Scientia Horticulturae 127 (2011) 488–493

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Involvement of a cucumber MAPK gene (*CsNMAPK*) in positive regulation of ROS scavengence and osmotic adjustment under salt stress

Huini Xu[a,b], Xudong Sun[a], Xiufeng Wang[a,*], Qinghua Shi[a], Xiaoyu Yang[a], Fengjuan Yang[a]

[a] State Key Laboratory of Crop Biology, College of Horticulture Science and Engineering, Shandong Agricultural University, Tai'an 271018, PR China
[b] Biotechnology Research Centre, Kunming University of Science and Technology, Kunming 650224, PR China

ARTICLE INFO

Article history:
Received 26 August 2010
Received in revised form 9 November 2010
Accepted 16 November 2010

Keywords:
Mitogen-activated protein kinase
Cucumber
Pollen-tube pathway
Salt stress

ABSTRACT

Mitogen-activated protein kinase (MAPK) cascades play a key role in plant growth and development as well as biotic and abiotic stress response. To unravel the roles of MAPK in cucumber, its expression in transgenic cucumber plants was reduced by an antisense approach. For this purpose, a 1113 bp cDNA fragment of cucumber MAPK gene (*CsNMAPK*) was expressed in antisense orientation driven by the 35S promoter of cauliflower mosaic virus. The pBI-CsNMAPK plasmid DNA was introduced into cucumber embryo by the pollen-tube pathway method. All seeds were sown in the soil and screened for transformants with kanamycin, polymerase chain reaction (PCR), Northern blot and real-time quantitative reverse transcriptative PCR (RT-PCR) analysis. Two independent transgenic plants were obtained and analyzed. The results showed that the transgenic cucumber plants exhibited retarded growth. The transgenic plant height was shorter and the leaves were smaller. Transgenic cucumber plants suppression *CsNMAPK* were more sensitive to salt stress than the wild-type (WT) plants. The fresh weight of shoot of the transgenic plants decreased more than WT after 50 mM NaCl treatment for 7 days. The malondialdehyde (MDA) content was higher, while the superoxide dismutase (SOD) activity and proline accumulation were lower in the transgenic plants than the WT plants after NaCl stress treatment. The evidence indicated that *CsNMAPK* was involved in positive regulation of reactive oxygen species (ROS) scavengence and osmotic adjustment under salt stress.

1. Introduction

Abiotic stresses are thought to be a major cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Bray et al., 2000). Among these abiotic stresses, soil salinity is the most significant one for plant agriculture and affects approximately 20% of global irrigated agricultural land (Flowers and Yeo, 1995). Salt stress signal transduction consists of ionic and osmotic homeostasis signaling pathways, detoxification response pathways, and pathways for growth regulation. For the ionic aspect of salt stress, a signaling pathway based on *SOS* (*salt overly sensitive*) genes has been established (Zhu, 2002). The *SOS* pathway was activated by excessive intracellular or extracellular Na^+. The osmotic stress or detoxification signaling may be mediated by several osmotic-activated, *SOS*-independent protein kinases such as the mitogen-activated protein kinase (MAPK) cascades.

MAPK cascades, which comprise three protein kinases of MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK), are known to be one of the major pathways by which extracellular signals such as growth factors, hormones, and stress stimuli are transduced into intracellular responses in plants (Zhang et al., 2006; Andreasson and Ellis, 2010). Many data suggest that MAPK is also rapidly activated in plants exposed to a variety of abiotic and biotic stresses including salt, cold, drought, UV-irradiation, wounding and pathogen (Seo et al., 1995; Ichimura et al., 2000; Samuel and Ellis, 2002; Droillard et al., 2004; Blanco et al., 2006; Shoresh et al., 2006). For example, in *Arabidopsis*, three MAPKs genes, ATMPK3, ATMPK4 and ATMPK6, were responsive to various forms of stresses (Ichimura et al., 2000; Droillard et al., 2004). In tobacco, it has been revealed that wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK), which were the orthologs of ATMPK3 and ATMPK6, respectively, were involved in the response to wounding and ozone (Seo et al., 1995; Samuel and Ellis, 2002).

