

CmNAC083* regulates resistance to *Alternaria alternata* via reactive oxygen species and jasmonic acid signaling pathways in *Chrysanthemum morifolium

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Abstract

Chrysanthemum morifolium is an ornamental plant of economic importance. Chrysanthemum black spot is caused by *Alternaria* fungi. Members of the NAC family regulate plant biological and abiotic stress responses. We screened and confirmed the target gene, *CmNAC083*, by transcriptome sequencing after inoculation with *Alternaria* fungi. In the present study, the *CmNAC083* gene was cloned from the chrysanthemum 'Jinba' leaves, and its protein structure was analyzed. *CmNAC083* exhibits transcriptional inhibitory activity, and a tobacco subcellular localization experiment revealed that *CmNAC083* is found in the nucleus. Compared with other tissues, *CmNAC083* was highly expressed in the stem and bud during the vegetative growth period and in the stems and roots during flowering. Inoculation with *Alternaria alternata* induced sustained high expression of *CmNAC083*, reaching its peak at 48 h after infection with the black spot pathogen. Overexpression of *CmNAC083* can enhance the resistance of chrysanthemums to black spot disease, and its transient silencing presents a susceptible phenotype. The results of RNA sequencing revealed that the transcription levels of jasmonic acid (JA) biosynthesis, response signal pathway, and reactive oxygen species (ROS) pathway genes in the *CmNAC083* overexpression lines were upregulated compared with those in the wild-type (WT) plants. Furthermore, the overexpressing strains exhibited a higher endogenous JA content than WT after treatment with *A. alternata*. Overall, *CmNAC083* overexpression positively enhances chrysanthemum resistance to black spot disease by regulating *A. alternata* infection by upregulating the transcription levels of the JA synthesis and ROS pathways.

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Introduction

Plants are often subjected to biotic stresses, such as pathogenic bacterial infections, during their growth and development, seriously affecting yield and quality. *Chrysanthemum morifolium* (chrysanthemum) is one of the top ten traditional flowers in China. However, many diseases affect chrysanthemum development and growth, including black spot disease caused by *Alternaria alternata*. Black spot disease severely affects chrysanthemum plants or cuttings and may cause chlorosis and necrosis of leaves. *Alternaria* fungi can produce pectinase and cellulase enzymes that degrade plant tissues, such as plant cell walls, thereby aiding the invasion and establishment of other disease-causing pathogenic fungi^[1,2]. Currently, chemical control is the primary control method for biotic stresses; however, it can harm plants, the environment, and humans. Therefore, using molecular biology techniques to develop new disease-resistant varieties is one of the most effective ways to control diseases and insect pests.

Plants have evolved a complex immune system to defend themselves against pathogenic infections through long-term development. When a pathogen invades a plant, it triggers a series of immune responses, and plants recognize pathogen-related molecular patterns through pattern recognition receptors on the cell surfaces (pathogen-related molecular pattern-triggered immunity [PTI])^[3,4]. PTI is the first line of defense

induced in response to pathogen invasion. However, pathogenic bacteria evolve and secrete a series of toxic effectors to disrupt the plant's PTI defense response mechanism or use the effector to mimic substances produced by plants to facilitate pathogen infection and reproduction in plants to suppress PTI immunity^[5]. Simultaneously, plants will configure various types of immune receptor proteins containing nucleotide binding domains and leucine repeat rich domains based on the PTI immunity to monitor and inhibit the activity of infected pathogenic factors, an immunity type called effector-triggered immune response (ETI)^[6]. PTI and ETI are primary immune defense mechanisms against plant pathogenic infections.

Reactive oxygen species (ROS) are a class of oxygen-containing molecules with more active chemical properties than oxygen. Various regions produce and remove ROS in plants, and each region has an antioxidant system that regulates the balance between local oxidation and reduction^[7]. In contrast, ROS can act as a signaling molecule to induce an immune response to pathogens. Furthermore, ROS can be toxic to plants, causing damage to plant cells. ROS is produced in the chloroplasts, mitochondria, peroxisomes, and ectoplasts. Furthermore, the superoxide anion generates H₂O₂ under the action of superoxide dismutase (SOD). ROS production in ectoplasts may play a major role in biological and abiotic stresses^[8]. Respiratory burst oxidase homolog (RBOH) enzymes are the

most important extracellular (ROS)-related enzymes. Expression of the *RBOHD* gene containing a mutation in the bik1-specific phosphorylation site of the *rbohD* mutant inhibits excessive oxidative damage, and the mutant plants are more susceptible to pathogens, indicating that RBOHD and bik1-mediated phosphorylation contribute to plant immunity^[9]. The sensor kinase protein MoOsm1 in rice can maintain ROS balance by phosphorylation and dephosphorylation transformation, thereby improving the resistance of rice to rice blasts^[10]. The *RbohA* and *RbohB* expressions are upregulated to resist pathogen infection during invasion by *Magnaporthe oryzae* and powdery mildew (*Blumeria graminis* f. sp. *hordei*) in rice and barley, respectively. Furthermore, antisense strains of the *RBOH* gene render plants susceptible to diseases^[11,12].

Plants generate different hormone signaling pathways to resist pathogen infection. Jasmonic acid (JA) is an important lipid hormone in plant immunity against necrotrophic pathogens. JA can be catalyzed to form methyl jasmonate (MeJA) and other polymers and metabolites, and it can form derivatives through alkylation, glycosylation, or when combined with amino acids^[13]. The role of JA signaling pathway genes in resistance to biotic stresses has been widely reported. For example, deletion of the *GhOPR9* gene increased the susceptibility of cotton to *Verticillium dahliae*^[14]. Overexpression of *VvAOS* and *VvCOI* genes can significantly reduce the hyphal area of *Botrytis cinerea* after infecting grapes^[15]. Inhibition of the *LOX* gene expression in tobacco increased susceptibility to *Phytophthora nicotianae* infection^[16]. *LOX*, *COI*, and *OPR* genes in wheat are expressed in response to aphid invasion. Downregulation of *COI1* and *JAZ* in cotton increased susceptibility to aphid invasion^[17,18]. A previous study reported that JA signal expression correlates with temperature. Low temperatures reduce the resistance of rice to *M. oryzae* and inhibit JA biosynthesis and signal transduction, increasing the susceptibility of rice to diseases^[19].

The NAC transcription factor family is one of the largest transcription regulatory factors in plants and exists in various plant species, such as *Arabidopsis thaliana* (*Arabidopsis*), rice, tobacco, and chrysanthemums^[20–23]. Previous studies found a highly conserved amino acid sequence, named the NAC domain, at the N-terminus of the protein sequence encoded by the genes in *Petunia hybrida* (*NAM*), *Arabidopsis* (*ATAF1/2*), and *CUC2* (cup-shaped cotyledon)^[24,25]. The NAC is divided into five subdomains: A, B, C, D, and E. The B and E subdomains are not highly conserved, whereas the C and D are highly conserved and may be the primary structures involved in the DNA-binding process^[26–28]. The NAC domain has a symmetric dimer structure different from the general double-helix structure. The C-terminus of NAC transcription factors contains a transcriptional activation domain that regulates transcription. The C-termini of NAC proteins are diverse; however, some contain partially conserved motifs. NAC TFs can be divided into typical NAC TFs and atypical NAC TFs considering the characteristics of their C-terminal structure^[29,30]. The atypical NAC TFs contain conserved motif fragments or C-terminal deletions. NAC transcription factors with the same motif fragments are similar in their physical structure and the biological functions they perform^[31].

