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Research paper

Functional characterization of calcium-dependent protein kinase (CPK) 2 gene from oilseed rape (*Brassica napus* L.) in regulating reactive oxygen species signaling and cell death control

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ABSTRACT

Calcium-dependent protein kinases (CPKs), being Ser/Thr protein kinases found only in plants and some protozoans are calcium sensors that regulate diverse biological processes. However, the function and mode of CPKs in oilseed rape (*Brassica napus*) remain elusive. In this study, we identified CPK2 from oilseed rape as a novel regulator of reactive oxygen species (ROS) and cell death. BnaCPK2 was identified to be located at the endoplasmic reticulum membrane. Expression of *BnaCPK2* was induced during Bax-induced cell death. Overexpression of the constitutively active form of BnaCPK2 led to significantly more accumulation of ROS and cell death than the full-length CPK2, which is supported by various measurements of physiological data. In addition, a quantitative RT-PCR survey revealed that the expression levels of a few marker genes are significantly changed as a result of *CPK2* expression. Mating-based split ubiquitin system (mbSUS) and bimolecular fluorescence complementation (BiFC) were used to screen and confirm the BnaCPK2 interacting proteins. We identified and confirmed that CPK2 interacted with NADPH oxidase-like respiratory burst oxidase homolog D (RbohD), but not with RbohF. Based on its function and interacting partners, we propose that BnaCPK2 plays an important role in ROS and cell death control through interacting with RbohD.

1. Introduction

Calcium (Ca^{2+}) is a ubiquitous second messenger in both plants and animals. Calcium-dependent protein kinases (CDPKs/CPKs) are a group of Ser/Thr protein kinases that are broadly distributed in plants and some protozoans only and decode Ca^{2+} signals (Luan et al., 2002; Kudla et al., 2010). CPKs are composed of four characteristic functional domains, which include an N-terminal variable domain (V), a protein kinase domain (K), an autoinhibitory junction domain (J) and a C-terminal calmodulin-like domain (C) (Harmon et al., 2001; Cheng et al., 2002; Hrabak et al., 2003). The highly variable N-terminal domain may contain potential myristoylation or palmitoylation sites that are responsible for membrane association (Cheng et al., 2002). The calmodulin-like regulatory domain contains four EF-hand motifs for binding to Ca^{2+} and, and thus CPKs are directly activated by binding of Ca^{2+} to this regulatory domain (Sanders et al., 2002).

The Arabidopsis and rice genomes encode 34 and 31 *CPK* genes, respectively, which are clustered into four subgroups based on the sequence similarity (Cheng et al., 2002; Hrabak et al., 2003). CPKs in plants have been demonstrated to play important roles in diverse physiological processes, including accumulation of storage starch in immature seeds of rice (Asano et al., 2002), pollen tube elongation (Myers et al., 2009), root development (Ivashuta et al., 2005), cell division, differentiation and programmed cell death (PCD) (Yoon et al., 1999; Lee et al., 2003), hormone signalings, as well as in abiotic and biotic stress tolerance (Kudla et al., 2010). For instance, several CPKs have been shown to positively or negatively regulate stress tolerance by modulating ABA signaling and/or reducing the accumulation of reactive oxygen species (ROS) (Asano et al., 2012).

Abiotic and biotic stress induces the accumulation of ROS (Miller et al., 2008). ROS could be produced by mitochondria, chloroplasts and peroxisome in plants. Low concentrations of ROS could function as signal molecules, however excess ROS can cause oxidative damage to lipid and proteins (Jaspers and Kangasjarvi, 2010). ROS generated by

Abbreviations: BiFC, bimolecular fluorescence complementation; CPK/CDPK, calcium-dependent protein kinase; GFP, green fluorescence protein; mbSUS, mating-based split ubiquitin system; MC, metacaspase; MDA, malondialdehyde; PCD, programmed cell death; PR, pathogenesis-related; qRT-PCR, quantitative RT-PCR; Rboh, respiratory burst oxidase homolog; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; SAG, senescence-associated gene; VPE, vacuolar processing enzyme; ZEN1, Zinnia Endonuclease 1

