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Canola (*Brassica napus* L.) *NAC103* transcription factor gene is a novel player inducing reactive oxygen species accumulation and cell death in plants

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ABSTRACT

NAC transcription factors are plant-specific and play important roles in many processes including plant development, response to biotic and abiotic stresses and hormone signaling. So far, only a few NAC genes have been identified to mediate cell death. In this study, we identified a novel NAC gene from canola (*Brassica napus* L.), *BnaNAC103* which induces reactive oxygen species (ROS) accumulation and cell death in *Nicotiana benthamiana* leaves. We found that *BnaNAC103* responded to multiple signalings, including cold, salicylic acid (SA) and a fungal pathogen *Sclerotinia sclerotiorum*. BnaNAC103 is located in the nucleus. Expression of full-length *BnaNAC103*, but not either the N-terminal NAC domain or C-terminal regulatory domain, was identified to induce hypersensitive response (HR)-like cell death when expressed in *N. benthamiana*. The cell death triggered by *BnaNAC103* is preceded by accumulation of ROS, with diaminobenzidine (DAB) staining supporting this. Moreover, quantification of ion leakage and malondialdehyde (MDA) of leaf discs indicates significant cell membrane breakage and lipid peroxidation induced by *BnaNAC103* expression. Taken together, our work has identified a novel NAC transcription factor gene modulating ROS level and cell death in plants.

1. Introduction

The NAC [no apical meristem (NAM), *Arabidopsis thaliana* transcription activation factor [ATAF1/2] and cup-shaped cotyledon (CUC2)] proteins constitute one of the largest transcription factor (TF) families and are plant-specific [1]. NAC TFs are characterized by a well-conserved N-terminal NAC domain and highly divergent C-terminus. Based on its motif distribution, the NAC domain, which comprises nearly 160 amino acid residues, can be divided into five subdomains (A–E) [2]. There are 105 putative NAC genes in *Arabidopsis*, and 140–151 in rice [3]. NAC TFs are demonstrated to be involved in many different biological processes, such as maintenance of the shoot apical meristem, regulating cell division and cell expansion in flower organs, and promoting lateral root development as reviewed in [1]. Many other NAC genes have been implicated in diverse cellular processes in various plant species, such as hormone signal pathways [4], leaf senescence [5–7] and abiotic stress signaling and tolerance [8,9]. In addition, a few NAC TFs have also been identified to positively or negatively regulate plant defense responses. For instance, *Arabidopsis* ATAF1 and ATAF2 are negative regulators of defense responses against bacterial and fungal pathogens [10,11]. *Arabidopsis NAC019* and *NAC055* mediate drought tolerance, but their overexpression also decrease resistance to *Botrytis cinerea* [12]. However, the molecular mechanisms wait to be elucidated.

In recent years, studies also indicate that NAC TFs play a role in endoplasmic reticulum (ER) or osmotic stress-induced cell death in *Arabidopsis*, rice and soybean, possibly through regulating vacuolar processing enzyme (VPE) or caspase-like protein activity [13–16]. However, whether there are other NAC genes regulating this process is not known yet.

Although members of NAC gene family in *Arabidopsis*, rice, and a few other species have been studied [2], little is known with canola. Canola is a very important oil crop in China and worldwide. However its yield is frequently influenced by environmental factors including drought, salinity, cold and fungal pathogens. Up to now, 11 NAC genes have been cloned and studied in canola response to stresses [17–19]. It is therefore essential to clone and

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characterize the NAC gene family in canola before stress/disease tolerant canola species can be developed. In our recent transcriptomic profiling studies in canola seedlings treated with a fungal pathogen *Sclerotinia sclerotiorum*, we identified several NAC genes induced by *S. sclerotiorum* (unpublished data). We therefore initiated a systemic identification and cloning of canola NAC genes through mining the public expressed sequence tag (EST) database. In this study, we reported the identification of a canola NAC gene, *BnaNAC103* that could elicit reactive oxygen species (ROS) accumulation and cell death when expressed in *Nicotiana benthamiana* leaves.

2. Materials and methods

2.1. Plant materials and growth condition

Canola (double haploid DH12075) and *N. benthamiana* plants were grown in Pindstrup soil mix (Denmark) in a growth chamber at 22 °C with 14 h light/10 h dark, with a light intensity of ca. 100 μ Em^{-2} s^{-1}. The relative humidity was 60–70%.

2.2. RNA isolation and RT-PCR

Young leaves of canola seedlings were harvested for RNA isolation using the Plant RNA kit (Omega bio-tek, USA). First-strand cDNA synthesis and high-fidelity PCR amplification using PrimeSTAR HS DNA polymerase (TaKaRa, Japan) were performed as previously described [20]. Primers used were listed in Table S1. PCR products were purified and cloned into pJET1.2 vector supplied in CloneJET PCR cloning kit (Fermentas, USA) before sequenced. The sequence of *BnaNAC103* was deposited in GenBank under the accession number KF738277.

2.3. Phylogenetic tree reconstruction and bioinformatic analysis

The predicted amino acid sequences of *NAC* of canola and other species were aligned using ClustalX1.83 and then phylogenetic tree was reconstructed using the maximum parsimony (MP) algorithm implemented in MEGA6.06 (release 6140226). Motif analysis of BnaNACs was determined by using Prosite program (http://prosite.expasy.org/prosite.html). The respective domains of NAC proteins were aligned using ClutsalX1.83 and illustrated by Boxshade (http://www.ch.embnet.org/software/BOX_form.html).

