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Functional characterization of an apple (*Malus* x *domestica*) LysM domain receptor encoding gene for its role in defense response

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ABSTRACT

Apple gene, MD09G1111800, was identified as a chitin binding receptor-like kinase based on sequence similarity to AtCERK1 (chitin elicitor receptor kinase 1) from Arabidopsis. Sequence analysis on genomic structure, domain composition and transcriptional response to exogenous chitin treatment indicated that MD09G1111800 is an ortholog to AtCERK1 and was therefore named as *MdCERK1*. Tissue specific expression patterns indicated that *MdCERK1* is primarily functional in vegetative tissues of leaf and root, rather than flower, fruit and seed of apple plant. The transcriptional regulation patterns in response to infection by *Rhizoctonia solani* demonstrated that MdCERK1 is a functional pattern recognition receptor protein (PRR) in apple root tissues. The ability of purified GST-MdCERK1 fusion protein to bind chitin molecules added biochemical evidence for its role in chitin mediated immune responses. An untargeted proteomic approach was also employed for identifying its putative *in vivo* interaction partners in apple root cells, and results indicated the existence of a functional receptor complex. These data support the conclusion that MdCERK1 is a chitin binding receptor kinase functioning in apple vegetative tissues, which plays an important role in defense activation in response to pathogen infection.

1. Introduction

Plants are constantly exposed to a wide range of biotic and abiotic stresses. Due to their sessile nature, plants rely on their innate immune system to fend off potential pathogens. The plant innate immune system consists of a two-layered pathogen-detection system. Plant pattern recognition receptors (PRRs), located at the host cell surface, bind the highly conserved pathogen-associated molecular patterns (PAMPs), which triggers the initial defense responses [1]. The so-called PAMP-triggered immunity (PTI) represents the first layer of defense activation [1,2]. The typical immune responses include a spike of cytosolic Ca^{2+} concentration, production of reactive oxygen species (ROS), callose deposition, activation of mitogen activated protein kinase and calcium-dependent protein kinase signaling cascades, induction of defense genes, and generation and transportation of defense metabolites [1,3–7]. Successful pathogens secrete effector proteins to suppress PTI, leading to effector-triggered susceptibility (ETS) [8]. To combat pathogen progression, plants have evolved resistance proteins (R proteins) to recognize specific effectors, leading to effector-triggered immunity (ETI) [1,9], a more robust form of immune response than PTI. Nevertheless, recognition of PAMPs by plant PRRs initiates the defense activation to both non-pathogenic microbes and pathogens. PRRs from both monocots and dicots have been identified, which can perceive various PAMPs including bacterial flagellin, peptidoglycans, EF-Tu and chitin [10–12]. The functional receptor complex and the relationship among the components have been mostly studied in the model systems of rice and Arabidopsis. Chitin, a β (1,4)-linked homopolymer of *N*-acetylglucosamine, is an essential component of fungal cell walls. As a typical fungal PAMP, chitin can be perceived and therefore triggers defense responses [13]. OsCEBiP (chitin-elicitor binding protein) in rice (*Oryza sativa*), was the first identified chitin receptor. It carries an extracellular lysin motif (LysM), a transmembrane domain, but lacks an intracellular kinase domain; therefore, a co-receptor is required to transduce chitin-triggered signals within the cell. OsCERK1 (chitin

Abbreviations: CERK, chitin elicitor receptor kinase; PRR, pattern recognition receptor; GST, glutathione S-transferase; PAMP, pathogen-associated molecular patterns; PTI, PAMP-triggered immunity; ROS, reactive oxygen species; ETS, effector-triggered susceptibility; ETI, effector-triggered immunity; CEBiP, chitin-elicitor binding protein; LysM, lysin motif; RLCK, receptor-like cytoplasmic kinase; GEF, guanine nucleotide exchange factor; LYP, LysM-containing receptor-like protein; LYK, LysM-containing receptor-like kinase; ABRE, ACGT-containing abscisic acid response element; LTR, low temperature responsive elements; HSE, heat stress responsive element; MBS, MYB binding site; MeJA, methyl jasmonate; GFP, green fluorescent protein; LC/MS/MS, liquid chromatography tandem-mass spectrometry; PR, pathogenesis-related protein; Hsp, heat shock protein; Cpn60β2, chaperonin 60 subunit beta 2; PSM, peptide spectrum match; NF, rhizobial nodulation factor; PGN, peptidoglycan; NJ, neighbor-joining