The biological functions of the NAC transcription factor family mainly regulate plant growth and development, plant response to abiotic and biotic stresses, regulation of plant

secondary growth, and hormone signal transduction^[32]. *ATAF1* transcription factor in *Arabidopsis* is a negative regulator of the plant defense response to infection by biotrophic fungi and bacterial pathogens. According to a previous study, overexpression of *ATAF1* exhibited higher sensitivity to *B. cinerea*^[33]. Overexpression of *OsNAC6* exhibited significant effects on dehydration, high salt stress, and tolerance to *M. oryzae* in rice^[34]. Plants overexpressing *OsNAC111*, *OsNAC066*, and *OsNAC58* had increased resistance to *M. oryzae*, *Xanthomonas oryzae* causing rice bacterial blight, and the sensitivity of rice to *X. oryzae*, respectively^[35–37]. Furthermore, the *mOsNAC60* mutation increased the sensitivity of rice to *M. oryzae*^[35–37]. *GbNAC1* positively regulates verticillium wilt resistance in cotton^[38]. The regulation of pathogen invasion by NAC transcription factors often triggers plant defense responses through hormone-signaling molecules. For example, *AtNAC019/AtNAC055*, *RIM1*, and *TaNAC6s* regulate *B. Cinerea*, *Rice dwarf virus*, and *B. graminis* f. sp. *tritici*, respectively, via the JA signaling pathways^[39–41]. Significant progress has been made in research on the biological functions of NAC genes in model plants. However, there are few studies on the functions of NAC family genes in chrysanthemums and no reports on the regulatory processes of biotic stress.

Therefore, the present study aimed to investigate the regulatory mechanisms of *CmNAC083* in response to black spot infection in chrysanthemums. We cloned the *CmNAC083* gene and determined its response to *A. alternata* infection. Furthermore, we evaluated the effect of *CmNAC083* on black spot disease resistance of the genetically transformed chrysanthemum 'Jinba.' Through RNA sequencing, we found that *CmNAC083* enhances the regulatory mechanism of chrysanthemum resistance to black spot disease. The present study lays a theoretical foundation for analyzing the role of NAC family genes in the resistance of chrysanthemums to black spot disease, and provides primary information for the breeding of new resistant varieties.

Materials and methods

Plant materials and growing conditions

The chrysanthemum 'Jinba' used in the present study was obtained from the Chrysanthemum Germplasm Resources Conservation Center of the Nanjing Agricultural University, Nanjing, China. The material was grown under long sunshine (16 h light/ 8 h darkness) with temperature maintained at 25 °C and 70% relative humidity. Wild-type (WT) and transgenic materials were cut into a hole plate for 2 weeks and then transplanted into a small black square for cultivation for 2 weeks before treatment. *Nicotiana Benthamiana* was grown under long sunshine (16 h light / 8 h darkness), with temperature maintained at 25 °C and relative humidity of 70%.

CmNAC083 cloning and sequence analysis

In the present study, total RNA was extracted from chrysanthemum 'Jinba' leaves using the RNA isolation kit (Huayueyang, Beijing, China) according to the manufacturer's protocol. The PrimeScript reverse transcription kit (Takara Bio, Tokyo, Japan) was used to reverse-transcribe RNA into cDNA. The *CmNAC083* sequence was downloaded from the chrysanthemum genome database to design primers (*CmNAC083*-ORF-F: 5'-ATGACAAA

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GCTAAATTTCTTCG-3'; *CmNAC083*-ORF-R: 5'-CTA-TAAACTATTCACTTCATCTTCA-3'). The open reading frame (ORF) of *CmNAC083* was amplified, and the polymerase chain reaction (PCR) products were inserted into the Blunt plasmid of the cloning vector (Vazyme, Nanjing, China) for sequencing. The molecular weight and isoelectric point of *CmNAC083* were predicted using the SWISS-PROT database (<https://web.expasy.org/compu-tepi/>). The amino acid sequences of the AtNAC family members were downloaded from the Arabidopsis Information Resource website (www.arabidopsis.org/Blast/index.jsp). The protein homologs of the *CmNAC083* and AtNACs were compared using DNAMAN 9 software (Lynnon Biosoft Bioinformatic Solutions, San Ramon, CA, USA), and a phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) 5.1 software (MEGA, Mumbai, India) using the neighborhood connection method and 1,000 guided repeats. AtNAC genes includes the following: *AtNAC002* (AT1G01720); *AtNAC019* (AT1G52890); *AtNAC041* (AT2G33480); *AtNAC047* (AT3G04070); *AtNAC055* (AT3G15500); *AtNAC056* (AT3G15510); *AtNAC059* (AT3G29035); *AtNAC081* (AT5G08790); *AtNAC083* (AT5G13180); *AtNAC092* (AT5G39610); *AtNAC100* (AT5G61430) genes.

Prediction of *CmNAC083* subcellular localization

The subcellular location of *CmNAC083* was predicted using PlantPLOC (www.csbio.sjtu.edu.cn/bioinf/plant-multi). The *CmNAC083* ORF sequence was amplified with *CmNAC083*-R4-F/R primers (*CmNAC083*-R4-F: 5'-TCCCCCGGGATGACAAAGC-TAAATTTCTTCG-3'; *CmNAC083*-R4-R: 5'-CCATCGATTAACAATTCACCTTCATCTTCAT-3'), in which an SmaI recognition site was included at the 5' end of the forward primer, and a ClaI recognition site was included at the 5' end of the Reverse primer. After double-digestion purification, the amplified product was inserted into the double-digested pORE-R4 and the vector contained the CaMV2 × 35S promoter-driven reporter green fluorescent protein (GFP) gene. P35S::*CmNAC083*-GFP (pORE-R4-*CmNAC083*) and p35S:: GFP (pORE-R4) were transformed into *Agrobacterium* strains using an electric shock. The constructed fusion protein was introduced into tobacco epidermal cells through needle injection for infection, considering the *Agrobacterium* infiltration method. After culturing in the dark for 24 h, the material was stored for 48 h under long-term sunshine conditions. GFP activity of the material was observed and imaged using a laser scanning confocal microscope (Carl-Zeiss, Munich, Germany).

***CmNAC083* transcriptional activation activity**

The transcriptional activity of *CmNAC083* was examined using the yeast two-hybrid system. The *CmNAC083* ORF sequence was amplified using *CmNAC083*-BD-F/R primers (*CmNAC083*-BD-F: 5'-GGAATTCATGACAAAGCTAAATTTCTTCG-3'; *CmNAC083*-BD-R: 5'-CGGATCCGCTATAAATTCACCTTCATCT-3'), and then digested using EcoRI and BamHI restriction endonuclease and introduced into the pGBKT7 vector to construct pGBKT7-*CmNAC083*. The plasmids pGBKT7-*CmNAC083*, pCL1 (positive control), and pGBKT7 (negative control) were transformed into *Saccharomyces cerevisiae* strain Y2HGGold (Takara Bio, Tokyo, Japan) according to the manufacturer's instructions. The transformed strains containing pGBKT7-*CmNAC083* or pGBKT7 were grown on the synthetic-defined (SD)/-tryptophan (Trp) medium, and the transformed strains containing pCL1 were grown on the SD/-Leucine medium. The transformed yeast cells were grown in SD/-Histidine-Adenine medium with X- α -Gal and SD/-Histidine-Adenine

medium. Next, the transformed yeast cells were cultured at 30 °C for 3–4 d. Finally, the blueness of the yeast strain was observed. The present study also used the transient transformation of Arabidopsis protoplasts to verify the *CmNAC083* transcriptional activity. The *CmNAC083* ORF sequence was amplified using *CmNAC083*-pENTR1A-forward and -reverse primers (*CmNAC083*-pENTR1A-F: 5'-CGGGATCCGGATGACAAAGC-TAAATTTCTTCGAA-3'; *CmNAC083*-pENTR1A-R: 5'-ATAAGAAT-GCGGCCGCGATAAATTCACCTTCA-3'), and was digested using BamHI and NotI restriction endonuclease and then was introduced into the pENTR1A vector to construct pENTR1A-*CmNAC083*. The pENTR1A-*CmNAC083* plasmid was recombined using the GAL4-DB plasmid using the LR recombination method. GAL4-DB-*ARF5* and the empty vector GAL4-DB were used as the positive and negative controls. GAL4-*LUC* was used as the reporter co-transformation plasmid. High-concentration plasmids were extracted for protoplast transformation using a Plasmid Midi Kit (Qiagen, Hilden, Germany). The extraction and transformation methods for Arabidopsis protoplasts were according to Wang et al.^[42]. After incubation for 16–18 h overnight, 20 mM sodium fluorescein (Sigma-Aldrich, Mannheim, Germany) was added to each sample. The indicated luciferase (LUC) activity was measured using a GloMax enzyme marker (GloMaxR20/20; Promega, Madison, WI, USA), and a CCD camera (Tanon 5200; Tanon Science, Shanghai, China) was used to capture the images.

