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Ectopic expression of *ZmSIMK1* leads to improved drought tolerance and activation of systematic acquired resistance in transgenic tobacco

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ABSTRACT

The mitogen-activated protein kinase (MAPK) cascades play pivotal roles in diverse signaling pathways related to plant biotic and abiotic stress responses. In this study, a group B MAPK gene in *Zea mays*, *ZmSIMK1*, was functionally analyzed. Quantitative real-time PCR (qRT-PCR) analysis indicated that *ZmSIMK1* transcript could be induced by drought, salt, *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and certain exogenous signaling molecules. Analysis of the *ZmSIMK1* promoter revealed a group of putative *cis*-acting elements related to drought and defense responses. β-Glucuronidase (GUS) staining produced similar results as qRT-PCR. ZmSIMK1 was mainly localized in the nucleus, and further study indicated that the C-terminal domain (CD) was essential for targeting to the nucleus. Transgenic tobacco accumulated less reactive oxygen species (ROS), had higher levels of antioxidant enzyme activity and osmoregulatory substances and exhibited an increased germination rate compared with wild-type (WT) tobacco under drought stress. ROS-related and drought stress-responsive genes in transgenic tobacco were significantly upregulated compared with the same genes in WT lines under drought stress. Moreover, overexpression of *ZmSIMK1* promoted the hypersensitive response (HR) and pathogen-related gene (*PR*) transcription in addition to triggering systemic acquired resistance (SAR) in tobacco.

1. Introduction

Plants are exposed to diverse stress conditions, including drought, salt and pathogen infection, throughout their life cycles. To address various stresses, plants have evolved a variety of biochemical and physiological mechanisms. Among the stresses, drought and pathogens constitute major limits to crop productivity. MAPK cascades have been demonstrated to play roles in a myriad of cellular processes, including biotic and abiotic stresses, growth, differentiation and cell death (Nakagami et al., 2005; Rodriguez et al., 2010; Kosetsu et al., 2010). MAPK is activated by its upstream specific MAPKK via the phosphorylation of conserved threonine (T) and tyrosine (Y) residues in the catalytic subdomain. MAPKK itself is activated via the phosphorylation of two serine/threonine residues in a conserved S/T-X3-5-S/T motif by an upstream MAPKKK (Chang and Karin, 2001). After activation, the MAPK module is either translocated into the nucleus or maintained in the cytoplasm to initiate the cellular responses through the phosphorylation of downstream proteins (Qiu et al., 2008; Nadarajah and Sidek, 2010). Thus, MAPKs, as the last component of the MAPK cascade, play a major role in signal transduction from upstream components to the target. MAPKs are ubiquitous proteins in eukaryotes and exist as a gene family. For example, the *Arabidopsis thaliana* genome contains a total of 20 MAPK genes, and 17 MAPK genes have been identified in the rice genome (Rohila and Yang, 2007; Nadarajah and Sidek, 2010), indicating the complexity of the MAPK cascades in the plant kingdom.

Since the first report of a plant MAPK identified MsERK1 in alfalfa (Duerr et al., 1993) and D5 kinase in pea (Stafstrom et al., 1993), MAPK components have been isolated from many plant species (Mizoguchi et al., 1993; Wilson et al., 1993). Among these, specific MAPKs involved in drought and biotic stress signal transduction have been identified, including AtMPK4 and AtMPK6, which are activated by osmotic stress, and AtMEKK1-AtMKK1/AtMKK2-AtMPK4, which is involved in drought, cold and salt stress signal

Abbreviations: MAPK, mitogen-activated protein kinase; qRT-PCR, quantitative real-time PCR; RT-PCR, reverse transcription PCR; *Pst* DC3000, *Pseudomonas syringae* pv. *tomato* DC3000; ROS, reactive oxygen species; *PR*, pathogen related genes; SAR, systemic acquired resistance; ABA, abscisic acid; SA, salicylic acid; MeJA, methyl jasmonate; GFP, green fluorescent protein; DAPI, 4′6-dianidino-2 phenylindole; CD, C-terminal domain; KD, kinase domain; REL, relative electrolyte leakage; MDA, malondialdehyde; NBT, nitroblue tetrazolium; DAB, 3,3′-diaminobenzidine; APX, ascorbate peroxidase; CAT, catalase; POD, peroxidase; SOD, superoxide dismutase; DMTU, dimethylthiourea; H_2O_2, hydrogen peroxide; HR, hypersensitive response; CaMV, cauliflower mosaic virus; CTAB, cetyl-trimethyl-ammonium bromide; GUS, β-glucuronidase.

