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RESEARCH ARTICLE

Cloning and pharmaceutical analysis of *CaMK* gene of *Botrytis cinerea*

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Abstract **Using a PCR homology approach, DNA and cDNA sequences of calcium/calmodulin-dependent protein kinase (*CaMK*) gene of *Botrytis cinerea* were obtained. Southern blotting result displayed that *CaMK* was single copy in the genome of *B. cinerea*. The cDNA sequence of *CaMK* revealed an open reading frame of 2190 nucleotides encoding a 730 amino acid protein with predicted molecular weight of 81.8748 kDa. The genomic sequence of *CaMK* revealed the same ORF interrupted by six introns. Bioinformatics analysis showed that this protein had the distinctive features that characterize *CaMK* ATP binding region signature and serine/threonine protein kinase active-site signature. Pharmaceutical analysis displayed that the *CaMK* specific inhibitor, KN-62, could inhibit conidial germination, pathogenicity and herbicidal activity of *B. cinerea* BC4 strain. It was suggested that *CaMK* played an important role in regulating conidial germination, pathogenicity and herbicidal activity of *B. cinerea*.**

Keywords *Botrytis cinerea*, *CaMK*, pharmaceutical analysis, KN-62

Introduction

Botrytis cinerea is the causal agent of gray mold diseases on a wide variety of crop plants; at the same time, it can produce bioactive compounds when it is cultured under certain conditions (Chen et al., 2008). It was reported that mycotoxins from *B. cinerea* could be developed as microbial herbicides due to their high herbicidal bioactivity (Li, 2003; Zheng et al., 2008). Because chemical herbicides can cause pollution of environments, the use of microbial fungicides is a trend (Anand et al., 2010). For example, *Trichoderma* was used as commercial biofungicides for control of plant pathogens (Mukherjee and Kenerley, 2010). Using *B. cinerea* BC4 and its mutant isolates (strong and weak toxic isolates) as materials, herbicidal substances and gene differential expression in *B. cinerea* were studied by Ma (2006). Through the method of DDRT-PCR (differential display reverse transcription-PCR), a herbicidal activity-related fragment was obtained. Sequence analysis showed that it had high similarity to calcium/calmodulin-dependent protein kinase. However, the toxigenic heredity mechanism of *B. cinerea* is not clear at present. Based on known *CaMK* genes in GenBank database, DNA and cDNA sequences of *CaMK* of *B. cinerea* BC4 were cloned by using PCR homology approach in this study. Pharmaceutical analysis for the function of *CaMK* was performed to determine the effects of KN-62, a specific inhibitor of *CaMK* (Tokumitsu et al., 1990), on conidial germination, pathogenicity and herbicidal activity of the toxin of *B. cinerea* BC4. Results obtained would pave the way to study the regulating mechanism of *CaMK* gene in *B. cinerea* BC4 in this work.

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Materials and methods

Fungal growth conditions

The isolates of *B. cinerea* strain BC4 used in this study corresponded to the collection from the Molecular Plant Pathology Laboratory, Agricultural University of Hebei, China, which were obtained from infected fruit tissue of tomato. *B. cinerea* was grown on 2% PDA plates for 10 d at 20°C. Spores of *B. cinerea* were resuspended in sterile water.