A. *alternata* inoculation and disease severity assessment

A. alternata F20 was cultured on potato glucose agar medium (Sigma-Aldrich) at 28 °C in darkness for 4–5 d. An appropriate amount of bacterial cake was picked from the growth edge of the bacteria in the culture medium and put into the potato glucose broth medium (PDB [Sigma-Aldrich]) for shaking propagation for 24 h at 28 °C. Next, the cultivated F20 was broken, transferred into the new PDB shaking propagation for 24 h at 28 °C, and used for inoculation treatment. Next, 1 mL of mycelia from the culture medium was collected to form a ball and inoculated on the back of the third fully expanded leaf using a toothpick. Each leaf was inoculated twice. Each line was inoculated with nine strains, and the experiment was independently repeated in triplicate. The inoculated seedlings were placed in a storage box and cultured for 48 h in a long sunshine (16 h light / 8 h dark) light incubator at 28 °C and a relative humidity of 70%. ImageJ software (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation, University of Wisconsin, WI, USA) was used to measure the diseased area of the inoculated leaves 2 d after inoculation. Disease severity was evaluated by determining the average lesion area.

qRT-PCR analysis

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was carried out on a LightCycle 96 real-time PCR system (Roche, Basel, Switzerland) using the SYBR Premium Ex Taq II kit (Takara Bio, Tokyo, Japan) according to the manufacturer's instructions. The qRT-PCR reaction procedure includes 40 cycles of pre-denaturation at 95 °C for 2 min, and at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s. The qRT-PCR data were obtained from three biological and three technical replicates for each sample. The expression level was calculated using the 2^{- $\Delta\Delta$ Ct} method^[43]. *CmEF1 α* (GenBank: KF305681) was used as an

internal reference gene. The primer pairs used for the qRT-PCR analysis are presented in [Supplemental Table S1](#).

Chrysanthemum genetic transformation

The construction method for the pORE-R4 vector is described above. The constructed fusion plasmid was transformed into *Agrobacterium tumefaciens* EHA105 strain using the electric shock method and then transferred into chrysanthemum 'Jinba' using the leaf-disk infection method^[38]. After leaf disc sprouts and roots, the positive strains were identified at the DNA level using the primer pair pORE-R4-F/*CmNAC083*-R4-R (pORE-R4-F: 5'-GACGACAATC-CCACTATCC-3'), and the relative expression level of *CmNAC083* was determined using qRT-PCR analysis. Each sample was tested using three biological and three technical replicates. The transgenic chrysanthemum and WT tissue culture seedlings were cultured at 25 °C under long sunshine (16 h light / 8 h darkness) after growing in the hole plate for 2 weeks.

Virus-induced gene silencing

A previously reported virus-based miRNA expression system^[44–47] was used to silence *CmNAC083* in chrysanthemum. Web MicroRNA Designer (wmd3.weigelworld.org/) was used to design the miRNA sequences (TAAGTAATGTACCGCAAGCTC) for the target genes. RS300 plasmid was used as a template and the following primers (I miR-s: 5'-gaTAAGTAATGTACCGCAAGCTCtctctctttgtattcc-3'; II miR-a: 5'-gaGAGCTTGCGGTACATTACTTcaagagaga-atcaatga-3'; III miR*s: 5'-gaGAACTTGCGGTACTTACTTTtcacaggtcgtga-tatg-3'; IV miR*a: 5'-gaAAAGTAAAGTACCGCAAGTTCctacatatattcct-3') were used to amplify amiRNA precursors. RS300R-*CmNAC083* was digested using XbaI and KpnI restriction endonucleases, introduced into the cabbage leaf-curl gemini-virus vector (CaLCuV), and transformed into *Agrobacterium tumefaciens* strain GV3101. PCVB and CaLCuV (control) or CaLCuV-*CmNAC083* were mixed evenly in 1:1 ratio (v/v), incubated with infiltration buffer at 28 °C for 3 h and then vacuumed (0.7 MPa) for 5–10 min. The plants were placed in the dark at 10 °C for 1–3 d and then transferred to a flowerpot containing a mixture of nutrient soil and vermiculite 1:3 (v/v), and grown under long sunshine conditions (16 h light/ 8 h darkness) for 3 weeks. The primer pair (II miR-a; CaLCuV-R: 5'-GCAACGGACAGATCCTATGTAAC-3') was used to identify the positive strains of CaLCuV-*CmNAC083* by removing the newly grown third leaf from the top^[48]. Positive strains were screened for the determination of the *CmNAC083* expression. Two independent experiments were conducted, and at least nine positive WT or transiently silenced strains were used for inoculation.

Determination of physiological indexes

The contents of endogenous JA were measured using plant JA elisa kit from the Shanghai Yuanju Biotechnology Center (Shanghai, China). The anti-superoxide anions and SOD activities were measured using a test kit from the Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China, according to the manufacturer's instructions. Diaminobenzidine (DAB) staining was performed after inoculation with WT and transgenic strains. First, 0.1 g DAB powder was dissolved in 100 mL of 10 mM phosphoric acid buffer (pH 7.8 [FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan]), and the pH was adjusted to 3.8. The leaves were completely immersed in the dye solution, placed on a shaking table, and slowly shaken in the dark for 8–12 h until brown spots were observed. The dyed leaves were fully immersed in 80% alcohol for 15 min in boiling water. The

leaves were then completely immersed in 95% alcohol for 15 min in boiling water; this was repeated twice until the color of the tissue faded. The decolored tissue was completely soaked in anhydrous ethanol and stored in a refrigerator at 4 °C until imaging.

RNA extraction, transcriptome sequencing, and bioinformatics analysis

The third fully expanded leaf of WT and OE-*CmNAC083* (OE-128, OE-156) was inoculated with *A. alternata* for 48 h to study the possible signaling pathway of *CmNAC083* regulating black spot disease. Samples of the third fully expanded leaf after inoculation and those of the control group were subjected to RNA-sequencing analysis. Each sample contained three biological replicates. The transcriptome was sequenced using an Illumina sequencing platform (GeneDenovo Biotechnology Co., Ltd., Guangzhou, China). The functional annotation of single genes was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG; www.kegg.jp) and Gene Ontology (GO; www.geneontology.org). The fragments per kilobase of script per million fragments mapped (FPKM) method was used to calculate the expression level of a single gene^[49]. Differential expression analysis was performed using DESeq2 software. Genes with a false detection rate (FDR) lower than 0.05 and a single gene with $|\log_2 \text{FoldChange}| > 1$ were considered differentially expressed genes (DEGs)^[50]. FDR was obtained by correcting the p-value of the significant difference. Venn maps and KEGG pathway enrichment analyses were performed using the online Omic Intelligent tool (www.omicsmart.com).

Statistical analysis

SPSS v17.0 (SPSS Inc., Madison, IL, USA) was used for one-way analysis of variance in all statistical analyses, and Duncan's multiple range test was used to determine significant differences. Statistical significance was set at $p < 0.05$.

Results

CmNAC083 exhibits significant increase after *A. alternata* inoculation

After inoculation of chrysanthemum 'Jinba' with *A. alternata* at different times, the samples were sent for high-throughput sequencing. The results revealed that the effect of *A. alternata* inoculation on *CmNAC083* expression was significantly different (Fig. 1a). The *CmNAC083* ORF sequence (Fig. 1b) was cloned to 660 bp (Supplemental Material 1). Phylogenetic tree analysis of the AtNACs homologous to *CmNAC083* indicated that *CmNAC083* exhibited the closest homologous relationship with *AtNAC041* (AT2G33480) and *AtNAC083* (AT5G13180) in Arabidopsis (Fig. 1c). *CmNAC083*- protein domain analysis revealed that its N-terminus contains a highly conserved domain of NAC family genes (Fig. 1d). The *CmNAC083* expression at different stages of *A. alternata* infection was analyzed using qRT-PCR to explore whether *CmNAC083* was involved in the infection of the Chrysanthemum black spot pathogen. *CmNAC083* was significantly and continuously induced to express at 6 h and reached the expression peak at 48 h after inoculation with *A. alternata*. The *CmNAC083* expression in the inoculated sample was 8.65-fold higher than that in the control sample (Fig. 1e), indicating that *CmNAC083* may participate in the response to the chrysanthemum black spot disease. The qRT-PCR analysis of the *CmNAC083* expression pattern in different tissues revealed that

