

Agrobacterium-mediated transformation of cucumber (*Cucumis sativus* L.) using a sense mitogen-activated protein kinase gene (*CsNMAPK*)

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Abstract The objective of this study was to develop an efficient regeneration and transformation protocol for cucumber (*Cucumis sativus* L.). Cotyledonary node explants showed a very high regeneration frequency (94.3 %) when incubated on Murashige and Skoog medium (MS) supplemented with 1.5 mg l⁻¹ 6-benzyladenine (BA). An *Agrobacterium*-mediated transformation methodology for cucumber was developed using this protocol. A construct containing a sense mitogen-activated protein kinase gene (*CsNMAPK*) was used to transform cucumber cotyledonary nodes. Adventitious shoots were obtained in selection media containing 150 mg l⁻¹ kanamycin and 250 mg l⁻¹ carbenicillin 4 weeks after bacterial inoculation. A total of twelve transgenic plants were confirmed using PCR-specific analysis. The transgene copy number of four transgenic plants (random selection) was found to be a single gene copy using Southern blot analysis and the expression level of the *CsNMAPK* gene in the transgenic plants was significantly higher than in the wild-type plants. The transgenic rate was approximately 4.8 %.

Keywords Cucumber · Cotyledonary node · Regeneration · *Agrobacterium tumefaciens* · Transformation

Abbreviations

MS	Murashige and Skoog medium
1/2MS	Half-strength Murashige and Skoog medium
MAPK	Mitogen-activated protein kinase
<i>CsNMAPK</i>	MAPK gene for cucumber
BA	6-benzyladenine
NAA	Naphthalene acetic acid
<i>A. tumefaciens</i>	<i>Agrobacterium tumefaciens</i>
YEP	Yeast Extract Peptone medium

Introduction

Cucumber is an important crop and is widely cultivated throughout the world. Cucumber plants often suffer from salt stress, drought stress and other environmental stresses during their development, which lead to reductions in crop yield and quality. Mitogen-activated protein kinase (MAPK) cascades 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 cells (Emerling et al. 2005; Sumbayev and Yasinska 2005). To date, a variety of MAPKs have been identified from different plant species, such as *Arabidopsis* (Mizoguchi et al. 1994), petunia (Decroocq-Ferrant et al. 1995), alfalfa (Bögge et al. 1996), rice (Fu et al. 2002), oat (Huttly and Phillips 1995), barley (Knetsch et al. 1996), potato (Blanco et al. 2006), tomato

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(Holley et al. 2003) and cucumber (Shoresh et al. 2006). However, little information has been reported on the role of MAPKs at the molecular level in cucumber. In a previous study, MAPK cDNA was cloned from cucumber and named *CsNMAPK* (GenBank accession DQ812086) (Xu et al. 2008). In order to determine the roles of this MAPK gene in response to various stresses, this study attempted to increase its activity in cucumber plants using a sense approach.

One of the most effective means of gene transfer into dicotyledonous plants is to utilize *Agrobacterium tumefaciens* (*A. tumefaciens*) (Gasser and Fraley 1989). Utilization of this method for gene transfer requires both a susceptibility to infection by *A. tumefaciens* and an ability to regenerate plants from individual transformed cells via tissue culture (Fang and Grumet 1990). Genetic instability is often observed in regenerated plants derived from in vitro cultures and may limit the use of somatic embryogenesis, particularly where the plant propagation numbers and the genetic transformation are large (Evans et al. 1984). In order to establish a successful strategy for practical plant genetic engineering, it is important to develop systems for recovering large numbers of whole plants from primary explants (Vasudevan et al. 2007). In many instances, however, the lack of an efficient regeneration system limits the use of gene transfer technologies in vegetable crops. Nora et al. (2001) reported that young leaf explants were found to regenerate at a higher rate of diploidy (85 %) and showed a good transformation efficiency (~3 %), whereas the diploidy rate was only 25 % using cotyledons as the explant. An efficient regeneration system is therefore essential for transformation and propagation. Although multiple shoots were successfully produced from cucumber cotyledons (Chee 1990; Selvaraj et al. 2007), leaves (Seo et al. 2000) and embryonal axes (Nishibayashi et al. 1996; Vasudevan et al. 2007) using tissue culture methods, it has been proven that a high variation frequency in the regenerated cucumber plants occurs at the callus or somatic embryo stage (Malepszy and Nadolska-Orczyk

1989). In contrast, morphological or physiological variation was much lower in the shoots produced by the direct regeneration of explants (Burza and Malepszy 1995; Plader et al. 1998).

