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A cotton Raf-like MAP3K gene, *GhMAP3K40*, mediates reduced tolerance to biotic and abiotic stress in *Nicotiana benthamiana* by negatively regulating growth and development

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ABSTRACT

Mitogen-activated protein kinase (MAPK) cascades mediate various responses in plants. As the top component, MAP3Ks deserve more attention; however, little is known about the role of MAP3Ks, especially in cotton, a worldwide economic crop. In this study, a gene encoding a putative Raf-like MAP3K, *GhMAP3K40,* was isolated. *GhMAP3K40* expression was induced by stress and multiple signal molecules. The plants overexpressing GhMAP3K40 had an enhanced tolerance to drought and salt stress at the germination stage. However, at the seedling stage, the transgenic plants suffered more severe damage after drought, exposure to pathogens and oxidative stress. The defence-related genes and the antioxidant system were activated in transgenic palnts, suggesting that GhMAP3K40 positively regulate the defence response. The transgenic plants were less able to prevent pathogenic invasion, which was due to defects in the cell structure of the leaves. The root system of the control plants were stronger compared with the transgenic plants. These results indicated a negative role of GhMAP3K40 in growth and development and GhMAP3K40 possibly caused the defects by down-regulating the lignin biosynthesis. Overall, these results suggest that GhMAP3K40 may positively regulate defence response but cause reduced tolerance to biotic and abiotic stress by negatively regulating growth and development.

1. Introduction

Sessile plants constantly suffer from diverse environmental stresses. To survive, plants have developed complex and elaborate signalling networks to perceive external signals and lead to physiological and morphological changes. The mitogen-activated protein kinase (MAPK) cascade is a conserved pathway involved in the response of plants to various stresses.

Typical MAPK cascades are composed of three interlinked protein cascades, a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAP kinase (MAPK) [1]. A MAPK can be phosphorylated by a MAPKK, while a MAPKK can be activated by an activated MAPKKK. MAPKKK is a larger super family in *Arabidopsis* compared with MAPK and MAPKK. At the top of the signalling pathway, there are 80 MAPKKKs in *Arabidopsis*, while only 20 MAPKs and 10 MAPKKs have been identified. Recently, the diploid cotton *Gossypium raimondii* genome was sequenced [2]. Seventy-eight putative MAPKKK genes have been identified in the genome and are classified into three subfamilies, including 12 ZIKs, 22 MEKKs and 44 Raf-like MAPKKKs [3].

Although it is the largest MAPKKK subfamily, Raf-like MAPKKK studies have been limited. A growing body of evidence has shown that these Raf-like MAPKKKs may play significant roles in plant growth and development or in the response to biotic and abiotic stress of particular hormone signal transduction pathways. Among them, the Constitutive Triple Response1 (CTR1) and Enhanced Disease Resistance1 (EDR1) have been well studied. The *Arabidopsis* mutant *ctr1* exhibits seedling and adult phenotypes when treated with the plant hormone ethylene, revealing the position of CTR1 in the ethylene signal transduction pathway [4]. The *edr1*mutation in *Arabidopsis* mediates salicylic acid (SA)-induced defence resistance [5]. *OsEDR1*is induced by JA, SA and ET, and negatively regulates bacterial defence in rice [6,7]. In addition to these two Raf-like MAPKKKs, several other members have been characterised. Drought hypersensitive mutant1 (DSM1), a Raf-like MAPKKK in rice, mediates drought resistance through Reactive Oxygen Species Scavenging [8]. Overexpression of MAP3Kδ4 in *Arabidopsis* enhances the resistance to salt stress and plays a role in ABA signalling [9]. Moreover, MAP3Kδ4 regulates plant growth by affecting shoot branching [10]. LeCTR2 in tomato plays a role

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in ethylene signalling. LeCTR2 overexpression in tomato is associated with enhanced susceptibility to a fungal pathogen and altered growth phenotype [11]. Increased Leaf Angle1, another Raf-like MAPKKK in rice, regulates mechanical tissue formation in the lamina joint [12]. Growing evidence shows that Raf-like MAPKKKs participates in the response to biotic and abiotic stress and in regulating growth and development.

The MAPK cascade is a typical module that transmits signals from upstream receptors to downstream substrates in response to multiple stress responses, hormonal signalling, and growth and development [13]. Several MEKK-like MAP3Ks have been characterised in MAPK cascades in *Arabidopsis*. The MEKK1-MKK1-MPK4 cascade is involved in wounding stress pathways [14,15]. The MEKK1-MKK2 -MPK4/MPK6 cascade is stimulated following salt and cold stress [16]. The MEKK1-MKK4/MKK5-MPK3/MPK6 cascade plays a role in pathogen defence [17]. MEKK-like MAP3Ks activate the MKKs in the MAPK cascade, and AtCTR1-AtMKK9-AtMPK3/6 in *Arabidopsis* is the only cascade that involves a Raf-like MAP3K [18]. In other examples, the Raf-like MAP3Ks functions as an inhibitor or activator in the cascade. In *Arabidopsis,* EDR1 physically interacted with MKK4 and MKK5 and negatively regulated the MKK4/MKK5-MPK3/MPK6 kinase cascade [19]. In rice, 12 novel non-redundant interacting protein pairs (IPPs) representing 11 non-redundant interactors have been identified using 12 rice MAP3Ks as bait; however, none of these MAP3Ks interact with MKKs or MAPKs [20]. Beyond that, limited information is available. Despite their large numbers, very few MAP3Ks have been shown to be involved in MAPK cascades, which raise the question as to whether several of these MAP3Ks are redundant or expressed as functional genes.