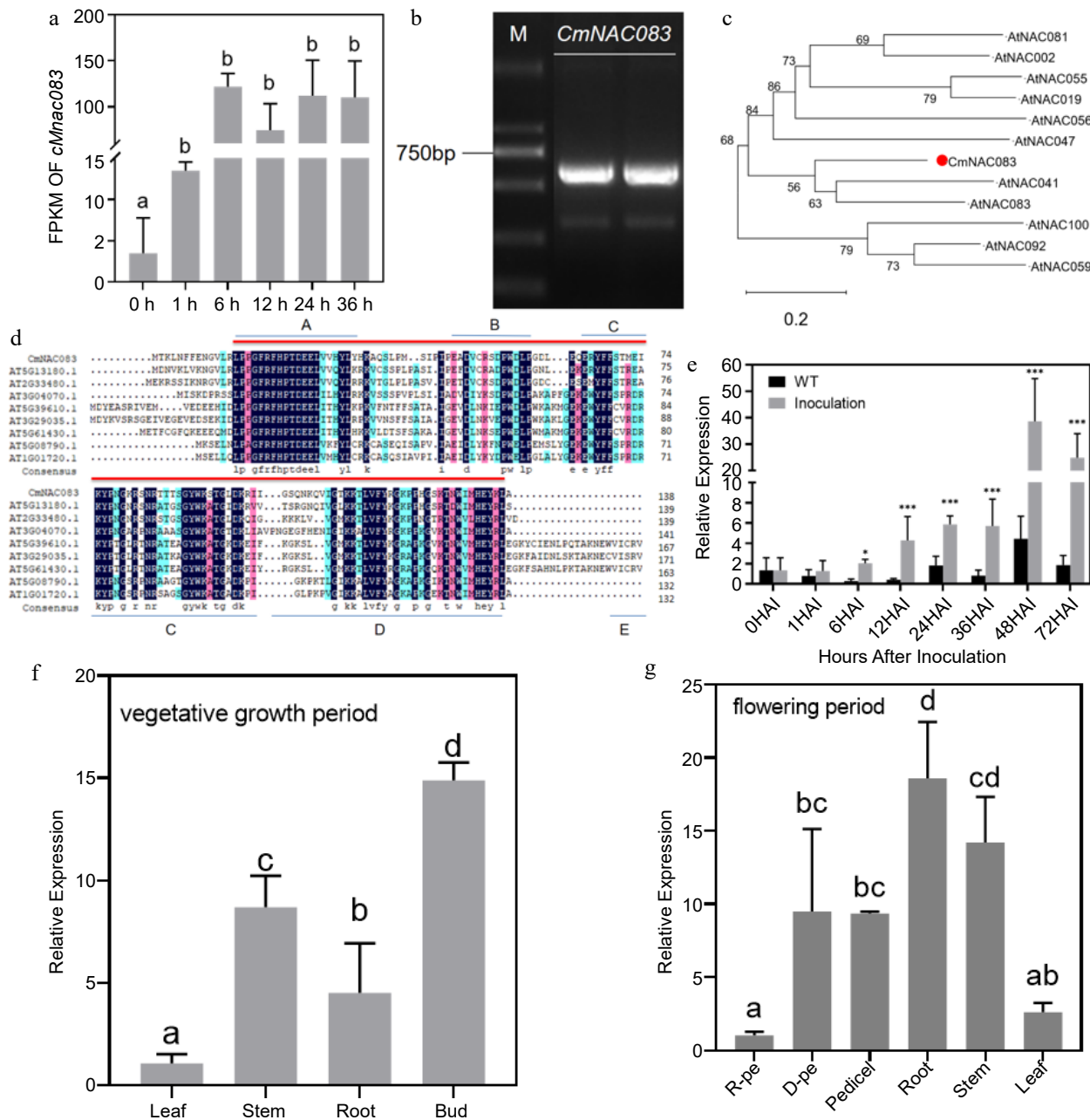


Fig. 1 Cloning and expression analysis of *CmNAC083*. (a) Analysis of the fragments per kilobase of script per million fragments mapped of *CmNAC083* at 0, 1, 6, 12, 24, and 36 h after inoculation with *Alternaria alternata*. (b) The results of electrophoresis of polymerase chain reaction products of the *CmNAC083* clone Note: M: DL2000. (c) The phylogenetic analysis of *CmNAC083* amino acid and Arabidopsis NAC family amino acid sequences. (d) Comparison of *CmNAC083* amino acid sequence with the homologous protein in Arabidopsis. (e) *CmNAC083* gene expression during *A. alternata* infection at different times. Wild-type not inoculated. The statistical analyses were performed using Student's test (* $p < 0.05$, *** $p < 0.001$). (f) *CmNAC083* gene expression in different tissues during the vegetative growth period. (g) *CmNAC083* gene expression in different tissues (R-pe, ray florets petals; D-pe, and disc florets petals) during the flowering period. Data are presented as the mean \pm standard error of the mean from the three biological replications. Statistical analyses were performed using Duncan's test. Different lowercase letters represent a significant difference between treatments at ($p < 0.05$).

CmNAC083 expression was not tissue-specific in chrysanthemum 'Jinba'. However, the *CmNAC083* expression level was higher in the stem and bud during the vegetative growth period than that in other tissues during the same period. Furthermore, *CmNAC083* expression was higher in the stems and roots during the flowering stage (Fig. 1f & g).

CmNAC083 as a transcription inhibitor

CmNAC083 was cloned into the p35S::GFP vector to confirm if it is a transcription factor. The fusion genes p35S::*CmNAC083*-GFP and p35S::GFP(control) were introduced into tobacco epidermal cells via *Agrobacterium* infection. The green fluorescence signal of the *CmNAC083*-GFP fusion protein was only

expressed in the nucleus, whereas the control GFP protein was expressed in the nucleus and the cytoplasm (Fig. 2a), consistent with the results of the subcellular location of *CmNAC083* predicted by the plantPLOC website. We performed a transcriptional activation validation experiment in yeast to detect the *CmNAC083* transcriptional activity. The results revealed that *CmNAC083* had no transcriptional activation (Fig. 2b). We further verified the *CmNAC083* transcriptional activity through transient transformation experiments using Arabidopsis protoplasts. The recombinant plasmid obtained by introducing *CmNAC083* into the GAL4-DB vector and the 5XGAL4-LUC plasmid were used as the effector and reporter genes, respectively (Fig. 2c). The GAL4-DB empty vector and GAL4-DB-ARF5 were used as negative and positive controls, respectively. Compared to the GAL4-DB empty vector and the positive control GAL4-DB-ARF5, the protoplast-transformed GAL4-DB-*CmNAC083* had lower LUC activity (Fig. 2d & e), indicating that *CmNAC083* may have transcriptional inhibition activity in plant cells and *CmNAC083* may act as a transcription inhibitor in chrysanthemum.

CmNAC083 overexpression increases chrysanthemum resistance to *A. alternata*

Overexpression transgenic lines (OE-*CmNAC083*) driven by the 35S promoter were obtained through chrysanthemum genetic transformation to study the role of *CmNAC083* in

response to *A. alternata* infection in chrysanthemums. Transgenic lines were used to study the effect of *CmNAC083* on Chrysanthemum resistance to black spot disease. qRT-PCR confirmed the transcript abundance of *CmNAC083* in the transgenic lines. The *CmNAC083* expression in the OE-128 lines was 11.6-fold higher than that in the WT, and the expression of *CmNAC083* in the OE-156 lines was 29.4 times as high as that in the WT (Fig. 3a). The results of the effect of *A. alternata* inoculation treatment on transgenic plants revealed that OE-*CmNAC083* plants were resistant to *A. alternata*. The disease severity was lower in the overexpressing strains than that in the WT (Fig. 3b). The lesion area of *CmNAC083* overexpression strains was significantly smaller than that of WT (Fig. 3c). The average lesion area of the WT plants was 0.946 cm², whereas that of the overexpression lines OE-128 and OE-156 were 0.282 and 0.283 cm², respectively, which was 0.3-fold that of the WT plant. There were no significant differences in root length and plant height between the overexpression lines and WT plants (Supplemental Fig. S1, Supplemental Table S2).