In this study, an efficient method for high-frequency shoot production via organogenesis from cotyledonary nodes of cucumber was established. Transgenic cucumber plants were successfully obtained using this regeneration protocol, demonstrating that this system can be successfully used for the transformation of cucumber.

Materials and methods

Plants materials

The seeds of the cucumber cultivar, 'Xintaimici', were provided by XiangYun Co., Ltd, Shandong Province, China.

Regeneration and selection

The cucumber seeds were soaked for 24 h in water, sterilized using 70–75 % (v/v) ethanol for 30 s and 4 % (v/v) sodium hypochlorite for 20 min and subsequently rinsed four or five times with sterilized distilled water. The seeds were cultured on half-strength Murashige and Skoog medium (1/2 MS) (Murashige and Skoog 1962) containing 3 % (w/v) sucrose without vitamins. The pH of the medium was adjusted to 5.8 before adding 7 g l⁻¹ agar and autoclaved at 121 °C for 20 min. The culture conditions were kept constant at 25 ± 1 °C under a 16 h photoperiod with an irradiance of 30 μmol (m² s)⁻¹ provided by cool-white fluorescent lamps in a culture room. The seeds were initially treated for 2 days in the dark to promote rapid germination. The regeneration media contained MS salts, 3 % sucrose, 0.7 % agar and different concentrations of 6-benzyladenine (BA), as described in Table 1. Antibiotics

Table 1 Influence of BA levels on shoot organogenesis in cotyledonary nodes of cucumber

BA concentration (mg l ⁻¹)	No. of explants	No. of explants developing shoots	No. of shoots	Regeneration frequency (%)	No. of shoots per explants
0	30	3.7	3.7	12.0g	1.0g
0.5	30	17.3	21.3	57.7d	1.23e
1.0	30	25.3	40.7	84.3b	1.61c
1.5	30	28.3	55.7	94.3a	1.97a
2.0	30	23.0	39.0	76.7c	1.69b
2.5	30	17.0	24.3	57.7d	1.42d

All media consisted of Murashige and Skoog (MS) medium and 3 % (w/v) sucrose. These experiments were repeated in triplicate. Different letters refer to significant differences at the 0.05 level (LSD test)

and BA were filter-sterilized and added to the autoclaved media that was to be used for transformation.

The cotyledonary node explants were obtained from 7 day-old aseptically germinated plants. The cotyledonary nodes were obtained according to the techniques described by Dang and Wei (2009), with minor modifications. The procedure involved slicing the embryonic axis (2 mm) while still attached to the cotyledons, removing the growing point, slicing the embryonic axis into two halves while still attached to the cotyledons and removing one-third of the cotyledon end and the cotyledon edge so that the explants contained one cotyledon with a small portion (2 mm) of split embryonic axis attached to it (including the axillary meristem). These explants were placed on 100 mm × 15 mm petri dishes filled with 30 ml of different regeneration media (Table 1) and maintained in a growth room at 25 ± 1 °C with a 16 h photoperiod and an irradiance of $30 \mu\text{mol} (\text{m}^2 \text{s})^{-1}$. For each treatment, 30 explants were used and each experiment was repeated three times. The subculture period was maintained at two-week intervals. Visual observations were conducted every week and the effects of the different treatments were quantified on the basis of the percentage of explants showing plant development responses and the number of shoots/culture after 4 weeks.