Cloning of *CaMK* in *B. cinerea* BC4

An appropriate method to extract DNA of CTAB was changed based on the description of Drenth et al. (1993). Total RNA was isolated with Trizol kit (TIANGEN) and then used to synthesize single-stranded cDNA. Using DNA and cDNA as template, respectively, *CaMK* gene was obtained by combining DC-F with DC-R primers. The sequences of DC-F and DC-R primers designed based on the CDS sequence of *B. cinerea* in GenBank were as follows: DC-F 5′-ATGTCTT-CATTCGCTGGCG-3′ and DC-R 5′-CTACTTCTCAGAGG-TAGCCCCTTC-3′. PCR reactions were carried out using 20 ng/μL DNA/cDNA, 1 × PCR buffer, 2.5 mmol/L dNTPs, 10 μmol/L primer each and 0.5 U *Taq* DNA polymerase in a total volume of 25 μL. PCR amplification was performed for 31 cycles (98°C for 10 s, 56°C for 30 s, 68°C for 2.5 min) followed by final extension for 10 min at 72°C. The PCR products were resolved on 1.2% TAE-agarose gel and purified from an agarose gel slice using the UNIQ-10 Gel Extraction Kit (Sangon). The purified PCR products were cloned into the plasmid pMD-19 simple vector (Takara) following the manufacturer's instructions. M13 primers were used to generate single pass partial sequences of the plasmid with the differential band inserts. The positive clones were sequenced using an automated sequencer (ABI PRISM 3730XL, Shanghai Sangon Biological Engineering Technology And Service Co., Ltd.).

Bioinformatics analysis

The cDNA and DNA sequences of *CaMK* were compared by DNAMAN software. The CaMK protein character was predicted by DNAStar. The conserved domain was analyzed by ScanPro site. The secondary structure of CaMK protein was analyzed by SOPMA, and the three-dimensional mode was constructed by SWISS-MODEL.

Southern blotting

Genomic DNA of *B. cinerea* strain BC4 was digested with *Eco*RV that was cut at a single site within the probe-specific *CaMK* gene sequence and with *Eco*RI, *Hin*dIII and *Kpn*I restriction enzymes that couldn't cut the probe-specific sequence. The digested DNA was electrophoresised on 0.8% TAE-agarose gel and transferred to a nylon membrane (AMRESCO). Hybridization, washes and hybrid detection were done according to the instructions provided with DIG DNA labeling and detection kit (Roche).

Assay of conidial germination, pathogenicity, herbicidal activity of the toxin extracted from *B. cinerea* BC4

KN-62 (purchased from ALEXIS Corporation), a specific inhibitor of CaMK, was used to determine the effects of CaMK on conidial germination, pathogenicity and herbicidal activity of the toxin of *B. cinerea* BC4. For the inhibition of pharmaceutical biosynthesis, spores of *B. cinerea* BC4 were suspended in KN-62 at the concentrations of 20 μmol/L, 40 μmol/L, 60 μmol/L, 80 μmol/L and 100 μmol/L, respectively, and then 50 μL conidial suspension (2×10^3 speres/mL) was titrated on the plastic film and cultured in Petri dishes containing moist bibulous paper for 11 h in the dark at 20°C. At the same time, sterilized ddH_2O was used as control, and the experiment was repeated at least three times with duplicates in each experiment. Conidial germination was observed using a microscope.

B. cinerea BC4 inoculated in PDA media (Φ = 90 mm) containing 80 μmol/L KN-62 and cultured in darkness at 20°C, with sterile water used as control, was inoculated for 10 d, and mature tomatoes were infected by placing a piece of agar of 0.8 cm diameter, containing *B. cinerea* mycelium. The lesion diameter of tomato was measured 2 days after inoculation to determine the statistical significance of the data. The experiment was repeated three times. Error bars were calculated as standard error.

PD liquid media were added with KN-62 at the final concentrations of 20 μmol/L, 40 μmol/L, 60 μmol/L, 80 μmol/L and 100 μmol/L, respectively, inoculated with mycelium plugs of *B. cinerea* BC4 (Φ = 6 mm) and cultured in darkness at 20°C for 21 d, with the toxin extracted, respectively. Stem-leaf dispose was used in this experiment. *Digitaria sanguinalis* was cultured at 25°C with 3800 lx of light and 70% RH. The toxin was sprayed on the weed using a sprayer till all the leaves were wet. Each treatment was repeated three times. After 12 h, weed growth was measured.