A modified CaCuV was used to silence the *CmNAC083* gene instantaneously through infection using Agrobacterium to determine the role of *CmNAC083* in response to *A. alternata* infection. The transcriptome abundance of the *CmNAC083* gene in the silenced lines was significantly lower than that in the WT plants (Fig. 3d). The *CmNAC083* gene silencing resulted in a susceptible phenotype (Fig. 3e). The spot area of the

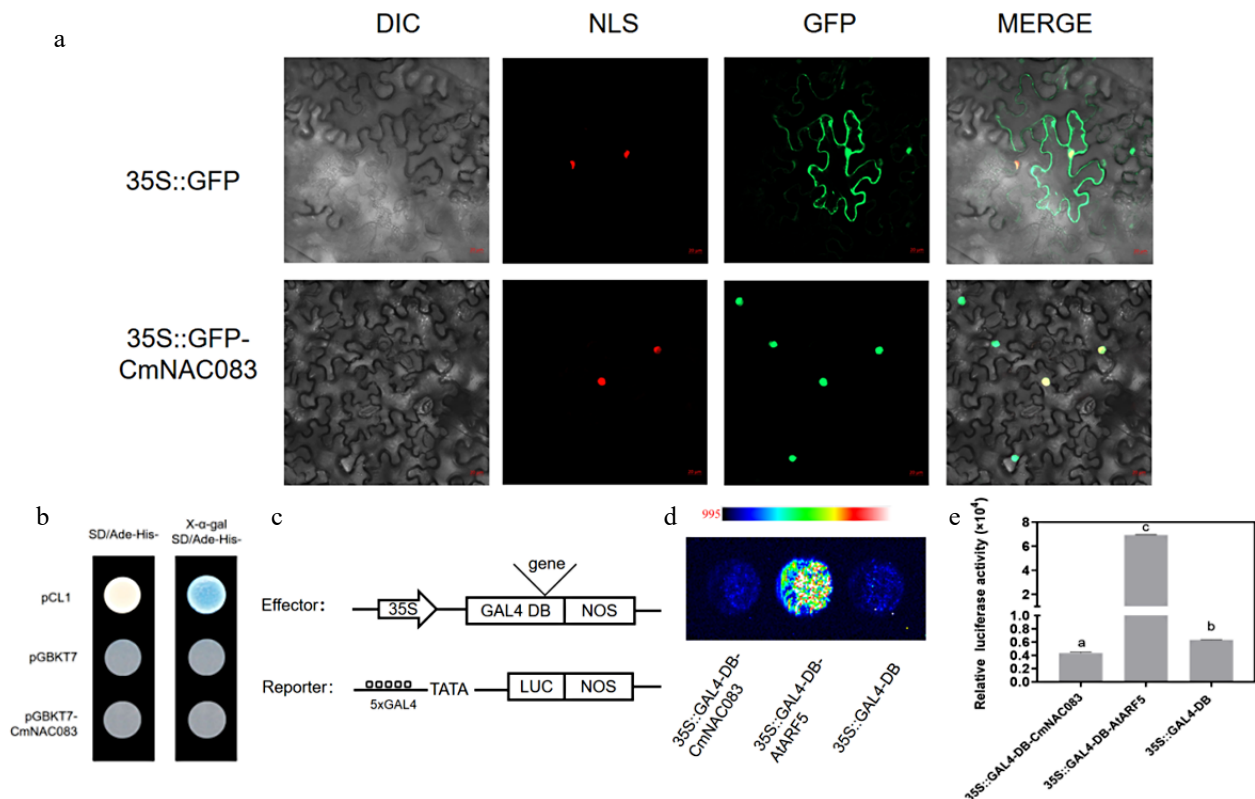


Fig. 2 *CmNAC083* subcellular localization and transactivation analysis. (a) The subcellular localization of *CmNAC083* in *Nicotiana benthamiana* cells. DIC, bright-field image; NLS, nuclear localization signal exhibited by the red fluorescent protein; GFP, green fluorescent protein; merged, overlay images. Scale bars = 20 μm. (b) *CmNAC083* transcriptional activation analysis in yeast cells. Positive control: pCL1; negative control: pGBKT7. (c) Simple structure diagrams of the effector and reporter in the protoplast transfection experiment. (d) Fluorescence images of protoplasts after the addition of 20 mM fluorescein sodium. The gradual increase in fluorescence signal from blue to red. Positive control: ARF5; negative control: GAL4-DB. (e) Quantitation of luciferase intensity of each sample. Positive control: ARF5; negative control: GAL4-DB. Data are presented as the mean ± standard error of the mean (n = 3). Significant differences were determined using Student's test at (***) $p < 0.001$.

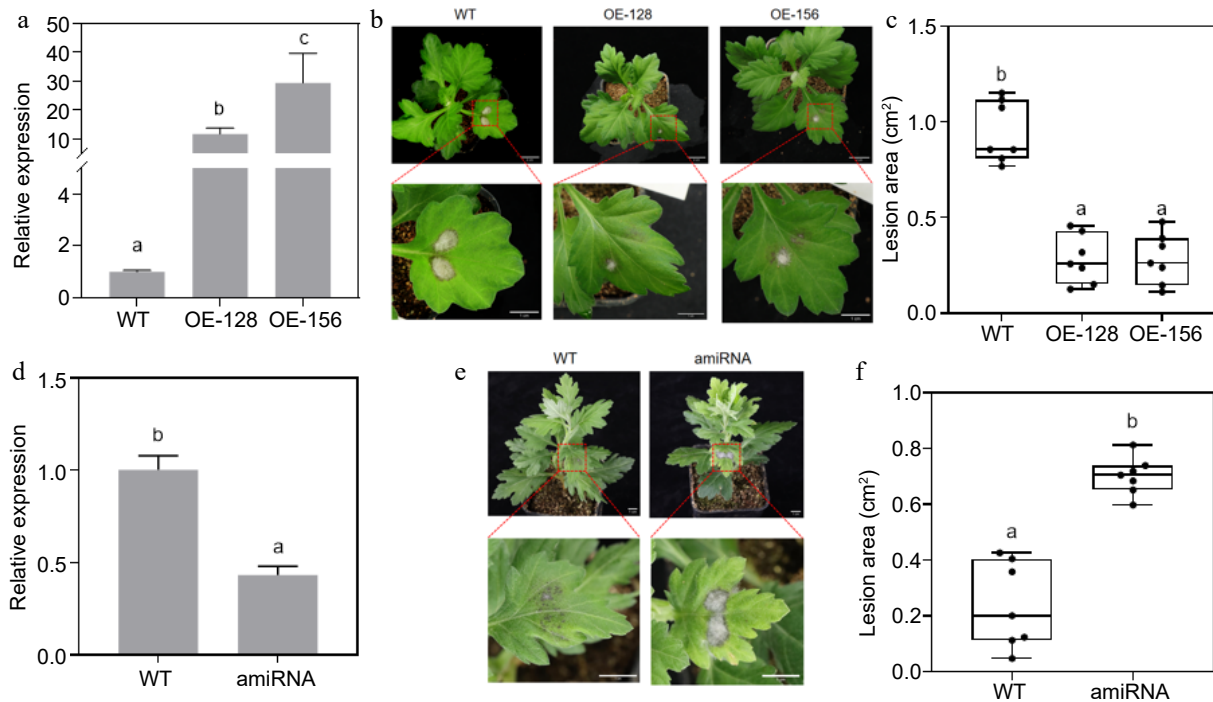


Fig. 3 Resistance analysis of *CmNAC083* overexpression and transient silencing lines. (a) Identification of *CmNAC083* overexpression transgenic lines at the RNA level. (b) Plant phenotype observation of transgenic lines and wild-type (WT) plants (1 month). Overall and drawings scale bars were = 2 cm and = 1 cm, respectively. (c) Area of diseased spots of the WT and transgenic lines inoculated with *Alternaria alternata*. (d) Identification of *CmNAC083* instantaneous silencing transgenic lines at the RNA level. (e) Plant phenotype observation of WT-CaLCuV and instantaneous silencing transgenic lines (2 months). Scale bars = 1 cm. (f) Area of diseased spots of the WT-CaLCuV and instantaneous silencing transgenic lines inoculated with *A. alternata*. WT: WT-CaLCuV; amiRNA: WT-CaLCuV- *CmNAC083*. Data represent the mean \pm standard error of the mean from three biological replications. Statistical analyses were performed using Duncan's test, and different lowercase letters represent a significant difference between treatments at ($p < 0.05$).