Recently, a cucumber cDNA designated as *CsNMAPK*, encoding a mitogen-activated protein kinase, has been isolated using RT-PCR, and 3′ and 5′ RACE. Characterization of *CsNMAPK* suggested that the full-length cDNA sequence contained 1636 bp and an open reading frame (ORF) of 1113 bp, which encoded 370 amino acid residues (Xu et al., 2008). Overexpression of *CsNMAPK* in tobacco plants significantly enhanced seed germination rate under high salt and osmotic stresses, indicating that *CsNMAPK* was involved in plant tolerance to both salt and osmotic stresses (Xu et al., 2010). Previous

* Corresponding author. Tel.: +86 538 8242456; fax: +86 538 8242456.
E-mail address: xfwang@sdau.edu.cn (X. Wang).

0304-4238/$ – see front matter.
doi:10.1016/j.scienta.2010.11.013

studies have shown that the tolerance to salt stress is usually correlated with a more efficient antioxidant system and better osmotic adjustment (Shalata and Tal, 1998; Lin and Kao, 2000). Therefore, we hypothesize that *CsNMAPK* may be a positive regulator for ROS scavengence and osmotic adjustment.

In order to certify our hypothesis, antisense vector of *CsNMAPK* was constructed and successfully transformed into cucumber via the pollen-tube pathway in this research. Using transgenic cucumber plants, we found that both SOD activation and proline accumulation were inhibited significantly in the antisense transgenic cucumber plants under NaCl stress. This evidence indicated that the *CsNMAPK* was involved in positive regulation of ROS scavengence and osmotic adjustment in cucumbers under salt stress.

2. Methods and materials

2.1. Transformation of cucumber

A 1113 bp fragment from the 5′ end of the *CsNMAPK* cDNA (GenBank accession no. DQ812086) was inserted in antisense orientation into the binary vector pBI121 (Clontech, Palo Alto, CA) behind the 35S promoter of cauliflower mosaic virus. The plasmid of pBI-CsNMAPK was extracted and purified. The plasmid DNA solution was adjusted to the final concentration of 200 $\mu g\,ml^{-1}$ in double-distilled water before use.

Cucumber pollen-tube pathway transformation was conducted according to the procedures described by Chen et al. (1998) with some modifications. 5 μl of the plasmid DNA was injected into the ovary of cucumbers (*Cucumis sativus* L. cv. Xintaimici) 24 h before hand-pollination in sunny days in May, 2007. Seeds from untreated plants were harvested as negative controls.

2.2. Screening of the transgenic plants

Seeds harvested from treated flowers were germinated in soil of the experimental farm of Shandong Agricultural University. Cucumber plants with one true leaf were screened for putative transformants that expressed the neomycin phosphotransferase II (*NPTII*) gene. The surface of the true leaf was painted with 2500 $mg\,L^{-1}$ kanamycin along the vein using a cotton bud for three times in sunny days. After 5–7 days, cucumber plants with yellow vein were removed. Kanamycin-resistant seedlings with green leaves were kept for further PCR, Northern blot and real-time quantitative RT-PCR analysis.

2.3. Total RNA extraction, Northern blot, and real-time quantitative RT-PCR analysis

Total RNA was extracted from cucumber leaves with Trizol reagent (Invitrogen) according to the supplier's recommendation. For Northern blot analysis, 15 μg of total RNA was electrophoresed on a 1.2% agarose–formaldehyde gel and transferred by capillary action overnight to Hybond-N^+ nylon membrane (Amersham, UK) with 20× SSC. The *CsNMAPK*-specific primers (tan1: 5′-CACCAAATAATCCGCTCCAAC-3′; tan2: 5′-TAATACGACTCACTATAGGGCGAGGATGTGGAGGTAGTTG-3′) were designed. The tan2 primer was added to a T7 RNA polymerase promoter with underline. The primer pair tanl/tan2 generated a probe of 468 bp from nucleotides 380 to 848 of the *CsNMAPK* gene. The PCR product was labeled with digoxygenin according to the DIG RNA Labeling Kit (Roche, Germany) manual. Hybridization, washing, and detection were performed using the DIG Northern Starter Kit (Roche, Germany) according to the manufacturer's instructions.