Cotton (*Gossypium hirsutum*) is one of the most important economic crops worldwide, and its growth and yield are severely inhibited under various environmental stresses and disease. However, previous studies were largely restricted to MKKs and MAPKs in cotton, and data regarding MAP3Ks, especially Raf-like MAP3Ks, are limited. In the present study, a Raf-like MAP3K, *GhMAP3K40*, was isolated and characterised, and its expression patterns under stress were detected. Ectopic GhMAP3K40 expression in *N. benthamiana* reduced resistance to biotic and abiotic stress by negatively regulating growth and development. However, GhMAP3K40, itself, positively regulated the plant tolerance to biotic and abiotic stress. The proteins that interacted with GhMAP3K40 were isolated, and the expression patterns of these interacting proteins were also investigated. These results broaden our knowledge of the role that Raf-like MAP3Ks may play in signal transduction.

2. Materials and methods

2.1. Plant materials and treatments

Cotton (*Gossypium hirsutum* L. cv. lumian 22) seeds were grown in a chamber at 28 °C with a 16 h light/8 h dark cycle. Seven-day-old cotton seedlings were collected for various treatments. For the tissue expression analysis, the roots, stems and leaves were obtained separately from seven-day-old cotton seedlings. For the salt and osmotic stress treatments, the seedlings were cultured in solutions containing 200 mM NaCl or 15% (w/v) PEG6000, respectively. For the temperature treatments, cotton seedlings were transferred to 4 °C or 37 °C for designated time periods. For wounding treatment, the seedling leaves were cut with scissors. For the fungal pathogen treatment, the roots of the cotton seedlings were dipped into suspensions of the bacterial pathogen *Ralstonia solanacearum* (OD_{600} = 0.6–0.8) or conidial suspensions of the fungal pathogen *Rhizoctonia solani* (10^5 spores/ml) in 1% glucose. For the hormone treatments, the leaves of uniformly developed seedlings were sprayed with 2 mM salicylicacid (SA), 100 μM abscisic acid (ABA), 100 μM methyl jasmonate (MeJA), ethylene (ET) released from 5 mM ethephon, 500 μM Gibberellic acid (GA_3), 0.5 mM Hydrogen peroxide (H_2O_2), 20 μM 6-benzylaminopurine (6BA) or 10 μM 1-naphthaleneacetic acid (NAA). The samples were frozen in liquid nitrogen and stored at −80 °C for later use.

2.2. RNA extraction and quantitative PCR

Total RNA was extracted from the cotton seedlings and *N. benthamiana* using the modified CTAB method and TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), respectively, as described by Lu et al. [21]. First-strand cDNA was synthesised using the EasyScript cDNA Synthetic SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer's protocol. Q-PCR was performed with the SYBR Premix Ex Taq (TaKaRa, Otsu, Japan) in the CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The cotton polyubiquitin gene (*GhUBI*) and the *N. benthamiana β-actin* gene were used to normalise RNA levels. The PCR amplifications were performed as follows: predenaturation 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. All the experiments were conducted triplicate. The data were analysed with the CFX Manager software program (version 1.1) using the $2^{-\Delta\Delta Ct}$ comparative CT method [22]. Differences among the samples were determined by one-way ANOVA using the Statistical Analysis System (SAS, Cary, NC, USA) software, version 9.1.

2.3. Cloning of GhMAP3K40, vector construction and plant transformation

The *GhMAP3K40* gene was isolated as previously described [23]. The full-length *GhMAP3K40*c DNA sequence under the control of the Cauliflower mosaic virus (CaMV) 35S promoter was inserted into the binary pBI121 vector via the XbaI and SalI sites. The recombinant plasmid was introduced into *Agrobacterium tumefaciens* (strain LBA4404) for *N. benthamiana* transformation using the leaf disc method [24]. The transgenic seedlings were selected on MS agar medium containing 100 mg/l of kanamycin, then transferred to soil and grown in a greenhouse before PCR confirmation. The plants transformed with only the pBI121 vector were used as the controls.

2.4. Relative water content and water loss rate analysis

Fully expanded leaves were detached from the plants, and the fresh weight (FW) was recorded immediately. The fresh parts were immersed in distilled water for 4 h, and the turgid weight (TW) was recorded. Finally, the dry weight (DW) was recorded after drying at 80 °C for 48 h. The relative water content was calculated as follows: RWC (%) = (FW−DW)/(TW−DW) × 100.

Leaves of the same size were excised from plants, submerged in distilled water and soaked for 2 h. Excess water was then removed with filter paper, and the leaves wereweighed (g_0). After drying at 80 °C, the leaves were weighed every hour. The water loss rate was calculated as follows: WLR (%) = $(g0 - g_t)/g_0 \times 100$.

2.5. 3, 3′-Diaminobenzidine (DAB) and Trypan blue staining assays

For DAB staining, the *N. benthamiana* leaves were incubated in DAB solution (1 mg/ml, pH 3.8) for 24 h at 25 °C in the dark. After staining, the leaves were soaked in 95% ethanol overnight to remove the chlorophyll. Trypan blue staining was performed based on the method of Pogaíny et al. [25]. The stock solution of Trypan