Results

Cloning of *CaMK*

Using single-stranded cDNA as template, cDNA sequence of *CaMK* was amplificated by DC-F/R primers. A special fragment about 2.1 kb was generated from *B. cinerea* BC4 (Fig. 1A). The special fragment was resolved, cloned and sequenced. A length of 2190 bp sequence was obtained and identified as the full-length cDNA complete sequence of *CaMK*. BLAST analysis result showed that it had 98% similarity to *CaMK* gene of *B. cinerea*. Using genomic DNA

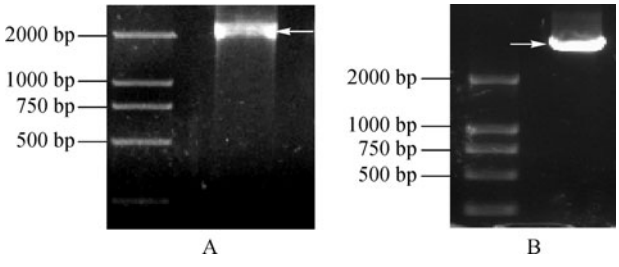

Figure 1 PCR amplification of full-length *CaMK*, cDNA and DNA of *B. cinerea* BC4. A is PCR amplification of *CaMK* cDNA. B is PCR amplification of *CaMK* DNA.

of *B. cinerea* BC4 as template, a special fragment about 2.5 kb was generated by DC-F/R primers (Fig. 1B). Cloning, sequencing and homologous analysis for the fragment indicated that the full-length DNA complete sequence of *CaMK* was 2540 bp.

Bioinformatics analysis

The bioinformatics character of *CaMK* gene was analyzed, indicating that 7 extrons and 6 introns existed in DNA of *CaMK*. The CaMK protein character predicted by DNAStar showed that the molecular weight and isoelectric point of the predicted protein was 81.8748 kDa and 6.22, respectively, with 730 amino acid residues including 95 alkaline amino acids (K, R), 116 acidic amino acids (D, E), 216 hydrophobous amino acids (AILFWV), and 160 polar amino acids (NCQSTY). The conserved domain was analyzed by ScanPro site, which indicated that the primary structure of CaMK contained ATP binding region signature and serine/threonine protein kinases active-site signature (Fig. 2).

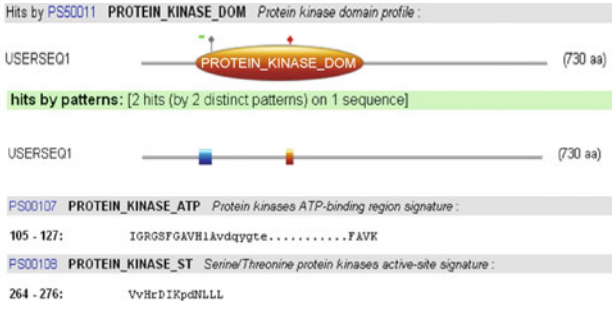

Figure 2 Conserved domain analysis of CaMK

CaMK protein had 51% homology to proteins containing the CaMK protein of *Setosphaeria turcica* by BLAST in NCBI results. The secondary structure of CaMK protein was analyzed by SOPMA (Fig. 3), which showed CaMK protein contained 37.26% alpha helix, 10.82% extend stand, 4.38% beta turn and 47.53% random coil.

Southern blotting

Southern blotting analysis was performed to determine the copy number of *CaMK* in the genome of *B. cinerea* BC4. Treatments with enzymes (*Eco*RI, *Hin*dIII and *Kpn*I) that were not cut within the sequence consistent with the probe produced single bands, and two bands were observed after the treatment with *Eco*RV that was cut at a single position within the probe sequence (Fig. 4). These results indicated that *CaMK* was single copy in the genome of *B. cinerea* BC4.

KN-62 inhibited conidial germination of *B. cinerea* BC4

CaMK inhibitor KN-62 was used to treat conidia of *B. cinerea* BC4. An inhibitory effect of KN-62 was positively related to concentrations of KN-62. Approximately 50% spore could not germinate at 60 μmol/L concentration of KN-62 (Fig. 5A). With the increasing concentration of KN-62, the inhibitory effect was enhanced gradually. The inhibition rate of conidial germination at 80 μmol/L KN-62 was approximately 100% (Fig. 5B). These results indicated that KN-62 was able to inhibit conidial germination.

