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Molecular cloning and expression characteristics of a novel MAPKKK gene, *GhCTR1*, from cotton (*Gossypium hirsutum* L.)

F. Yu, R. Guo, C. Wu, H. Li *, X. Guo

State Key Laboratory of Crop Biology, Shandong Key Laboratory of Crop Biology, Shandong Agricultural University, Taian, Shandong 271018, People's Republic of China

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Abstract

Signaling through MAPK (mitogen-activated protein kinase) cascades plays critical roles in plant development, defense and stress responses. In the present study, a novel *MPKKK* gene, nominated as *GhCTR1*, was isolated from the economic crop cotton (*Gossypium hirsutum* L.). Sequence analysis revealed that the full-length cDNA of *GhCTR1* is 3347 bp in length and encodes an 851-amino acid protein with the conserved ATP-binding site and Ser/Thr kinase active site. Fourteen introns were observed in the genomic DNA sequence, and the last one was located in the 3′ untranslated region (UTR). To investigate the expression patterns of *GhCTR1*, semi-quantitative RT-PCR was performed. The expression of *GhCTR1* was higher in the roots and stems than in the leaves. Moreover, *GhCTR1* was upregulated by signaling molecules, including salicylic acid (SA), gibberellins (GAs) and abscisic acid (ABA). Drought, wounding and *Rhizoctonia solani* infection also increased *GhCTR1* transcription. However, there was no remarkable difference in *GhCTR1* expression after treatment with methyl jasmonate (MeJA), ethylene (ET), H_2O_2, salt or cold. Additionally, analysis of a 439-bp *GhCTR1* promoter fragment revealed several putative *cis*-acting elements that may be responsible for the enhanced response to phytohormones and stress. These results suggest that *GhCTR1* may be involved in SA-, GA- and ABA-mediated signaling pathways to provide resistance to biotic and abiotic stress.

Keywords: Biotic and abiotic stresses; *Cis*-acting; Cotton; *MAPKKK*; Phytohormones; RACE

1. Introduction

Various signaling pathways that transduce external signals to cellular responses have evolved in plants to enable responses to biotic and abiotic environmental stresses (Champion et al., 2004; Hahn and Harter, 2009; Schenk and Snaar-Jagalska, 1999). Protein phosphorylation by protein kinases plays an essential role in many signaling transduction mechanisms. The genes coding for protein kinases account for about 5% of the genome of green plants (Colcombet and Hirt, 2008). Moreover, roughly 10% of all plant kinases participate in mitogen-activated protein kinase (MAPK) pathways (Colcombet and Hirt, 2008).

The MAPK cascade is a fundamental and conserved transduction mechanism in all eukaryotes (You et al., 2007). Genetic and biochemical studies have revealed that the MAPK cascade is composed of at least three sequentially activated protein kinases: MAPK kinase kinases (MAPKKKs, also known as MAP3Ks), MAPK kinases (MAPKKs) and MAPKs (Rodriguez et al., 2010). Compared with MAPKs and MAPKKs, the MAP3K class includes more members. Approximately 60 different MAP3Ks have been identified in the *Arabidopsis* genome, thus forming the most complicated and largest group of MAPK pathway components (Gao and Xiang, 2008). These kinases can be classified into Groups A, B and C. Groups B and C are also called Raf-like kinases (Kazuya et al., 2002) and are more similar to mammalian Raf1 (Rodriguez et al., 2010).

MAP3Ks are located at the top of the MAPK signaling pathway and are associated with developmental processes and

* Corresponding author. Tel.: +86 538 8245679; fax: +86 538 8226399.
E-mail address: lihan@sdau.edu.cn (H. Li).

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environmental stress responses (Del Pozo et al., 2004; Mizoguchi et al., 1996; Nakagami et al., 2004). MAP3Kε1 and MAP3Kε2 are functionally redundant genes that together are essential for pollen development but not necessary for female gametophyte function (Chaiwongsar et al., 2006). Further study revealed that some MAP3Kε1 proteins were localized to the plasma membrane in *Arabidopsis* cells, which suggests that MAP3Kε1 may participate in a process required for normal plasma membrane function in *Arabidopsis* pollen (Chaiwongsar et al., 2006). The high tolerance of AT6, a strain with a mutation in a putative MAP3K locus (At1g73660), to salt stress during germination and seedling growth strongly indicates that this locus negatively regulates salt tolerance in *Arabidopsis* (Gao and Xiang, 2008). OMTK1 plays a role in MAPK scaffolding and activation of H_2O_2-induced cell death in plants (Nakagami et al., 2004). MEKK1, whose kinase activity is regulated by H_2O_2, functions in integrating ROS homeostasis with plant developmental and hormonal signaling (Nakagami et al., 2006).