CmNAC083 transient silenced strain was significantly increased compared with that of the control WT-CaLCuV (Fig. 3f). The average lesion area of the control WT-CaLCuV was 0.204 cm², whereas that of the instantly silenced strain was 0.731 cm², which was 3.6-fold that of the control. Overall, the results indicated that *CmNAC083* conferred resistance to *A. alternata* infection in chrysanthemums.

Role of *CmNAC083* in regulating black spot disease in chrysanthemums

One-month-old transgenic seedlings (OE-128, OE-156) and WT seedlings were inoculated with *A. alternata* for 48 h to determine the possible regulatory mechanism of *CmNAC083* affecting the infection of black spot pathogens. DEGs were analyzed by comparing the control group with the WT and overexpression groups. The sequencing results revealed that there were 519 DEGs (456 upregulated and 63 downregulated) in WT compared with those of OE-128 and 803 DEGs (648 upregulated and 155 downregulated) in WT compared with those of OE-156 (Fig. 4a). After inoculation with *A. alternata*, 8,926 genes (5,366 upregulated and 3,560 downregulated) were differentially expressed in WT plants (WT-vs-WT-Aa). Furthermore, 10,505 genes (5,708 upregulated and 4,797 down-regulated) and 8,732 genes (4,613 upregulated and 4,119 downregulated) were differentially expressed in transgenic lines OE-128 (OE-128-vs-OE-128-Aa) and OE-156 (OE-156-vs-OE-156-Aa), respectively. After inoculation with *A. alternata*, 1,230 genes (840 upregulated and 390 downregulated) of the overexpression lines OE-128 and WT (WT-Aa-vs-OE-128-Aa)

were differentially expressed, and 1,049 genes (617 upregulated and 432 downregulated) of the overexpression lines OE-156 and WT (WT-Aa-vs-OE-156-Aa) were differentially expressed. Additionally, 320 DEGs were found in WT-vs-OE-128 and WT-vs-OE-156 pairwise comparisons (Fig. 4b). DEG analysis results of the control and treatment groups of the WT and overexpression strains are presented in Supplemental Fig. S2. The results revealed 48 (40 upregulated and eight downregulated) of the 519 (456 upregulated and 63 downregulated) genes differentially expressed in the WT and the overexpression line OE-128 were induced by the inoculation with *A. alternata*. Furthermore, 240 (163 upregulated and 77 downregulated) of the 803 (648 upregulated and 155 downregulated) genes differentially expressed in the WT and OE-156 were induced by inoculation with *A. alternata*, indicating that *CmNAC083* affects the expression of genes related to disease response.

KEGG enrichment analysis was performed on the differential genes obtained from the seven comparative groups (WT-vs-OE-128, WT-vs-OE-156, WT-vs-WT-Aa, WT-Aa-vs-OE-128-Aa, WT-Aa-vs-OE-156-Aa, OE-128-vs-OE-128-Aa, and OE-156-vs-OE-156-Aa). The results exhibited that plant hormone signal transduction, plant-pathogen interaction, mitogen-activated protein kinase signal pathway-plant, alpha-linolenic acid metabolism, and other signaling pathways related to disease resistance were significantly enriched (Fig. 4c & d, Supplemental Fig. S3). Six differentially expressed transcription factors were randomly selected, and the transcriptome data were validated using qRT-PCR (Fig. 4e, Supplemental Table S3). The quantitative results

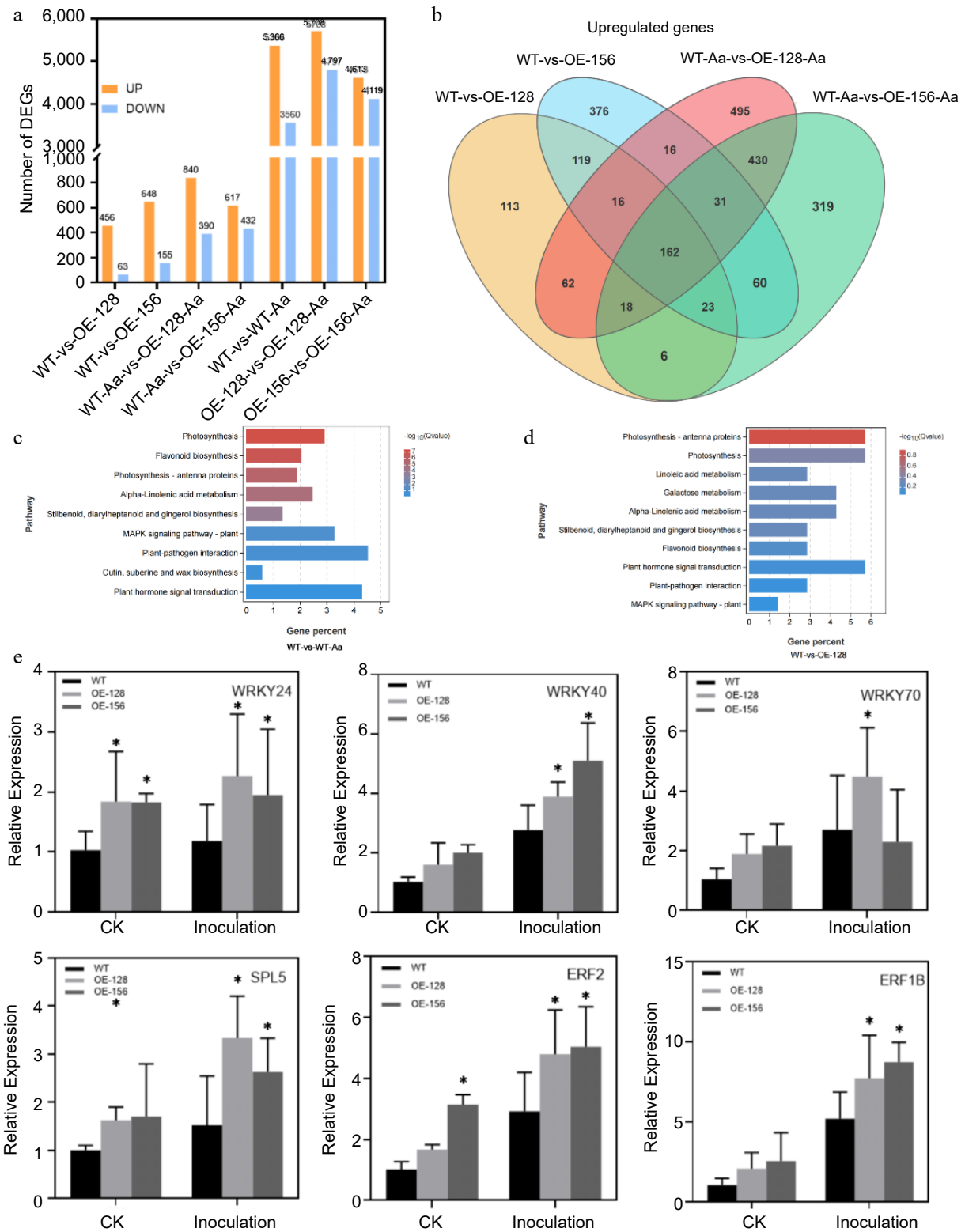


Fig. 4 Analysis of *CmNAC083* transcriptome sequencing data. (a) The number of upregulated or downregulated genes in seven different comparison groups. (b) The Venn map reveals the overlap of differentially expressed genes among WT-vs-OE-128, WT-vs-OE-156, WT-Aa-vs-OE-128-Aa, and WT-Aa-vs-OE-156-Aa. (c) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the differentially expressed genes in two comparisons (WT-vs-WT-Aa, WT-vs-OE-128) of the WT and overexpression transgenic lines under contrast and inoculation. (e) Verification of six differentially expressed transcription factor genes between WT and overexpression strains. Data are presented as the mean \pm standard error of the mean from the three biological replications. Statistical analyses were performed using Duncan's test. Different lowercase letters represent a significant difference at ($p < 0.05$).

obtained were consistent with the transcriptome analysis results, indicating the reliability of the transcriptome data.

