Journal of Plant Physiology 216 (2017) 108–117

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Transcriptional response of a novel *HpCDPK1* kinase gene from *Hippeastrum x hybr.* to wounding and fungal infection

Agnieszka Pawełek*, Maria Duszyn, Brygida Świeżawska, Adriana Szmidt-Jaworska, Krzysztof Jaworski

Nicolas Copernicus University, Chair of Plant Physiology and Biotechnology, Lwowska St. 1, PL 87-100 Torun, Poland

ARTICLE INFO

Keywords:
Calcium ions
CDPK
Hippeastrum x hybr.
Wounding
Fungal infection
Stress response

ABSTRACT

Calcium dependent protein kinases (CDPK) are well established plant sensor and effectors for calcium ions and participate in regulation of multiple abiotic and biotic stress responses in plant cells. Here we present the identification and characterization of a new CDPK kinase gene from bulbous plant *Hippeastrum x hybr.* and examine the role of this kinase in stress responses leading to phytoalexin (PA) production in plant tissues. In the previous research, it was shown that *Hippeastrum* bulbs mechanically wounded or infected with *Peyronellaea curtisii* (=*Phoma narcissi*) are inducted to an antifungal red substance synthesis. In this research, we demonstrated Ca^{2+} dependence of the phytoalexin production by wounded bulbs. Furthermore, the isolated *HpCDPK1* cDNA for ORF was found to be 1596 bp long and encoded 531 amino acid protein with CDPK kinase activity, as was shown by recombinant GST-HpCDPK1 enzyme production and analysis. *HpCDPK1* transcript was present in all vegetative and chosen generative organs of *Hippeastrum* plant. The dynamics of the observed *HpCDPK1* mRNA changes in bulbs depended on stressor type. The mechanical injury caused one wave of transcript increase while more complex transcript changes were observed within 48 h after *Peyronellaea* inoculation. In plant bulbs already accumulating red phytoalexin, increases in *HpCDPK1* mRNA level were observed at certain intervals within 48 h whereas, in the case of fungal infection, only one big increment in the transcript amount at the 10th minute after inoculation was detected. The observed transcriptional response of *HpCDPK1* gene to wounding and pathogen infection stress suggests a positive correlation with phytoalexin synthesis and maintenance in bulb tissues and puts more light on CDPK kinase role in the plant stress response regulation. This also bears some potential for understanding the mechanism of a phytoalexin formation.

1. Introduction

Calcium as a signaling molecule plays a central role in a living cell's response to almost every endogenous and external signal. The role of calcium ions in signaling of plant defense is also well proven (Ma and Berkowitz, 2007; Kudla et al., 2010). Changes in plasma membrane permeability leading to calcium and proton influx and potassium and chloride efflux are the earliest reactions of plant cells to elicitors. These ion fluxes are essential for induction of the oxidative burst, defense gene activation and phytoalexin production (Scheel, 1998). An elevation in cytosolic and nuclear Ca^{2+} concentration caused by various stress factors are sensed by different calcium binding proteins, including calmodulins/calmodulin-like proteins (CaMs/CMLs), calcineurin B-like proteins (CBL) and CDPK kinases, which are three major types of calcium decoders in plant cells (Lecourieux et al., 2006).

CDPK kinases, named lately CPKs are commonly found in plants and so far, not discovered in the animal kingdom. They contain highly conserved protein domains, including serine/threonine kinase domain, an auto-inhibitory region and calmodulin-like domain (CLD) which most often contains 4 EF hand motives (Klimecka and Muszyńska, 2007). At N-terminal part of CDPKs, a highly variable domain occurs carrying myristoylation and/or palmitoylation sites, which enable the kinase association to the cell membranes (Hrabak et al., 2003). The binding of Ca^{2+} ions to helix-loop in EF hands of CLD domain causes conformational changes and activation of CDPK kinase which by a substrate phosphorylation, relays a signal into downstream response processes such as regulation of gene expression (Liese and Romeis, 2013). In plants, CDPKs are coded by multi-gene families (e.g. 34 isoforms in *Arabidopsis*, 31 in rice) and are divided into four evolutionary subgroups (Boudsocq and Sheen, 2013).