In order to determine the concentration of kanamycin in the regeneration media needed to suppress the regeneration of the cucumber cultivar, ‘Xintaimici’, uninfected cotyledonary node explants were placed on MS media with 1.5 mg l^{-1} BA containing 50, 100, or 150 mg l^{-1} kanamycin. The minimum lethal dose was used for selecting the transformed shoots. A positive control without kanamycin was also maintained. The growth conditions were similar to those described above (Fig. 1)

Bacterial strain and plasmid type

The strain of *A. tumefaciens* used in this study was LBA4404 (Hoekma et al. 1983) harboring pBI121, which is a binary vector that contains an *NPTII* gene for kanamycin resistance. The *CsNMAPK* coding sequence was inserted into the sense orientation of the pBI121-based binary vector, pHAGSK, downstream of the cauliflower mosaic virus 35S promoter (35S:*CsNMAPK*). The GUS gene of the vector was replaced with *CsNMAPK* at the *XbaI* and *Sall* restriction sites (Fig. 2).

Plant transformation

The *A. tumefaciens* strain, LBA4404, harboring the binary plasmid, pBI121, was used to inoculate 50 ml of liquid Yeast Extract Peptone (YEP) medium (An et al. 1988) containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} rifampicin

in an Erlenmeyer flask. The culture was then shaken at 100–110 rpm overnight in the dark at 28 °C. The overnight culture of *A. tumefaciens* ($\text{OD}_{600} = 1\text{--}1.5$) was diluted 1:5 with liquid MS medium.

Agrobacterium tumefaciens transformation rate of the cucumber cotyledonary nodes

On the basis of the selected medium, physical conditions for plant regeneration and the concentration of kanamycin, 250 explants were selected and used to determine the *A. tumefaciens* transformation rate of the cucumber cotyledonary nodes. The cotyledonary node explants were obtained from 7 day-old aseptically germinated plants and pre-cultured in MS medium containing 1.5 mg l^{-1} BA for 2 days in the dark. The explants were then immersed in the bacterial suspension for 20 min under constant shaking, following which, the explants were removed, blotted dry with sterile filter paper to remove excess bacteria and placed on petri plates filled with MS medium containing 1.5 mg l^{-1} BA. The plates were sealed with parafilm and the co-cultivation was performed at 25 °C for 2–3 days in the dark.

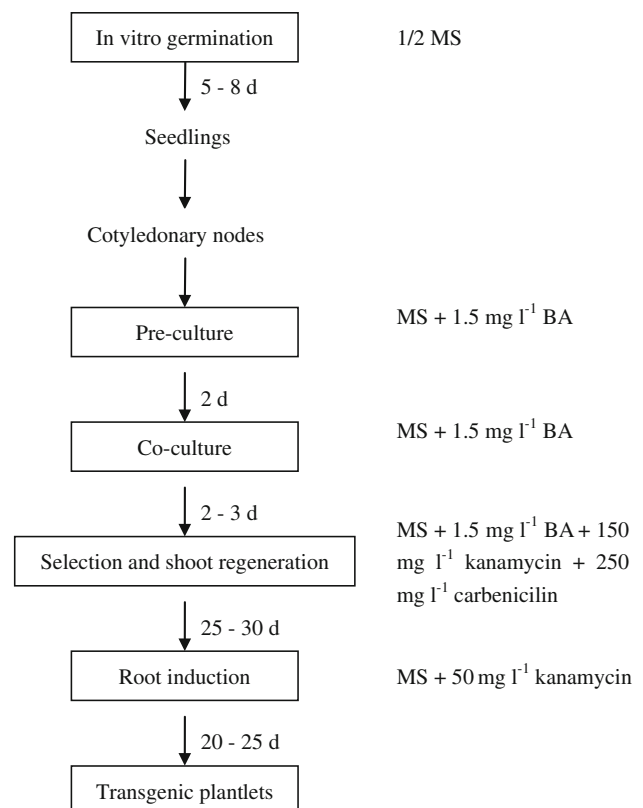


Fig. 1 Tissue culture medium and transformation protocols in cucumber. All media consisted of Murashige and Skoog (MS) medium and 3 % (w/v) sucrose. Antibiotics and BA were filter-sterilized and added to the autoclaved media that was to be used for transformation