Most significantly DEGs were enriched in the linolenic acid metabolism pathway and pathways related to disease resistance. Linolenic acid is a substrate in JA synthesis, and many transcription factors regulate JA biosynthesis. The JA response signaling pathway was also found in the plant hormone signal transduction pathway (Fig. 5a). The expression of several JA-related genes was detected in overexpression lines and WT plants using qRT-PCR. The results revealed that the expression of genes increased after inoculation with *A. alternata*, and the expression in the overexpression lines was significantly higher than that in the WT after inoculation (Fig. 5b). Then we measured endogenous JA content of the overexpression lines and the WT. The results revealed that the endogenous JA content of the overexpressing strains were higher than those of

the WT after treatment with *A. alternata* (Fig. 5c). Additionally, we found that *RBOHD*, the key gene encoding respiratory burst oxidase in the ROS pathway of the plant-pathogen interaction signaling pathway, was different between the WT and overexpression strains before and after inoculation with *A. alternata* (Fig. 6a & b). DAB staining was performed on the overexpressing strains and WT plants after inoculation with *A. alternata*. The results revealed that the staining degree and range of the WT were deeper and larger, respectively, than that of the overexpression lines after inoculation (Fig. 6c). Furthermore, we measured anti-superoxide anion and SOD activities of the overexpression lines and the WT. The results revealed that the anti-superoxide anion and SOD activities of the overexpressing strains were higher than those of the WT after treatment with *A. alternata* (Fig. 6d & e).

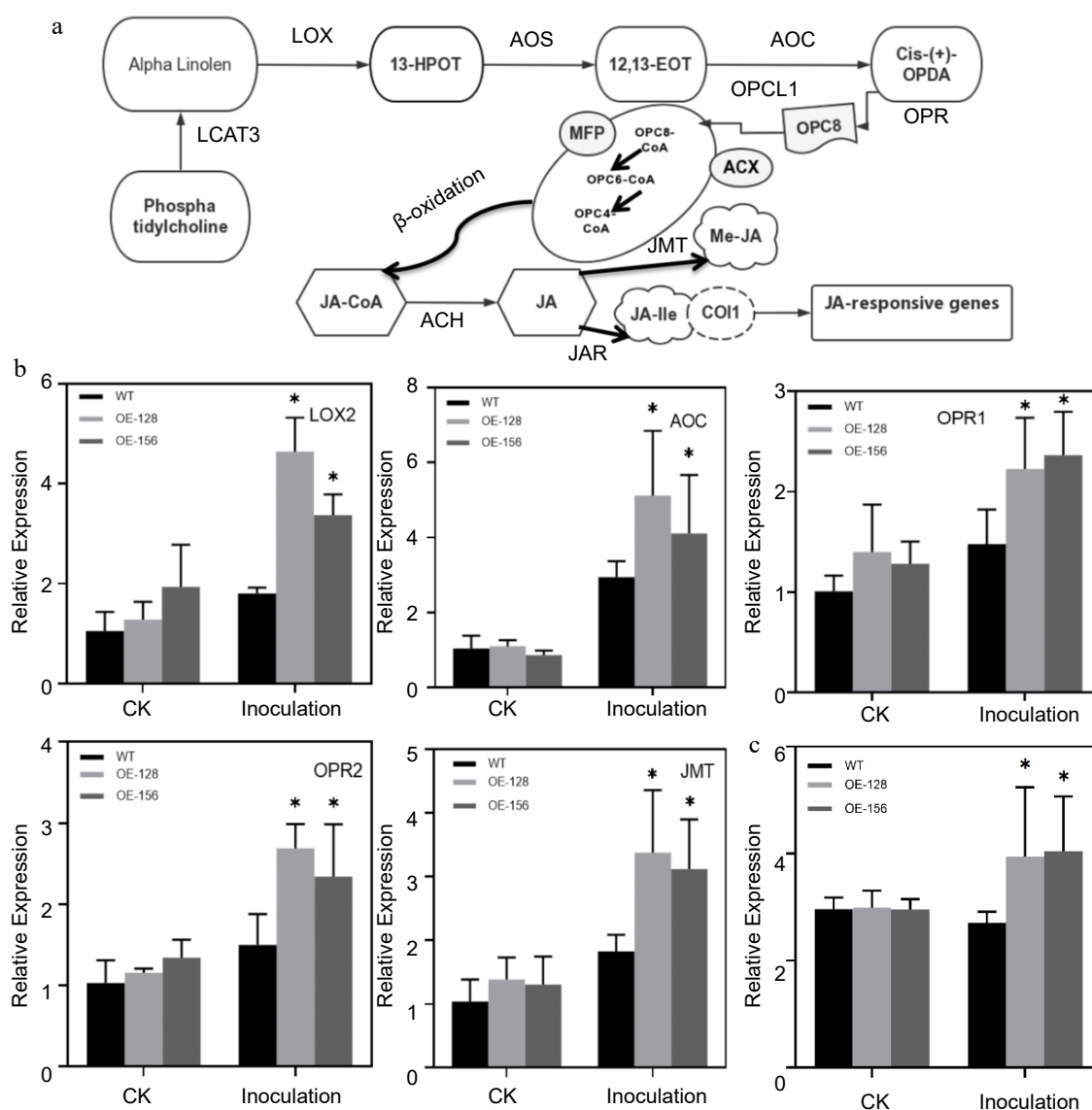


Fig. 5 Analysis of the involvement of JA signaling pathway in *Alternaria alternata* infection. (a) Simplified diagram of the JA signaling pathway. (b) Quantitative analysis of the JA Signaling pathway genes between WT and overexpression strains. Data are presented as the mean \pm standard error of the mean from the three biological replications. Statistical analyses were performed using Duncan's test. Different lowercase letters represent a significant difference between treatments at ($p < 0.05$). (c) Determination of endogenous JA content after inoculating with *A. alternata*.

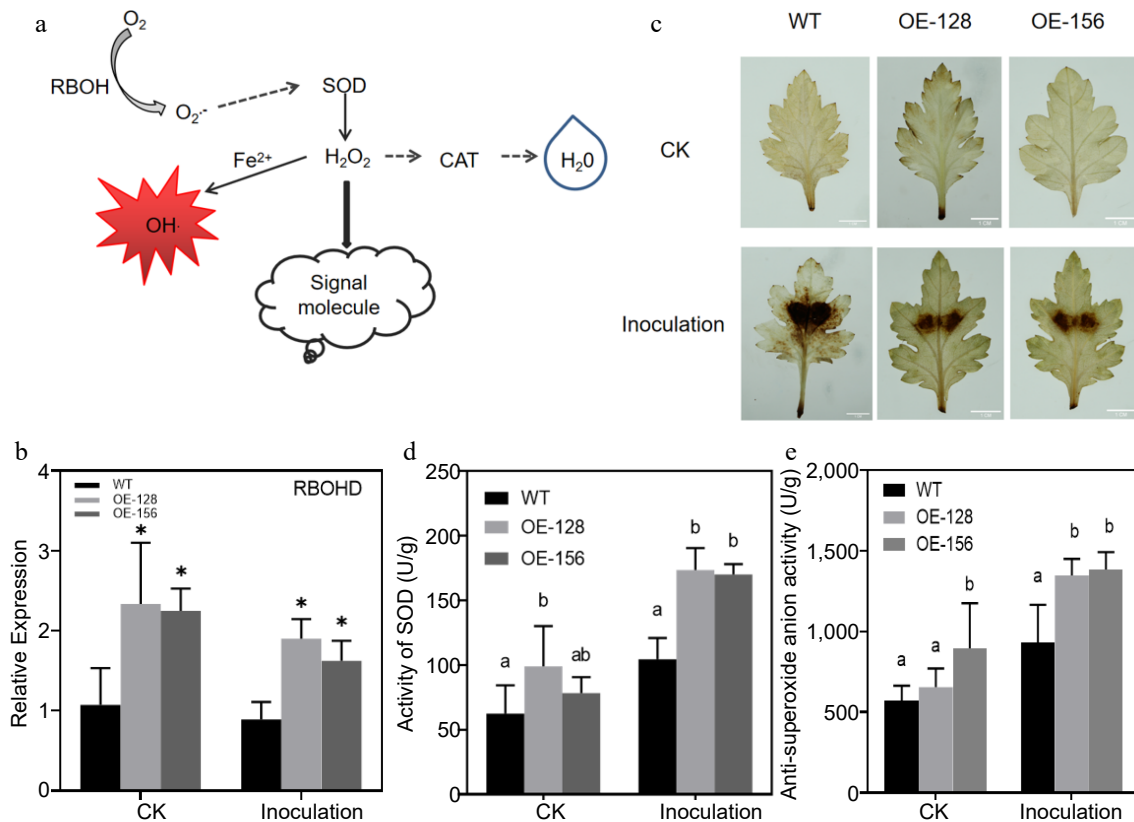


Fig. 6 Analysis of the role of the ROS signaling pathway in *Alternaria alternata* infection. (a) Simplified diagram of the ROS signal pathway. (b) Quantitative analysis of *CmRBOHD* between the wild-type (WT) and overexpression strains. (c) Diaminobenzidine staining for the transgenic and WT plants inoculated with *A. alternata*. (d) Determination of the superoxide dismutase activity in transgenic and WT plants inoculated with *A. alternata*. (e) Determination of anti-superoxide anion activity for the transgenic and WT plants inoculated with *A. alternata*. Values represent the mean \pm standard error of the mean from three biological replications. Statistical analyses were performed using Duncan's test. Different lowercase letters represent a significant difference between treatments at ($p < 0.05$).