* Corresponding author.
E-mail addresses: apawelek@umk.pl (A. Pawełek), duszyn@doktorant.umk.pl (M. Duszyn), swiezawska@umk.pl (B. Świeżawska), asjawors@umk.pl (A. Szmidt-Jaworska), jaworski@umk.pl (K. Jaworski).

http://dx.doi.org/10.1016/j.jplph.2017.05.025
Received 30 January 2017; Received in revised form 2 May 2017; Accepted 8 May 2017
Available online 01 June 2017

CDPKs participate in the regulation of multiple growth and development processes as well as different stress responses, including mechanical injury and pathogen attack (Szczegielniak et al., 2005; Jaworski et al., 2010; Asano et al., 2012).

Mechanical wounding of plant tissues can be caused by harsh weather conditions like wind and rain or by living organisms like herbivores and results in tissue injury and opening the way for pathogen attack. Important roles during plant response to wounding are played by phytohormones such as jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA) and ethylene (Kunkel and Brooks, 2002; Wilmowicz et al., 2012; Wilmowicz et al., 2014). Participation of CDPK in post-wounding responses was observed in many plant species, including CPK3 from *Arabidopsis* (Kanchiswamy et al., 2010), NaCDPK4 and NaCDPK5 from *Nicotiana attenuata* (Yang et al., 2012) and GmCPK3, GmCPK31 from *Glycine max* (Liu et al., 2016).

CDPKs are also crucial calcium sensors in plant responses to pathogen infection. AtCPK1 was identified as a component of the innate immune system of *Arabidopsis* against fungal (*F. oxysporum* and *B. cinerea*) and bacterial (*P. syringae*) pathogens, modulating SA-dependent defense signaling pathway (Coca and San Segundo, 2010). In potato, CDPK mediates production of reactive oxygen species (ROS) and has a crucial role in defense signaling. This is illustrated when constitutively-active form of StCDPK5 (StCDPK5VK) phosphorylates the N-terminal part of NADPH oxidase (StRBOHB) which activates ROS signaling (Kobayashi et al., 2007).

The survival of plants in a changing environment and their battle with different threats, including pathogens are also firmly determined by chemical compounds which are products of plant's secondary metabolism. One of the secondary metabolites are phytoalexins which production is induced in plant cell after a stressor action. Phytoalexins are low molecular weight compounds produced on different biosynthetic pathways. They are characteristic for certain plant species but their antimicrobial activity has broad pathogen spectrum (Piasecka et al., 2015).

In our study, we examined *Hippeastrum x hybr.* plant response to mechanical and fungal stresses. One of the most common disease of *Hippeastrum* is red spot caused by *Phoma narcissi* infection. The disease causes economic losses in the ornamental bulb business. The tissues of *Hippeastrum x hybr.* organs as a response to wounding, pathogen fungi or mites attack, produce characteristic red substance. This substance inhibits the spread of fungal infection (Saniewska, 1992; Saniewska et al., 2005), confirming its antimicrobial (phytoalexin) nature. The chemical structure of *Hippeastrum* phytoalexin is not fully known. It was revealed that phytoalexin of *Hippeastrum x hortorum* bulbs is a mixture of an orange-coloured chalcone and 3 different flavans, and the colourless flavans can be oxidized to red-coloured dimers or polymers (Wink and Lehmann, 1996).

Here, we present Ca^{2+} dependence on the phytoalexin biosynthesis and the transcriptional response of a novel *Hippeastrum x hybr. CDPK* to mechanical wounding and *Peyronellaea curtisii* (=*Phoma narcissi*) infection.

2. Materials and methods

2.1. Plant material and stress conditions

In this study, ornamental bulbous *Hippeastrum* × *hybr.* plant from Amaryllidaceae family was used. The plants were grown in a growth chamber under light/dark conditions (12 h:12 h) at 21 °C. Nine different organs from *Hippeastrum* plant- leaves, flower stem, bulbs, roots, petals, stamens, pollen, pistils and ovaries- were collected. Different stress conditions were induced by either: (i) mechanically wounding bulb scales by cutting them into small pieces of approximately 4 × 4 mm, (ii) wounding and then immediately infecting with *Peyronellaea curtisii* (=*Phoma narcissi*) P2 strain inoculum (Hrynkiewicz et al., 2015) or (iii) infecting with *Peyronellaea curtisii* 48 h after mechanical wounding. The plant material was collected for 4 days (time points), frozen in liquid nitrogen and stored at −80 °C.