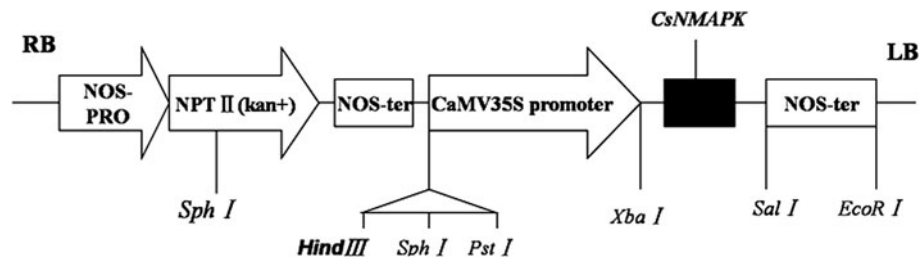


Fig. 2 Construction of the sense-*CsNMAPK* expression vector. *CsNMAPK* MAPK cucumber gene, *NOS-PRO* nopaline synthase promoter, *NPTII* gene for neomycin phosphotransferase, *NOS-ter*

terminator for nopaline synthase, *CaMV35S promoter* 35S cauliflower mosaic virus promoter

After co-cultivation, the infected explants were washed with liquid MS medium containing 250 mg l⁻¹ carbenicillin, in order to stop the growth of any *A. tumefaciens* attached to the explants, and then transferred to a selective medium consisting of shoot regeneration medium supplemented with 150 mg l⁻¹ kanamycin, 250 mg l⁻¹ carbenicillin and 1.5 mg l⁻¹ BA for 25–30 days. The subculture period was maintained at two-week intervals.

Plantlets regenerated from the cotyledonary nodes were transferred into rooting medium (MS medium + 50 mg l⁻¹ kanamycin) for 3–4 weeks. The rooted plantlets were then transplanted into pots (10 cm × 14 cm) with a sterile media of 1 vermiculite : 1 perlite : 1 turf (v/v/v) and grown under high relative humidity in an illuminated incubator for 2 weeks. After this, the plantlets were moved to a greenhouse.

PCR analysis

Genomic DNA from the young leaves of regenerated plants was extracted following the method used by Doyle and Doyle (1990). For the PCR analysis, two specific primer sequences for the *NPTII* coding region were designed to amplify a 668 bp fragment. The primers (forward, 5'-CTGGGCACAACAGACAATC-3'; reverse, 5'-TACCGTA AAGCACGAGGAA-3') were synthesized by the Sunny Biotech Company (Shanghai). The PCR was performed using the following program: 94 °C pre-denaturation step for 3 min; 35 cycles of amplification (94 °C for 30 s; 58 °C for 30 s and 72 °C for 1 min) followed by 72 °C for 10 min. The PCR products were separated by electrophoresis in a 1.0 % agarose gel.

Southern blot analysis

Southern blot analysis was performed in a manner similar to that previously described by Al Abdallat et al. (2011) and Zia et al. (2010) with some minor modifications. For Southern blot analysis, the primers for the specific genes of *CaMV35S/CsNMAPK* were synthesized downstream of the

cauliflower mosaic virus 35S promoter and the internal conservative fragment of *CsNMAPK* (Fig. 2). The primers set generated a 781 bp amplicon (forward, 5'-AGGTGGCTCTACAAAT-3' and reverse, 5'-TGCTCGTTTCAAGTC-3'), which was synthesized by the Sunny Biotech Company (Shanghai). About 30 µg genomic DNA was extracted from the leaves of four randomly selected transgenic plants and was digested overnight at 37 °C with BamHI, which did not cut within the probe region, separated on a 0.8 % (W/V) agarose gel and subsequently transferred to a nylon membrane (Roche). The nylon membrane was fixed by baking at 80 °C for 90 min. The probe was labeled using digoxigenin (DIG)-dUTP with DIG High Prime DNA Labeling reagents II (Roche, Mannheim, Germany). Hybridization was carried out at 47 °C for 20 h. Washing, blocking and detection were carried out according to the manufacturer's instructions.

RNA isolation and quantitative real-time PCR analysis

Fresh leaf tissue (100–200 mg) was ground in liquid nitrogen and extracted with Trizol reagent (TaKaRa) according to the manufacturer's instructions. First-strand cDNA was synthesized using the PrimeScript™ RT Reagent Kit (TaKaRa), following the manufacturer's instructions.