There are two interesting Raf-like MAP3Ks: ENHANCED DISEASE RESISTANCE 1 (EDR1) and CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1). Previous studies showed that these MAP3Ks participate in ethylene (ET)-mediated signaling in *Arabidopsis thaliana* (Frye and Innes, 1998; Huang et al. 2003). However, a report showed that *enhanced disease resistance 1* (*edr1*) mutants depend on salicylic acid (SA)-induced defense responses that are independent of jasmonate (JA)- and ET-induced defenses (Frye et al., 2001). In rice, OsEDR1 plays an important role in the negative regulation of bacterial resistance (Shen et al., 2011). Furthermore, transcript levels of *OsEDR1* can be increased by all three phytohormones, including JA, SA and ET (Kim et al., 2003). In *Cucurbita pepo*, the expression of *CpCTR1* and *CpCTR2* is higher in male floral organs than in female organs in the early stages of flower development, when ET production is extremely low (Manzano et al., 2010). Using the yeast two-hybrid assay, a specific interaction was detected between the CTR1 amino-terminal domain and the predicted histidine kinase domain of the ethylene receptors ETR1 and ERS, which implies that CTR1 acts in the ETR1/ERS pathway (Clark et al., 1998; Gao et al., 2003). Previous studies also revealed that LeCTR1 plays a pivotal role in plant defense responses. Compared with wild-type tomatoes, tomatoes overexpressing *LeCTR1* displayed enhanced susceptibility to infection by the fungal pathogen *Botrytis cinerea* and higher transcript levels of pathogenesis-related genes such as *PR1b1* and *chitinase B* (Lin et al., 2008). However, there are limited reports about the function of CTR1 in response to abiotic stresses, such as cold, wounding and drought, or in non-ET signaling pathways.

Thus far, three MAPK members from cotton have been reported in our laboratory (Shi et al., 2010; Shi et al., 2011; Wang et al., 2007), but the sequence information for MAP3Ks in *Gossypium hirsutum* L., which is an important economic crop, is not available. In the present study, we isolated a novel MAP3K gene, nominated as *GhCTR1*, from cotton. The expression analysis shows that *GhCTR1* transcription can be increased by SA, GA or ABA but not by ET, MeJA or H_2O_2. *GhCTR1* is upregulated by various biotic and abiotic stresses, including the fungus *Rhizoctonia solani*, wounding and drought. A partial 5′-flanking region was obtained by TAIL-PCR, revealing a number of predicted *cis*-acting elements related to SA, GAs and MeJA. The cloning and characterization of *GhCTR1* is an important step in the further study of the function of *CTR1* in different signaling pathways and various plant defense responses.

2. Materials and methods

2.1. Plant materials and treatments

The cotton cultivar (*G. hirsutum* L. *cv* Lumian 22) was used throughout these experiments. Seeds were germinated in the dark, and germinated seedlings were incubated by aquaculture in a greenhouse under a 16 h light/8 h dark cycle. For tissue-specific expression analysis, the roots, stems and leaves were harvested from seven-day-old cotton seedlings. Seedlings were used for expression analysis under the following treatments. Seedling leaves were sprayed with 2 mM SA, 100 μM ABA, 100 μM MeJA, ethylene released from 5 mM ethephon, 500 μM GA_3, or 10 mM H_2O_2. Seedling roots were exposed to NaCl (200 mM) or 15% (w/v) PEG6000 solutions for salt and drought treatments. Cold, wounding and fungal treatments were performed as described in Guo et al. (2010). Seedlings without any treatment were used as controls for all of the above treatments. Seedling leaves (just the cotyledons) were harvested, frozen in liquid nitrogen, and stored at −80 °C for further use.

2.2. RNA extraction, cDNA synthesis and DNA preparation

Total RNA was extracted from the frozen tissue samples with Trizol reagent (Invitrogen, USA), treated with RNase-free DNaseI (Promega, USA) and used as a template for first-strand cDNA synthesis using the EasyScript First-Strand cDNA Synthesis SuperMix (Transgen, China) following the manufacturer's protocol. Genomic DNA was isolated from seedling leaves via the revised CTAB method (Saghai-Maroof et al., 1984).

2.3. Isolation of the full-length cDNA of GhCTR1

To obtain internal conserved gene fragments, two pairs of degenerate PCR primers KP1/KP2 and KP3/KP4 were designed and synthesized according to the conserved amino acids of CTR1 proteins in *Arabidopsis*, parsley, tobacco and sweet potato. Reverse-transcription PCR (RT-PCR) was carried out using the first-strand cDNA as template. The PCR program was as described in Supplementary Table S1. The PCR product was purified, cloned into the pMD18-T vector, and then transformed into *Escherichia coli* competent cells for sequencing.

For 5′ RACE, first-strand cDNA was purified using the Wizard DNA Clean-up System (Promega, USA), and the purified cDNA was homopolymerically tailed at its 5′ end with dCTP by terminal deoxynucleotidyl transferase (TaKaRa, Japan) according to the manufacturers' instructions. Two primers were designed and synthesized based on the obtained sequence of the internal conserved fragment, and two rounds of PCR amplification were performed. The primary PCR was performed with primer 5KP1 and Abridged Anchor Primer (AAP), and the 50-fold diluted PCR product was used as the template for nested