2.2. EGTA and $CaCl_2$ treatment of Hippeastrum bulb scales and phytoalexin quantification

The bulbs, mechanically wounded by cutting into 4 × 4 mm pieces were treated with 0.25, 0.5 and 1 mM water solution of EGTA or $CaCl_2$. The control samples were treated with water only. The plant material was collected at 0, 24, 48, 72 and 96 h after treatment, frozen in liquid nitrogen and stored in −80 °C. Afterwards, phytoalexin (PA) was isolated and its concentration was assessed. For this purpose, the bulb scales (0,3 g) were ground in liquid nitrogen with a mortar and pestle. The powder was then extracted with 1 mL of 90% methanol and centrifuged at 10,000 x g for 30 min. The absorbance of the resulted supernatant was analysed spectrophotometrically (UV-160 1PC, SHIMADZU) at 495 nm based on red colour intensity (Wink and Lehmann, 1996; Saniewski et al., 2006). Three biologically independent treatments and PA measurements were performed and the data are presented as mean ± standard error (SE).

2.3. HpCDPK1 cDNA identification

mRNA from *Hippeastrum* roots was isolated using Dynabeads® mRNA Purification Kit (Life Technologies). RT-PCR was done with a pair of degenerate primers (for catalytic kinase domain) (forward: 5′-GT(CT)GG(GACT)AGTGC(AT)TA(CT)TATGT(TG)GC-3′ and reverse: 5′-(GA)TTCTTC(AG)TA(GTCA)GT(GAT)AT(AGCT)GT(ACTG)CC(AG)CT-3′). The RT-PCR followed these cycling parameters: 95 °C for 2 min followed by 30 cycles of 95 °C for 60 s, 60 °C for 60 s, and 72 °C for 210 s, with a final extension of 7 min at 72 °C. The total volume of 50 μL PCR reaction solution contained: 5 μL PCR buffer, 3.5 μL 50 mM $MgCl_2$, 1 μL 10 mM dNTP mix, 1.5 μL 10 μM of each pair, 0.5 μL DMSO, 1U Platinum DNA polymerase (Invitrogen) and 3 μL of cDNA. The PCR product was cut from agarose gel, the DNA was extracted and cloned into pGEM-T Easy vector and then sequenced. A full length *HpCDPK1* ORF cDNA was obtained using BD SMART RACE cDNA Amplification Kit (Clontech) with 5′RACE and 3′RACE primers (5′-GGTGC CGCTCTTGTCGGTGTCCATATTGG-3′ and 5′-CTCCTTAGTGGT GTCCCTCCATTTTGGGC-3′, respectively). The RACE-PCR products were cloned into pGEM-T Easy and transformed to JM107 *E. coli* cells. Plasmids carrying the cloned cDNAs were purified with GeneMATRIX Plasmid Miniprep DNA Purification Kit (Eurx) and sequenced. The full-length ORF for *HpCDPK1* cDNA assemblage was confirmed by PCR amplification during *HpCDPK1* ORF cloning into pGEX-6P-2 expression vector.

2.4. HpCDPK1 mRNA quantification

Total RNA was extracted from the tissues using TriPure and the RNA samples (1 μg) were treated with RNase-free DNase I and reverse transcribed using Transcriptor High Fidelity cDNA Synthesis Kits (Roche) following the manufacturer's instructions.

Quantitative real-time PCR (qPCR) was performed using the LightCycler 2.0 Instrument, and the Universal Probe Library, UPL, (Roche). A *Hippeastrum* actin 1 gene (*HpACT1*) (GenBank accession no. KY856960) was used as an internal control. For *HpCDPK1* mRNA quantification, gene specific primers 5′-CAGCCGAACCAACTAAACCT-3′ (forward) and 5′-TGATGACTTTGTGGTGGTTGTT-3′ (reverse), and the specific #10 hydrolysis probe, CCACCTCC were used. However, for *HpACT1* mRNA quantification, gene specific primers 5′-ACTTTCCAGCAGATGTGGATT-3′ (forward) and 5′-AAAATTA GAAGCATTTCCTA-3′ (reverse), and the specific #13 hydrolysis probe, AGGCAGAG were used. The reaction mixture contained the following: 2 μL of LightCycler® TaqMan® Master Mix, 0.3 μL of each primer (10 μM), 0.1 μL of UPL probe (10 mM), 6.3 μL of RNase-free water and