For the quantitative real-time PCR analysis, the primers of the specific genes: *CsNMAPK* (forward, 5'-AAGCGT-TAGCACATCCGTACCT-3'; reverse, 5'-CATCTCCTT-CATCTGTTCTTCGTCT-3') and β -actin (forward, 5'-CCACGAACTACTTACAACCTCCATC-3'; reverse, 5'-GGGCTGTGATTCCTTGCTC-3') were designed using Primer Premier 5.0. The length of the *CsNMAPK* fragment and the β -actin gene was 122 and 137 bp, respectively. The PCR cycles were as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 56 °C for 30 s and 72 °C for 15 s, followed by 1 cycle of 72 °C for 3 min and 81 cycles of 55 °C for 7 s. The analysis of the relative mRNA expression data was performed using the 2^{- $\Delta\Delta$ Ct} method (Livak and Schmittgen 2001).

Statistical analysis

The values presented are the means of three replicates. The data were analyzed using factorial analysis of variance (ANOVA) with SAS (SAS Institute, Cary, NC) software. The differences between the treatments were separated by the least significant difference (LSD) test at the 0.05 probability level.

Results and discussion

Direct shoot regeneration from cotyledonary nodes

When shoots were produced by direct regeneration from explants, the morphological and physiological variation was found to be much lower than was the case with other tissue types (Burza and Malepszy 1995; Plader et al. 1998). Cotyledonary node explants obtained from 5 to 8 day old cucumber seedlings were tested for regeneration responses. The use of cotyledonary node explants in this study was based on earlier experiments using bottle gourd (Saha and Kazumi 2007), okra (Rajan and Markose 2007), castor (Alam et al. 2010) and leguminosae (Dang and Wei 2009; Mahmoudian et al. 2002; Margie et al. 2006). The results from these previous studies proved that cotyledonary nodes were efficient explants. The cytokinin-induced activation of totipotent cells has been shown to be restricted only to the proximal half of the cotyledons of cucumber (Gambley and Dodd 1990). In this study, maximum callus induction was also observed when the cut ends of the proximal half of the cotyledonary nodes were used in the presence of BA. BA, a potent cytokinin, has been used in tissue culture for the induction of shoots in various plant species. Vasudevan et al. (2007) has reported that a combination of 4.44 μM BA and 1.59 μM naphthalene acetic acid (NAA) in MS medium triggered the initiation of adventitious shoot buds from embryonal axis explants. The explants with shoot buds produced the highest number of shoots (10.6 per explant) in MS medium supplemented with 4.44 μM BA and 0.065 mM L-glutamine. Selvaraj et al. (2007) found that MS medium supplemented with 1.34 mM NAA, 8.88 mM BA, 0.91 mM zeatin and 136.85 mM L-glutamine produced the highest number of adventitious shoots from cotyledon explants, with a shoot induction frequency of 75.6 %. BA is very effective in inducing cucumber shoot regeneration at a very low concentration, yet the shoot induction frequency was significantly reduced at a higher concentration of BA (Hu and Wang 1983). Saha and Kazumi (2007) showed that the yield of shoots from cotyledonary node explants of bottle gourd was low in MS medium containing 5 mg l^{-1} BA. In this study, as the concentration of BA increased, the regeneration frequency

of the cotyledonary node explants initially rose and then declined and the highest regeneration frequency was observed on MS regeneration medium supplemented with 1.5 mg l^{-1} BA, where 1.97 shoots per explant were produced (Table 1).

Kanamycin selection

Kanamycin selection of transformed cucumber shoots has been adopted previously with successful results (Chee 1990; Kose and Koç 2003; Raharjo et al. 1996). The effective concentration of kanamycin for selecting transformed cells was examined in this study. As shown in Table 2, approximately 50 % of the explants showed necrosis at 50 mg l^{-1} kanamycin, whereas the shoot bud production decreased to 15.4 % at 100 mg l^{-1} kanamycin. A concentration of 150 mg l^{-1} kanamycin caused the total inhibition of bud production and regeneration by the cotyledonary node explants. Therefore, 150 mg l^{-1} kanamycin was used for transformant pre-selection.