KN-62 inhibited pathogenicity of *B. cinerea* BC4

To analyze the effect of *CaMK* on virulence, conidial suspension of *B. cinerea* BC4 treated with *CaMK* inhibitor KN-62 was inoculated on the acupuncture surface of tomato. Lesion sizes were determined 3 days post inoculation (dpi). In the wild-type control, water-soaked lesions with a diameter of approximately 3 cm were formed 3 dpi. The *B. cinerea* treated with 80 μmol/L KN-62 was able to infect tomato, but it was strongly delayed in primary lesion formation and lesion diameters were smaller at 3 dpi compared with the wild type (Fig. 6). These results indicated that KN-62 was able to inhibit pathogenicity of *B. cinerea*.

KN-62 inhibited herbicidal activity of the toxin of *B. cinerea* BC4

Different toxins of *B. cinerea* BC4 treated with various KN-62 concentrations were isolated and sprayed on the *D. sanguinalis*. The result indicated that the herbicidal activity of toxins from *B. cinerea* treated with KN-62 was reduced, especially the herbicidal activity of *B. cinerea* treated with 80 μmol/L KN-62 was remarkably weakened (Fig. 7), indicating that KN-62 was able to inhibit herbicidal activity of the toxin of *B. cinerea* BC4.

Discussion

Calcium/calmodulin-dependent protein kinase (CaMK) is a

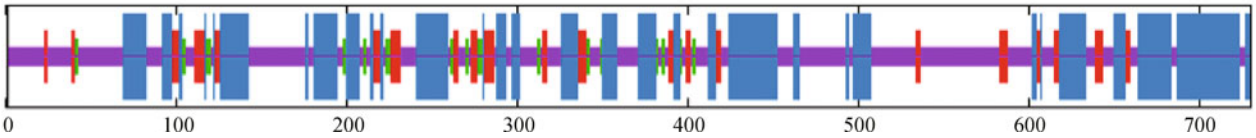

Figure 3 Predicted secondary structure for CaMK protein from *B. cinerea*. Blue, red, green and purple represent alpha helix, extended strand, beta turn and random coil, respectively.

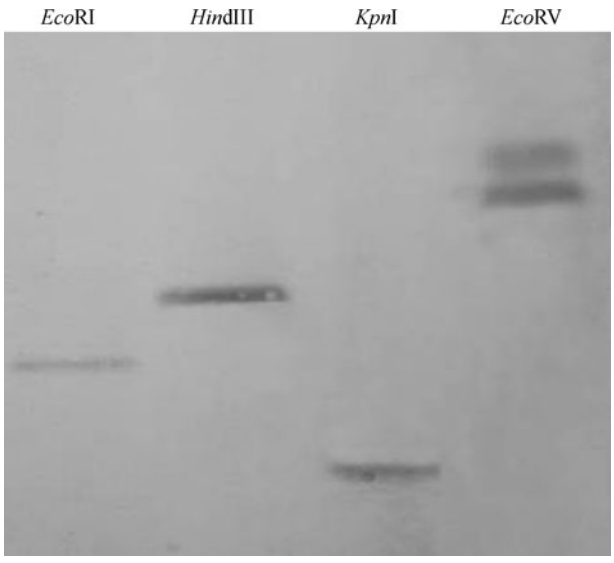

Figure 4 Southern blotting of *CaMK* in the genome of *B. cinerea* BC4.