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plasma membrane-localized respiratory burst oxidase homologs (Rbohs), homologs of mammalian NADPH oxidase (NOX), play a pivotal role in both development and defense against pathogens. In Arabidopsis, RbohD and RbohF participate in ABA signal transduction in guard cells (Kwak et al., 2003) and are also required for the accumulation of reactive oxygen intermediates during plant defense responses (Torres et al., 2002). Recent studies showed that Rbohs could be regulated at transcriptional and post-translational levels. For instance, two potato (*Solanum tuberosum*) CPKs, StCDPK4 and StCDPK5 have been shown to regulate oxidative burst via phosphorylating NADPH oxidase StRbohB (Kobayashi et al., 2007). In Arabidopsis, two paralogous pairs, CPK5/CPK6 and CPK4/CPK11 also appear to regulate ROS production (Boudsocq et al., 2010). AtCPK5 was recently shown to phosphorylate RbohD and to regulate its activity (Dubiella et al., 2013). However, the function and mechanisms of many CPKs in plants remain elusive.

Excess ROS can induce PCD in plants and it has been proposed that ROS act as key inducers of PCD in plants (De Pinto et al., 2012). PCD fulfills similar roles in both animals and plants, including elimination of unwanted cells during development and sacrifice of diseased cells (Pennell and Lamb, 1997). Even if it is clear that PCD occurs in plants, the signaling pathways implicated in the triggering of this cell suicide remain unclear. A more widely-known phenomenon in the triggering of PCD in plants upon challenged by some invading pathogens is hypersensitive response (HR), which results in the formation of a zone of dead cells in the vicinity of the infection site (Mittler et al., 1997). It is believed that the HR could boost plants' resistance to further pathogen multiplication and spread (Mittler et al., 1997). Moreover, an evidence to support that plants and animals share some commonalties in PCD is that the proapoptotic mammalian protein Bax can induce cell death both in mammalian cells (Oltvai et al., 1993) and plants (Lacomme and Cruz, 1999). Besides, the cell death induced by *Bax* expression in tobacco is similar to the HR (Lacomme and Cruz, 1999). It is also reported that production of ROS was involved in cell death triggered by expression of *Bax* in Arabidopsis (Kawai-Yamada et al., 2004). However, whether and how a CPK links ROS and PCD in plants await to be explored.

Despite the extensive studies of CPKs in many plants (Cheng et al., 2002; Hrabak et al., 2003; Asano et al., 2005; Liu et al., 2006; Ray et al., 2007; Yu et al., 2007; Li et al., 2008; Ye et al., 2009), little is known about the role and molecular mechanism of CPKs in oilseed rape or canola (*Brassica napus* L.), which is an important oil crop in the world. Previously, we systematically identified and cloned the cDNA sequences of over 23 CPK genes from oilseed rape by mining the expressed sequence tag (EST) database (Zhang et al., 2014). Here, we investigated the role and putative mechanism of BnaCPK2.

2. Materials and methods

2.1. Plant materials and growth conditions

Wild type oilseed rape (double haploid DH12075) and *Nicotiana benthamiana* plants were grown in a soil mix in two separate growth chambers with a photoperiod of 14 h light (light intensity of 120 $\mu E\, m^{-2}\, s^{-1}$)/10 h dark, and a temperature of 22 °C day and night.

2.2. Phylogenetic tree construction and bioinformatics

The CPK sequences of representative plants were retrieved from the TAIR (www.arabidopsis.org) and NCBI databases. Multiple alignments of protein sequences were done using ClustalX. A phylogenetic tree was inferred using maximum parsimony method in MEGA6 program. The myristoylation and palmitoylation motifs were predicted by Myristoylator (http://web.expasy.org/myristoylator/) and CSS-Palm 3.0 (http://csspalm.biocuckoo.org/), respectively.