Agrobacterium-mediated transformation

Explants began to develop into greenish plants on regeneration medium supplemented with 150 mg l^{-1} kanamycin and multiple shoot formation was achieved after 4 weeks. Twenty explants developed shoots out of 250 explants and, after these shoots were transferred to rooting medium supplemented with 50 mg l^{-1} kanamycin, 12 plants rooted normally. The survival rate of the rooted plantlets transferred to sterile media (Fig. 3a) and grown in the greenhouse was 100 % and these were fertile with normal growth and flower development (Fig. 3b).

PCR and Southern blot analysis

To prove the integration of the foreign DNA into the cucumber genome, PCR amplification of the *NPTII* gene (lanes 1–12) was conducted and yielded the correct band size (668 bp) corresponding to the transformed *NPTII* gene (Fig. 4). No

Table 2 Effect of kanamycin on uninfected explants of cucumber

BA concentration (mg l^{-1})	Kanamycin concentration (mg l^{-1})	No. of explants	No. of explants developing shoots	Regeneration frequency (%)
1.5	0	27	25	92.6a
1.5	50	29	14	48.3b
1.5	100	26	4	15.4c
1.5	150	30	0	0

All media consisted of Murashige and Skoog (MS) medium and 3 % (w/v) sucrose. Different letters refer to significant differences at the 0.05 level (LSD test)

PCR products were detected in the samples from the control plants. To confirm the integration of the transgene into these transgenic lines, Southern blot analysis was performed using four randomly selected transgenic lines. Figure 6 shows that the copy number of all tested transgenic lines (Lines 1–4) was a single gene copy. Furthermore, there was a common cross belt in all the cucumber (Line N, 1–4) genomic DNA due to a certain homology between the probe and the endogenous MAPK of the genomic DNA. The transformation efficiency was approximately 4.8 %. The transformation efficiency can be influenced by many factors, such as the plant variety,

selection regime, transgene cassette, *Agrobacterium* strain and culture conditions (Fang and Grumet 1990). Ntui et al. (2010) reported that the melon cultivar, NHC1-130, showed a higher transformation frequency (9.9 %) than the cultivar, ‘Ejagham’ (5.7 %). Sri Shilpa et al. (2010) suggested that in seven safflower genotypes (A-1, A-2, HUS-305, JLSF-414, JSF-1, NARI-6 and Sharda), the HUS-305 genotype showed the highest transformation frequency from both of the explants evaluated (51.4 % from the roots and 47.6 % from the hypocotyls), whereas genotypes: Sharda and JLSF-414, exhibited low transformation efficiencies of less than 10.0 %. These



Fig. 3 Plant regeneration from cotyledonary nodes of cucumber: **a** in vitro prolific shoot induction on cotyledonary node explants. **b** regenerated plant established using the described media, growing in an illuminated incubator; **c** mature regenerated plant in the greenhouse

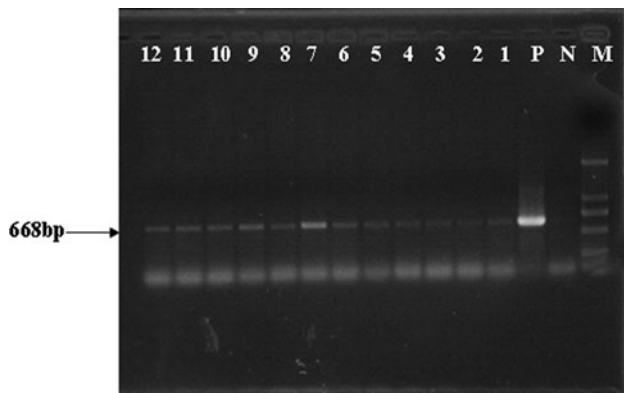


Fig. 4 PCR confirmation of transformed shoots. *Arrows* indicate the amplification of the NPTII gene (668 bp). *Lane M* DL 2000™ DNA Marker, *Lane N* DNA samples from non-transformed shoots (negative control), *Lane P* plasmid DNA (pBI-*CsNMAPK*) (positive control), *Lanes 1–12* DNA samples from transformed plants

results indicated that transformation efficiency is cultivar and genotype dependent.