multi-functional serine/threonine protein kinase, and it is a major member of Ca^{2+}/CaM-regulated protein family. The multifunctional CaMK family includes CaM kinases I, II, III and V on the basis of different arrangement of the catalytic, regulatory and associated domains, whose activity is highly dependent on Ca^{2+}/ calmodulin (Hanks and Quinn, 1991). In this study, we cloned a *CaMK* gene in *B. cinerea*, and the effects of inhibitors of the encoded protein on the conidial germination, pathogenicity and toxin activity were analyzed. *CaMK*s are key components in many calcium/CaM mediated pathways in animals and yeast (Ma et al., 2004; Timmins et al., 2009). *CaMK* function is various in different fungi. In the filamentous fungus *Aspergillus nidulans*, calcium/calmodulin-dependent protein kinases, CMKB was expressed on the nuclear division and affected conidia germination (Joseph and Means, 2000). The disruption of calcium/calmodulin-dependent protein kinases, CMKA and CMKB in filamentous fungus *A. nidulans* was reported to be lethal (Joseph and Means, 2002). In another filamentous fungus, in *Sporothrix schenckii*, SSCMKI inhibited effective mycelium transition (Valle-Aviles et al., 2007). The *MoCMK1* gene encoding a putative Ca^{2+}/calmodulin-dependent kinase plays key roles in the pathogenicity of the rice blast fungus (Liu et al., 2010). In *Neurospora crassa*, a Ca^{2+}/calmodulin-dependent protein kinase regulated aflatoxin production and suppressed growth of *CaMK-1* null strains (Jayashree et al., 2000; Yang et al., 2001). In *Coprinus cinereus*, a Ca^{2+}/calmodulin-dependent protein kinase played crucial roles in mycelial growth (Kameshita et al., 2007). CaMK protein of *B. cinerea* was 71% similar to Ca^{2+}/calmodulin dependent protein kinase A of *A. nidulans* and 53% similar to CaMK-II of *Saccharomyces cerevisiae*. Tsai et al. (2002) described a Ca^{2+}/ calmodulin-dependent protein kinase gene in the filamentous fungus, *Arthrobotrys dactyloides*, which encoded a protein with significant homology to mammalian *CaMK*s. Our study is the first report about cloning of *CaMK* gene in *B. cinerea*. Analysis result showed that conserved domain of Ca^{2+}/ calmodulin-dependent protein kinase such as ATP binding region signature and serine/threonine protein kinases active-site signature existed in *CaMK* of *B. cinerea* BC4. Southern blotting indicated that *CaMK* was single copy in the genome of *B. cinerea* BC4. To further elucidate the physiologic role of *CaMK* in *B. cinerea*, we examined the effect of KN-62, a membrane-permeable CaM kinase inhibitor (Praskova et al., 2002). After treatment by KN-62, conidial germination, pathogenicity and herbicidal active substances of *B. cinerea* were affected. Therefore, *CaMK* played an important regulation role in *B. cinerea*. The molecular mechanism of *CaMK* in conidial germination, pathogenicity and herbicidal activity of *B. cinerea* should be identified in the future. Using *B. cinerea CaMK* gene deletion mutants obtained by building