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transduction (Nadarajah and Sidek, 2010; Ichimura et al., 2000). In rice, OsMAPK5 positively regulates drought, salt and cold tolerance and negatively regulates *PR* expression and broad-spectrum disease resistance (Xiong and Yang, 2003). OsMPK6 functions as a repressor to regulate SAR in response to *Xanthomonas oryzae*; however, it also regulates local resistance to *X. oryzae* as an activator (Shen et al., 2010). Maize (*Zea mays*) is one of the most important crops in the world. Its growth and yield are severely limited by drought and disease. Recently, some MAPK genes have been cloned from maize, including *ZmSIMK1*, *ZmMPK3*, *ZmMPK5*, *ZmMPK6*, *ZmMPK7* and *ZmMPK17* (Alexandrov et al., 2009; Wang et al., 2010; Zhang et al., 2007; Lalle et al., 2005; Zong et al., 2009; Pan et al., 2011). However, current studies on MAPK genes from maize are focused on cDNA cloning and expression analysis under various abiotic conditions. For *ZmSIMK1*, Gu et al. studied that ectopic expression of *ZmSIMK1* in *Arabidopsis* increases tolerance to salt stress (Gu et al., 2010). The dual functionality of MAPKs under abiotic and biotic stresses has been poorly studied. Additionally, certain differences in the composition and function of a specific component in MAPK cascades might exist (Morris, 2001) despite the evolutionary conservation of the MAPK cascades in eukaryotes. It is possible that the array of potential functions of ZmSIMK1 is larger than previously thought.

Here it is shown that ZmSIMK1 is involved in both responses to drought stress and *Pst* DC3000 infection, with putative involvement of hormonal signals such as abscisic acid (ABA) and salicylic acid (SA). Overexpression of *ZmSIMK1* in tobacco confers drought stress tolerance by affecting the antioxidant defense system. Additionally, ZmSIMK1 may be involved in triggering SAR, which strengthens the disease resistance of transgenic tobacco.

2. Materials and methods

2.1. Plant materials, growth conditions and treatments

Maize seedlings (*Z. mays* L. Zhengdan 958) were grown in Hoagland's solution (pH 6.0) under greenhouse conditions at 22/26 °C (night/day) with a photosynthetic active radiation of 200 μmol m^{-2} s^{-1} and a photoperiod of 16/10 h (day/night) for two weeks.

Two-week-old maize seedlings were incubated in Hoagland's solution containing 20% PEG6000 (w/v), 250 mM NaCl, 100 μM ABA, 100 μM SA, 100 μM MeJA, 20 mM H_2O_2 or 5 mM $CaCl_2$ (the control was Hoagland's solution without additives) at 26 °C with a continuous light intensity of 200 μmol m^{-2} s^{-1}. For pathogen treatment, *Pst* DC3000 suspensions (4×10^8 cfu ml^{-1}) were used to infect the leaves of maize seedlings. The inoculated seedlings were maintained in a moist chamber. The leaves were harvested at the indicated time and frozen in liquid nitrogen for later use.

The transgenic and WT plants were grown for six weeks in quartz sand with a photoperiod of 16/8 h (day/night), a temperature of 25/20 °C (day/night) and photosynthetic active radiation of 200 μmol m^{-2} s^{-1}.

Six-week-old transgenic and WT tobacco plants grown in quartz were treated with 20% PEG 6000 for five days to analyze drought stress tolerance. The capacity to resist disease was assessed by the inoculation of plant leaves with *Pst* DC3000 suspensions (4×10^8 cfu ml^{-1}) under the conditions described above, after which samples were obtained.