Expression of sense *CsNMAPK* in transgenic plants

As sessile organisms, plants have evolved a complex signaling network that mediates the perception of and responses to different environmental cues. Recent studies have shown that MAPK cascades are evolutionarily conserved signaling modules that play a pivotal role in plant responses to multiple biotic and abiotic stresses. The roles of the MAPK pathway in salt stress and osmotic stresses have been studied in a wide range of organisms. A Northern blot analysis showed that *CsNMAPK* in transgenic tobacco was induced by salt stress and osmotic stress (Xu et al. 2010). This result was consistent with previous

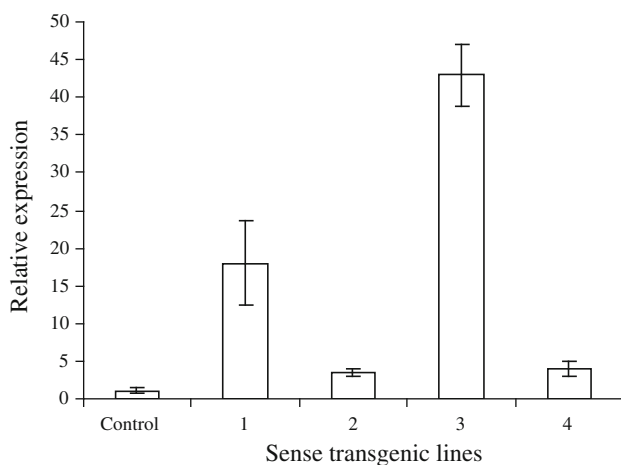


Fig. 5 *CsNMAPK* gene transcript abundance in transgenic and control plants was determined by quantitative real-time PCR analysis. Four transgenic plants (random selection) were examined. The experiment was repeated in triplicate per sample

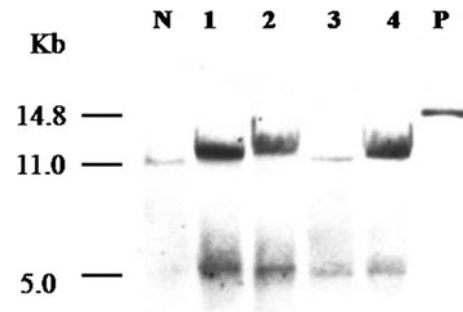


Fig. 6 Southern blot analysis of DNA isolated from putative transgenic lines. Genomic DNA (30 μ g) was isolated from the leaves of four randomly selected transgenic plants and digested with BamHI and a CaMV35S/*CsNMAPK* was used as the probe (a 781 bp PCR product) that was downstream of the cauliflower mosaic virus 35S promoter and the internal conservative fragment of the *CsNMAPK* sequences (see Fig. 2). *Lane N* DNA samples from non-transformed plants (negative control) had a single band, *Lane 1–4* DNA samples from transformed plants had two bands of different lengths, *Lane P* plasmid DNA (pBI-*CsNMAPK*) (positive control)

studies (Fu et al. 2002; Mizoguchi et al. 1996; Jeong et al. 2006; Yu et al. 2005), which indicated that salt and osmotic stresses might be activators of *CsNMAPK*. Previous studies demonstrated that plant MAPKs may positively or negatively regulate plant defense responses to environmental stresses (Xiong and Yang 2003). In order to clarify the role of *CsNMAPK* in cucumber plants, 12 transgenic cucumber plants harboring the sense *CsNMAPK* gene were obtained by this study using *Agrobacterium*-mediated transformation. To verify the efficiency of the MAPK-sense strategy, the mRNA expression levels of four of the transgenic plants (randomly selected) were determined. The quantitative real-time PCR results showed that the mRNA levels of *CsNMAPK* in transgenic plants varied but was enhanced 3.43–42.91 times compared to the control plants (Fig. 5). Future work will focus on the responses of transgenic plants under different stresses, such as salt and drought (Fig. 6).

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