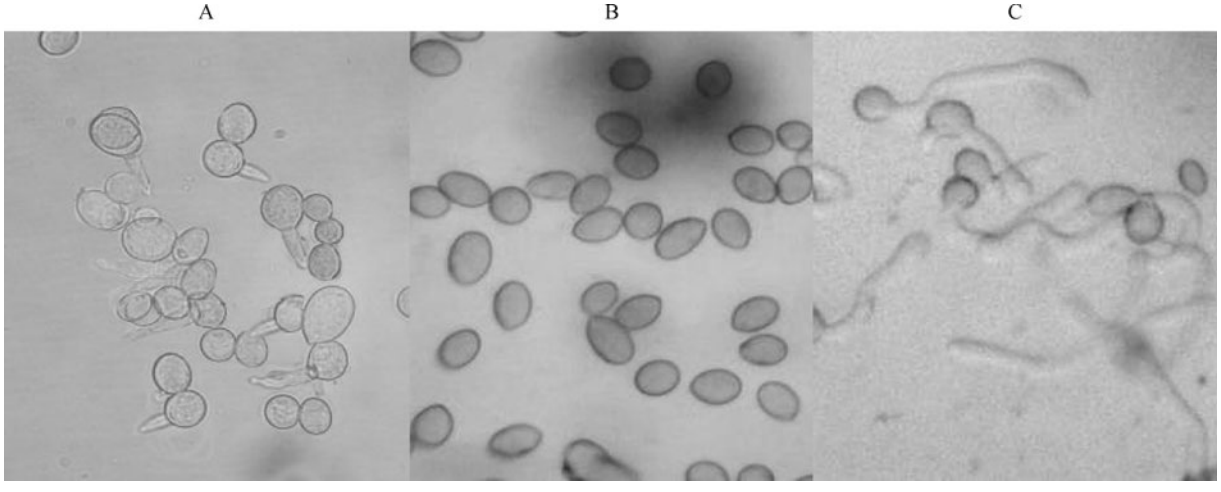

Figure 5 The inhibition of KN-62 on the conidial germination of *B. cinerea*. A–C represent concentrations of KN-62 at 60 μmol/L, 80 μmol/L and control, respectively.

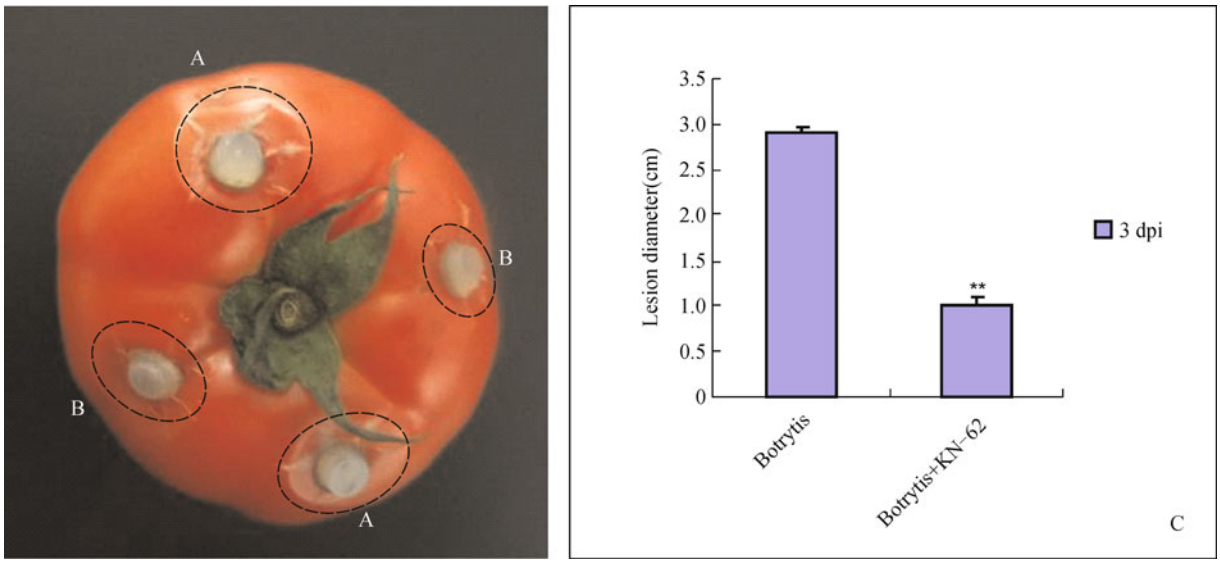

Figure 6 The inhibition of KN-62 on the pathogenecity of *B. cinerea*. A: *B. cinerea* mycelia plugs without KN-62 treatment. B: *B. cinerea* mycelia plugs with 80 μmol/L KN-62 treatment. C: Lesion areas were measured 3 d after inoculation. Values are averages from at least eight lesions. Asterisks indicate statistically significant differences between control and treatment, according to Student's *t*-test.