Reverse transcription of RNA was carried out according to the instruction of the SYBR® PrimeScript™ RT-PCR Kit II (Takara, Japan). Real-time quantitative PCR was performed using the iCycler iQ Real-time PCR detection system (Bio-rad). A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified using software provided with the iCycler iQ real-time PCR detection system. To minimize sample variations, mRNA expression of the target gene was normalized relative to the expression of the house-keeping gene *Actin* (GenBank accession no. DQ641117). The primers used were: *Actin* (forward): 5′-CCACGAAACTACTTACAACTCCATC-3′; *Actin* (reverse): 5′-GGGCTGTGATTTCCTTGCTC-3′; MAPK (forward): 5′-AAGCGTTAGCACATCCGTACCT-3′; MAPK (reverse): 5′-CATCTCCTTCATCTGTTCTTCGTCT-3′. The size of the product generated was 136 bp from nucleotides 392 to 528 of the *Actin* gene. The MAPK primers generated a product of 121 bp from nucleotides 950 to 1071 of the *CsNMAPK* gene. Three replicates were run for each sample.

2.4. NaCl stress treatment

Cucumber seeds of the transgenic plants of the T_2 generation and WT plants were germinated on moisture filter paper in an incubator at 28 °C for 2 days. The germinated seeds were sown in sands in the greenhouse of Shandong Agricultural University. After 10 days, the cucumber seedlings of the T_2 generation were analyzed by PCR selection for transgenic cucumber plants with the *NPTII* gene. 20 transgenic and 20 WT cucumber plants were chosen for further analysis. Ten cucumber seedlings each were transferred to plastic tank (40 cm × 30 cm × 12 cm) with 10 L nutrient solution with pH 6.0–6.5 containing aerated full nutrient solution according to Shi et al. (2007) with some modifications: $Ca(NO_3)_2$ 3.5 $mmol\,L^{-1}$, KNO_3 7 $mmol\,L^{-1}$, KH_2PO_4 0.78 $mmol\,L^{-1}$, $MgSO_4$ 2 $mmol\,L^{-1}$, H_3BO_3 29.6 $\mu mol\,L^{-1}$, $MnSO_4$ 10 $\mu mol\,L^{-1}$, Fe-EDTA 50 $\mu mol\,L^{-1}$, $ZnSO_4$ 1.0 $\mu mol\,L^{-1}$, H_2MoO_4 0.05 $\mu mol\,L^{-1}$, and $CuSO_4$ 0.95 $\mu mol\,L^{-1}$. The experiment was carried out under natural conditions with the air temperature of 25–30 °C during the day and 18–25 °C during the night. When the cucumber seedlings were at three-leaf-stage, NaCl was added into the nutrient solution to form a final NaCl concentration of 0 and 50 mM. The samples were collected after 7-day treatment.

2.5. Determination of plant growth

Plant height, stem diameter, and leaf area of WT and transgenic cucumbers grown hydroponically were determined. After 7-day treatment, plants were sampled and divided into shoots and roots. The fresh weight (FW) was directly determined.

2.6. Lipid peroxidation assay

The leaf lipid peroxidation of both transgenic and WT plants was determined after 0 and 50 mM NaCl treatment for 7 days by estimating the malondialdehyde (MDA) formation using the thiobarbituric acid method described by Madhava Rao and Sresty (2000).

2.7. SOD activity assay

Leaf samples of transgenic plants and WT plants treated with 0 and 50 mM NaCl for 7 days were used for enzyme analysis. 0.5 g leaves homogenized in 4 ml of 0.05 M sodium phosphate buffer (pH 7.8) including 1 mM EDTA and 2% (w/v) PVP. The homogenate was centrifuged at 10,000 × *g* for 20 min at 4 °C. Supernatant was used for analysis of enzyme activity. All steps in the preparation of the enzyme extract were carried out at 4 °C. All spectrophotometric analyses were conducted on a Shimadzu (UV-2450PC)