2.4. Quantitative RT-PCR (qRT-PCR) assay

Seven-day-old canola seedlings grown vertically on 1/2 MS plate medium supplemented with 1% sucrose in a growth chamber under a photoperiod of 14 h light /10 h dark with a relative humidity of 60% were transferred onto a variety of stress medium plates containing different chemical solutions, including 200 mM NaCl (MP Biomedicals), 50 μM abscisic acid (±-ABA, Sigma, USA), 10 μM methyl viologen (MV, Sigma), 12.5% PEG8000 (MP Biomedicals), 2 mM SA (Sigma) and 25 μM 1-aminocyclopropane-1-carboxylic acid (ACC, Sigma). Control, cold and heat treatments were set up by transferring seedlings onto normal 1/2 MS medium plates and placed in the same growth chamber, 4 °C, 37 °C, respectively. For fungal pathogen *S. sclerotiorum* and its virulence factor oxalic acid (OA) treatments, 18-d old canola seedlings were used as described previously [20]. Whole seedlings were collected at 1 h and 24 h post treatments, flash frozen in liquid nitrogen and stored at −80 °C. Total RNA samples were isolated and the first-strand cDNAs were synthesized from 2.5 μg of total RNA as described previously [20]. Three independent biological replicates of each sample were prepared at different times.

Quantitative reverse transcriptase PCR (qRT-PCR) was carried out using 10-fold diluted cDNA and SYBR Premix Ex Taq II (TaKaRa, Japan) on the CFX96 real-time PCR machine (Bio-Rad, USA). Primers used for qRT-PCR were designed using PrimerSelect program (DNASTAR Inc. USA), which targeted mainly at 3′UTR with an amplicon size of 75–200 bp (Table S1). The specificity and amplification efficiency of each pair of primers were examined through both BLASTn search in NCBI database and by running standard curves with melting curves. Three independent biological replicates and two technical replicates for each biological replicate were run and the significance was determined through *t*-test of SPSS statistic software ($p \leqslant 0.05$).

2.5. Subcellular localization and confocal microscopy

To examine the localization of BnaNAC103 *in planta*, the coding region was amplified using *Pfu* polymerase (Bioer, China) with primers listed in the Table S1. After purification, PCR products were restricted and then fused upstream of green fluorescent protein gene (*GFP*) in the pYJGFP vector. Agroinfiltration of *N. benthamiana* leaves was performed as described previously [20]. Two days later, leaf discs were observed of GFP under confocal microscope LSM510 (Zeiss, Germany).

2.6. Agroinfiltration and physiological assay

The full-length coding region or different fragments of *BnaNAC103* were amplified by high-fidelity *Pfu* polymerase using primers listed in Table S1. After digestion, the PCR products were inserted downstream of a double CaMV 35S promoter in the binary vector pYJGFP. Recombinant plasmids were transformed into *Agrobacterium tumefaciens* GV3101 and infiltrated into the lower epidermal side of five-week-old leaves of *N. benthamiana* plants. For each construct, 15 independent leaves of five independent plants (three leaves per plant) were used for each time-point tested. After that, infiltrated plants were kept under normal growth condition with the phenotype observed and recorded daily. To quantify the degree of cell death, electrolyte leakage was measured according to [21]. Distribution of hydrogen peroxide (H_2O_2) was detected by 3,3′-diaminobenzidine (DAB, MP Biomedicals, USA) staining according to the previously described protocol [21].

2.7. Lipid peroxidation assay and DNA ladder detection

For malondialdehyde (MDA) content determination, nearly 100 mg of leaf discs were homogenized in 4 mL of 0.1% trichloroacetic acid (TCA). After centrifuging the extract at 10,000×*g* for 15 min, the supernatant was collected and 1 mL of supernatant was mixed with 2 mL of 20% TCA and 2 mL of 0.5% thiobarbituric acid (TBA). Then the mixture was heated at 95 °C for 30 min in a fume hood and later cooled on ice. The absorbance of supernatant at 532 nm and 600 nm was read. *A*600 is the nonspecific absorbance and is subtracted from the values for *A*532.

DNA ladders were detected by extracting total DNA from homogenized leaf discs. Samples were incubated for 5 min at 65 °C in DNA extraction buffer (2%(w/v) CTAB, 1.4 M NaCl, 20 mM EDTA (pH8.0), 100 mM Tris–HCl (pH8.0), 0.2% β-mercaptoethanol) and mixed with an equal volume of chloroform/isoamyl alcohol (24:1, vol/vol). The mixture was centrifuged for 10 min at 12,000×*g*. The supernatant was precipitated with a 0.7 volume of isopropanol, washed with 70% ethanol, and dissolved in Tris-EDTA buffer containing RNase A (40 μg/ml). Eight micrograms of DNA samples from each genotype were separated on a 1.5% agarose gel in 1 × Tris-Acetate-EDTA, stained with ethidium bromide, and visualized under UV light in GelDoc imager system (Bio-Rad).