Discussion

Abiotic stresses, such as diseases and insect pest invasion, affect the growth and development of plants. Chrysanthemum black spot is one of the fungal diseases of economic importance that seriously affects the ornamental value of chrysanthemums. Previous studies have revealed that the NAC transcription factor family genes play an important role in plant resistance to different pathogens.

In the present study, WT plants were inoculated with *A. alternata*, and high-throughput sequencing was performed. There was a significant difference among the WT plants in the expression of one NAC family member, the TF *CmNAC083*. Furthermore, we isolated and identified the *CmNAC083* gene from the chrysanthemum 'Jinba' leaves and analyzed its protein structure, and the N-terminal had a conserved domain of the NAC family. Phylogenetic tree analysis of the NAC gene in Arabidopsis revealed that *CmNAC083* has close homology with *AtNAC041* and *AtNAC083*. In Arabidopsis, the interaction between *AtNAC083* and *AtNAC030* regulates the synthesis of xylem vessels synthesis of xylem vessels^[51,52], whereas *AtNAC041*, *bZIP1*, and *MYB46* directly regulate the expression of the cellulose synthase gene *CSLA9*^[53]. However, the effects of the *NAC083* gene on disease resistance mechanisms in different species have not yet been reported. We found that *CmNAC083* was located in the nucleus through subcellular localization in

tobacco, providing the first evidence that *CmNAC083* is a transcription factor. The results of yeast transcriptional activation revealed that *CmNAC083* does not exhibit transcriptional activation activity. We further verified the *CmNAC083* transcriptional activity through the transient transformation of Arabidopsis protoplasts and found that *CmNAC083* had transcriptional inhibitory activity. Therefore, we conclude that *CmNAC083* is a transcription inhibitor that may affect disease resistance by directly binding to downstream genes and negatively regulating their expression. However, the exact underlying regulatory mechanisms require further investigation. Quantitative analysis of different tissues revealed that the *CmNAC083* expression was higher in the stem and bud during the vegetative growth period and in the stem and root during the flowering period. This could be related to the regulation of xylem vessel development by *AtNAC083* in Arabidopsis^[54,55]. Xylem vessel development in the stems and roots at the flowering stage is relatively advanced, increasing the *CmNAC083* expression. Inoculation with *A. alternata* to WT plants at different time points induced the continuous expression of *CmNAC083*, indicating that *CmNAC083* responds to *A. alternata* infection. Therefore, we constructed the *CmNAC083* overexpression lines and found that the overexpression conferred resistance to *A. alternata*, and the disease severity of the transgenic lines was significantly lower than that of the WT. We transiently silenced *CmNAC083* to determine the resistance phenotype. The results

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revealed that *CmNAC083* had contrasting phenotypes before and after silencing, consistent with the finding that *CmNAC083* improves the resistance to black spot disease in chrysanthemums.

Plants respond to pathogens through various large-scale transcriptional changes, including increased synthesis of stress hormones when infected by pathogens. Plant hormone signaling pathways activate the co-expression of many defense response genes, thereby activating plant defense response^[56,57]. Analysis of the transcriptome data revealed that the expression of some JA biosynthesis genes (*LOX1*, *LOX2*, *AOC*, *OPR1*, *OPR2*, and *JMT*) in the overexpression lines were significantly higher than those in the WT after inoculation with *A. alternata*. JA generally plays a positive regulatory role in plant disease resistance. Knockout of the *GhOPR9* gene increased sensitivity to *V. dahlia* in cotton^[13], where low temperatures inhibit JA biosynthesis and signal transduction and reduce rice resistance to rice blasts^[18]. We measured the endogenous JA content of the overexpression lines and the WT after treatment with *A. alternata*. The results revealed that inoculation with *A. alternata* significantly increased the endogenous JA content of the overexpressing strains, consistent with the increased expression of JA biosynthetic genes observed above. Plants infected with pathogens can accumulate ROS to resist infection. When ROS accumulates to a certain limit, it activates the enzyme defense system of the plant, increased SOD enzyme activity, and removes excess ROS from the plant tissue^[58,59]. The respiratory burst oxidase encoded by the *RBOH* gene is the primary source of ROS production in plants, and plants resist the invasion of pathogens by regulating ROS content. In a previous study, the expressions of *RbohA* and *RbohB* genes were upregulated, improving the rice resistance to *M. oryzae* infection^[55]. Through transcriptome analysis, we found that the key gene for the ROS pathway, *RBOHD*, was more highly expressed in the overexpression line than that in the WT before and after inoculation with *A. alternata*, which may provide an immune effect for plants against pathogenic bacterial infection at the early growth stage. However, when ROS exceeds the range that a plant can withstand, it can cause irreversible mechanical damage^[60]. DAB staining can be used to locate the tissue producing H₂O₂ in plants. After inoculation, the staining degree and range of the WT were deeper and larger than that of the overexpression lines, respectively, probably owing to the overexpression lines undergoing less mechanical damage after inoculation with the pathogen. The results of anti-superoxide anion and SOD enzyme activities in the overexpression lines and WT plants revealed that their activities in the WT plants after inoculation were significantly lower than those in the overexpression lines, indicating that the SOD activity of the overexpression lines increased after inoculation to remove excess ROS and anti-superoxide anion activity was enhanced, maintaining the ROS balance in the plant, and causing less mechanical damage. Overall, *CmNAC083* overexpression affected the expression of JA synthesis and ROS regulation pathway genes in response to *A. alternata* infection.

We speculate that *CmNAC083* may indirectly regulate JA biosynthesis and ROS outbreaks by inhibiting the expression of downstream JA and ROS signaling negative regulators as a transcriptional repressor, and regulates the defense response of chrysanthemums to black spot disease through the coordination of these two biological pathways. We conducted a prelimi-

nary study on the expression pattern of *CmNAC083* under *A. alternata* treatment and its positive regulatory role in chrysanthemum black spot disease. Furthermore, we provided a theoretical basis for analyzing the mechanism of chrysanthemum resistance to black spot disease.

Conclusions

In conclusion, we isolated and identified the NAC transcription factor *CmNAC083* from the chrysanthemum 'Jinba' leaves which conferred resistance to *A. Alternata* infection. The present preliminary study revealed that the regulatory effect could be related to the JA biosynthesis and ROS pathways. Although the specific *CmNAC083* molecular regulatory mechanism in chrysanthemum black spots still needs to be studied, the present study provides a theoretical basis for improving the breeding of chrysanthemums for resistance to black spot disease.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Fang W, Liu Y; data collection: Huang G, Dong B; analysis and interpretation of results: Jiang J, Chen S, Huang G, Dong B; draft manuscript preparation: Huang G, Liu Y. All authors reviewed the results and approved the final version of the manuscript.

Data availability

Available data and materials can be obtained from the corresponding author according to reasonable requirements.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest. Jiafu Jiang and Sumei Chen are the Editorial Board members of *Ornamental Plant Research* who were blinded from reviewing or making decisions on the manuscript. The article was subject to the journal's standard procedures, with peer-review handled independently of these Editorial Board members and their research groups.

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