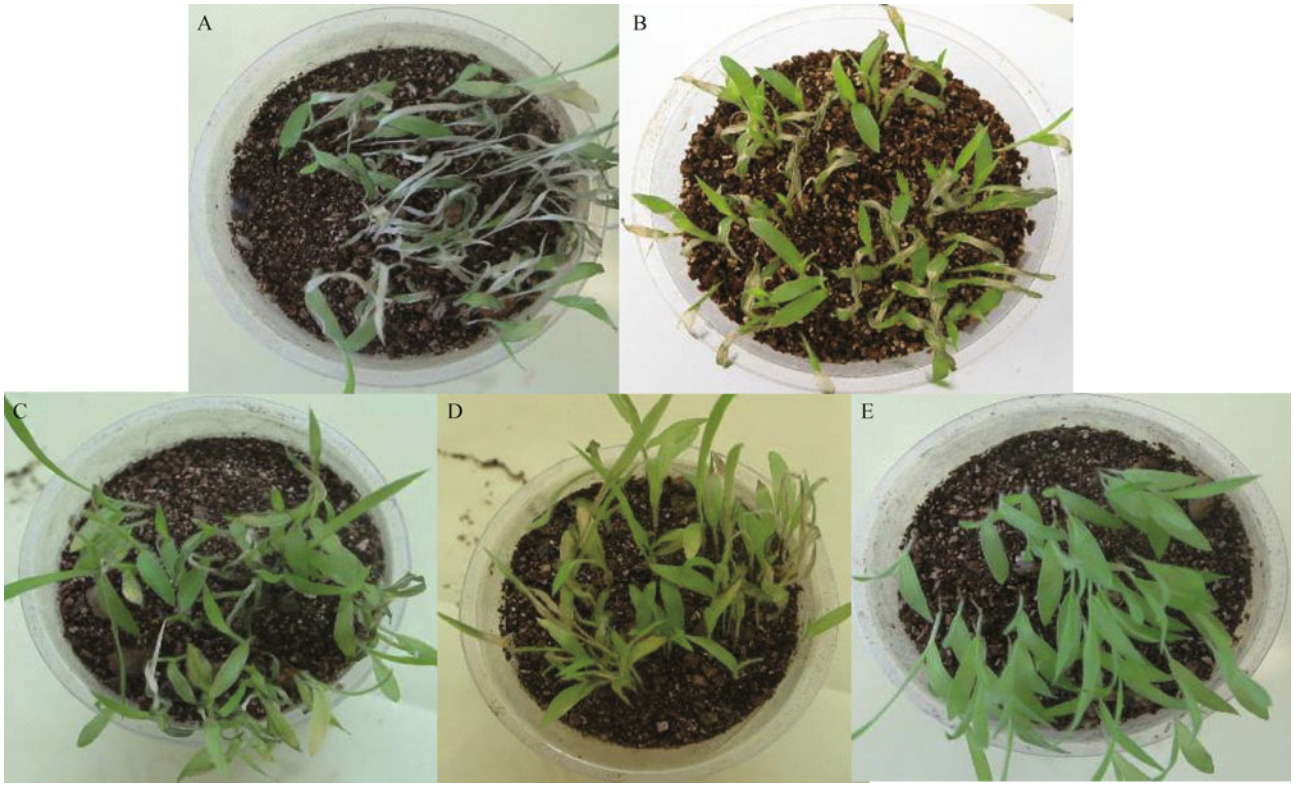

Figure 7 The herbicidal activity of the toxin of *B. cinerea* BC4 treated with various KN-62 concentrations. A–E are concentrations of KN-62 at 0 μmol/L, 20 μmol/L, 60 μmol/L, 80 μmol/L and control, respectively.

the gene homologous recombination vector and transformation of *B. cinerea* protoplast, regulation mechanism of *CaMK* could be identified. Results about regulation mechanism of *CaMK* in *B. cinerea* can provide theoretical basis for controlling gray mold and developing new herbicides, etc.

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