2.2. Amplification of ZmSIMK1 and generation of transgenic plants

Total RNA were extracted from the leaves of maize seedlings using the Trizol reagent according to the manufacturer's instructions (Invitrogen, USA). The first-strand cDNAs were synthesized using the First-Strand cDNA Synthesis Kit (Fermentas, USA). Primers (SK-F/R) were designed according to the known maize *ZmSIMK1* sequence (GenBank accession number: EU959799) (Alexandrov et al., 2009) to amplify *ZmSIMK1* with reverse transcription PCR (RT-PCR). The expected PCR fragment was cloned into the pMD18-T vector and sequenced. The fragment was then sub-cloned into the pBI121 vector under the control of the CaMV35S promoter. The recombinant plasmid, pBI121-*ZmSIMK1*, was introduced into tobacco plants through *Agrobacterium*-mediated transformation (Wu et al., 1995). The transgenic tobacco plants were verified by kanamycin resistance and PCR. T_2 transgenic tobacco plants were used in the stress tolerance analysis. The transcriptional levels of *ZmSIMK1* in six T_2 transgenic lines were examined with qRT-PCR (the primer sequences used in this study are provided in Table 1).

2.3. ZmSIMK1 promoter amplification and GUS staining analysis

Total genomic DNA was extracted from maize seedlings using the cetyl-trimethyl-ammonium bromide (CTAB) method and

Table 1
Primer sequences used for cloning, subcellular localization, vector construction, transgenic confirmation, and expression analysis.

Description/gene	Abbreviation	Forward sequence(5′–3′)	Reverse sequence(5′–3′)
Amplification of *ZmSIMK1* encored region	SK-F/R	GGATCCGCGAAAGGA ACCATGGATT	GTCGACCCTCTAGCAGATAACTCATCC
Forward primer of CaMV35S Promoter	35S-F	TACGCAGCAGGTCTCATCAAGACGAT	
For over-lap PCR	KD-F/R	CCATACTTGGCTTCACCTGCACCTTTCAGC	GCTGAAAGGTGCAGGTGAAGCCAAGTATGG
Used for subcellular location	GSK-F/R	TCTAGAGCGAAAGGA ACCATGGATT	GGTACCGTAGGGAGGCTCTG
EU959799	ZmQSK-F/R	CCAGCGGACGCAAGAACT	ATGCCCGTATGCCCAACA
NM_001156990.1	ZmActin-F/R	CCACGAGACCACCTACAACT	CCACGAGACCACCTACAACT
EU959799	NtQSK-F/R	ATCCGCGAAAGGAACCA	GGTGGGGCGTACTTGGA
U66264.1	NtUbiquitin-F/R	TCCAGGACAAGGAGGGTAT	CATCAACAACAGGCAACCTAG
U15933.1	NtAPX-F/R	CAAATGTAAGAGGAAACTCAGAGGA	CAGCCTTGAGCCTCATGGTACCG
EU998969.1	NtCAT-F/R	AGGTACCGCTCATTCACACC	AAGCAAGCTTTTGACCCAGA
AY206007.1	NtGST-F/R	CCCCTAGTTTGCTCCCTTCT	TTCTTAGCTGCCTCCTGCTC
AY639146.1	NtPOX2-F/R	CTTGGAACACGACGTTCCTT	TCGCTATCGCCATTCTTTCT
X14482.1	NtSOD-F/R	AGCTACATGACGCCATTTCC	CCCTGTAAAGCAGCACCTTC
HM068892.1	NtNCED1-F/R	AAGAATGGCTCCGCAAGTTA	GCCTAGCAATTCCAGAGTGG
AF053076.1	NtLEA5-F/R	TTGAATCTGGGGTTTTGGTT	GGAAGCATTGACGAGCTAGG
X12737	NtPR1a-F/R	TCTCTACACTTCTCTTATTC	GTTCTACACCTACATCTG
M60460.1	NtPR2-F/R	CATAACCTTCCACTCTTA	CATAACCTTCCACTCTTA
X58546	NtPR4a-F/R	AACAGTGAGAATAGTAGAT	CATAGTTGACAGTAAGGT
AF480488.1	NtNPR1-F/R	GAATGATACGGCAGAAGA	AGATGAGGAGATGTTGTTAG
AB022693.1	NtWRKY1-F/R	ATCAGTAAGTCAGGAGAAGA	ACCGTCATCAAGAATATCAAT
DQ460475.1	NtWRKY12-F/R	GCAACAAGAGATAAGCACTA	TCCTCCATTTGTATCCATCA
Amplification for *ZmSIMK1* promoter	Pro-F/R	CCAAGCTTGGCCAGACGAGCTTTCACAC	CCGGATCCTTTTGACTTGGGCGGCGA