2.3. Subcellular localization and confocal microscopy

The coding region of *BnaCPK2* was subcloned into pYJGFP vector. After confirmed, the recombinant plasmid and P19 strain of tomato bushy stunt virus were transferred into *Agrobacterium tumefaciens* GV3101 for infiltrating into the leaves of 28-d-old *N. benthamiana* (Waadt and Kudla, 2008). GFP signals were observed on the TCS SP8 confocal microscope (Leica, Germany) after 2 d.

2.4. Expression profiling

Oilseed rape seedlings of 12-d-old grown in a growth chamber under a 14-h light/10-h dark photoperiod at a temperature of 22 °C were used to induce cell death for expression profiling. Binary plasmids of pVX-Bax and control p35SFC (harbors *GFP* expression cassette) were transformed independently into agrobacteria GV3101 via freeze-thaw method. Overnight cultures of agrobacteria transformed with p35SFC- and pVX-Bax were separately pelleted, resuspended in an infiltration medium (10 mM MES-KOH (pH 5.6), 10 mM $MgCl_2$ and 0.15 mM acetosyringon) to an optical density of 1.5, before mixed with equal amount (identical optical density)of agrobacteria culture of P19 silencing suppressor. 200 µl of mixed agrobacterial culture were infiltrated into each leaf with two leaves per plant. After that, plants were covered with a plastic dome to keep high humidity and placed under moderate light intensity overnight in a growth chamber before uncovered. Infiltrated leaves were harvested at 24, 25, 27 and 30 hpi and flash frozen in liquid nitrogen. Three biological replicates were prepared. Total RNA was extracted using the Plant RNA kit (Omega bio-tek, USA) with on-column digestion of contaminated genomic DNA following the manufacturer's manual.

2.5. Quantitative RT-PCR (qRT-PCR)

For relative quantitative reverse transcriptase PCR (qRT-PCR), 5 µg RNA was transcribed into cDNA by using MMLV (RNase H-) reverse transcriptase and Oligo$(dT)_{18}$ primers (TaKaRa). 10-fold diluted cDNA samples were subjected to assay using *SYBR* Green I premix (CWBio, China) on a CFX96 thermocycler (Bio-Rad, USA). The amplification efficiency (E) of each primer pair was calculated following that described previously (Zhang et al., 2014). Data were normalized to the two endogenous genes (*BnaUP1* and *BnaUBC9* for oilseed rape, and *NbL23* and *NbPP2A* for *N. benthamiana*) and fold changes were calculated according to (Pfaffl, 2001). Three independent biological replicates (two technical replicates for each biological replicate) were assayed and the significance were determined with SPSS ($P \leq 0.05$).

For absolute qRT-PCR, quantified and serially diluted plasmid DNA of pJET1.2-BnaCPK2 was used as templates individually; in parallel, cDNA samples prepared from oilseed rape leaves of different developmental stages were also used as the template for qRT-PCR. Copies of transcript were calculated against the standard curve generated from plasmid DNA. Three biological replicates were run and the data were subjected to ANOVA test in SPSS software.

2.6. Overexpression and physiological measurements

The coding regions of *BnaCPK2, BnaCPK2VK* and *GFP* were subcloned into the binary vector p35SFC using primers listed in Table S1. Agroinfiltration into the lower epidermal side of 30 d old leaves of *N. benthamiana* plants were performed as described previously (Sun et al., 2014). Electrolyte leakage was measured according to (Sun et al., 2014). Qualitative and quantitative assays of hydrogen peroxide (H_2O_2) were performed as previously described (Sun et al., 2014; Chen et al., 2016). Quantitative assays of chlorophyll, anthocyanin and malondialdehyde (MDA) were performed as previously described (Niu et al., 2014; Niu et al., 2016).