*) and endogenous cloroplastos alterados 1 gene (*GhCLA1*) were used as the controls in all VIGS experiments, which encode a target protein of the chloroplast thioredoxin [27,46]. Each experiment was performed at least triplicate with more than 15 plants for each construct per repeat.

However, some types of cotton MKKs have many homologous genes. In such case, it is impossible to silence all member genes only by single silencing vector. So merged vector silencing approach was conducted, in which, two or three silencing vectors were mixed with an equal volume of *Agrobacterium* harboring *pCLCrVB*, and then were agroinfiltrated into a single plant [47]. For example, six GhMKK2 genes were divided into three silencing groups of GhMKK2-1, GhMKK2-2 and GhMKK2-3 and formed