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elicitor receptor kinase 1), a receptor-like kinase, was then identified which forms a receptor complex with OsCEBiP and helps to transduce the chitin signal to downstream components [14–16]. The knock-out mutants for either *CEBiP* or *CERK1* showed the suppression of chitin-induced defense responses in rice [14,15]. However, OsCERK1 itself does not have chitin binding ability. Besides OsCEBiP, two other LysM-containing receptor-like proteins (LysM RLPs), OsLYP4 and OsLYP6, were also reported to involve in chitin perception [17]. Upon chitin perception, OsCERK1 phosphorylates OsRLCK185 (receptor-like cytoplasmic kinase 185) and OsRacGEF1 (Rac GDP/GTP exchange factor 1) [18,19]. The phosphorylation of OsRacGEF1 in turn actives OsRac1 [19]. They are both required for chitin-triggered responses and resistance to fungal pathogens in rice [19–21]. Moreover, OsCERK1 and OsRacGEF1 can form protein complex with the heat shock protein 90 (Hsp90) and the co-chaperone Hop/Sti1. The Hop/Sti1-Hsp90 chaperone complex contributes to the maturation and intracellular transport of the OsCERK1 complex [19,22]. In *Arabidopsis thaliana*, there are five LysM-containing receptor-like kinase (LYKs): LYK1/CERK1 and LYK2-5 [23,24]. AtCERK1 directly binds chitin molecules and has been thought to be an "all-in-one" receptor for chitin signaling [5,25,26]. However, recent research showed that, in addition to AtCERK1, both AtLYK4 and AtLYK5 are required for chitin responsiveness and they may share overlapping function with each other in mediating the chitin response [27]. AtLYK5 forms a receptor complex with AtCERK1 to recognize chitin [27]. PBL27 is the downstream component for AtCERK1 after chitin perception, and it can be phosphorylated by AtCERK1 [16]. Tremendous progress has been achieved in understanding the functional receptor complex, though mostly from model plant pathosystems of foliar pathogens.

For a non-model plant, such as the perennial tree fruit apple (*Malus* x *domestica*), the roles of chitin-related PRRs in defense activation and disease resistance are poorly defined. In this study, the objective was to identify the orthologous PRR genes from apple genome and analyze their functional roles in chitin signaling pathway. Specifically, sequence features, cellular localization, chitin binding ability and transcriptional responses to pathogen infection as well as cellular interaction partners were examined during interactions between apple roots and a soilborne pathogen *Rhizoctonia solani*.

2. Materials and methods

2.1. Sequence features analysis of apple MdCERK1 gene

A local BlastP search against the apple genome sequences using AtCERK1 protein sequence as the query was performed to identify the apple homologous *CERK* encoding genes using default expectation value of < e-10^6 (http://pfam.xfam.org/). The Arabidopsis AtCERK1 protein sequence was retrieved from TAIR (www.arabidopsis.org). The genomic sequences, the coding region and the predicted amino acid sequences of MdCERKs were downloaded from The Apple Genome and Epigenome (https://iris.angers.inra.fr/gddh13/jbrowse). SMART (http://smart.embl-heidelberg.de/) was used to identify the conserved LysM domain for selected MdCERKs [28–30]. Genes homologous to *AtCERK1* from other plant species, such as rice, populus, strawberry, tomato, tobacco and potato, were identified by BlastP with a search using an online service at Plaza (http://bioinformatics.psb.ugent.be/plaza/). A phylogenetic tree was generated using the Neighbor-Joining (NJ) method with the program MEGA5 [31,32]. The reliability of the phylogenetic tree was tested by bootstrapping with 1000 replicates.

2.2. Plant materials and tissue collection

Tissue culture technique based micro-propagation procedures were used to obtain genetically uniform and developmental stage defined young apple plants as described previously [33]. The genotypes included apple scion cultivar 'Gala', and selected apple rootstock germplasm lines (#115, #132, #161, G.935 and #5257) from a cross population between O3x R5 (Ottawa 3 *x* Robusta 5). Many agronomical traits such as dwarfness, cold hardness, and disease resistance are known to be segregated among the progeny from this cross population [34,35]. The numeral identifiers used for describing the plant genotypes, such as #115, #132 and #5257, represent the individual progeny from this cross population. Plants at 4–5 weeks after root induction were used for infection assay. For studying tissue-specific expression pattern of *MdCERK1*, root tissues were collected from micropropagated plants. Leaf, flower, fruit and seed tissues were collected from 10-year old 'Gala' trees at Columbia View experimental station (Orondo, WA). The tissues were collected by flash frozen in liquid nitrogen and stored at −80 °C until RNA isolation.

2.3. Inoculum preparation and plant inoculation

Inoculum of *R. solani* AG-5 strain 1007 was prepared by growing the pathogen in 500 mL beakers containing 250 mL of oat bran and 80 mL of distilled water [36]. The beakers were sterilized by autoclaving for 90 min in two consecutive days and inoculated with a piece of agar culture of *R. solani* growing on a 1/5th strength potato dextrose agar (PDA) plate. Beakers were incubated at 20 °C for 14 days then the inoculum were air dried in a fume hood. Ground oat grain inoculum of *R. solani* AG-5 were homogeneously mixed into soil at a rate of 0.8% (dry weight/dry weight) and soils were planted after 24 h of incubation at 20–23 °C [37]. Inoculation was performed by planting apple plants into the infested soil or un-infested soil as mock inoculation. Root tissues were collected at 0, 24, 48 and 72 h post inoculation (hpi), by excavating root from soil at the designated time point. After quick rinse under tap water, root tissues were flash frozen in liquid nitrogen for RNA isolation. Genotype-specific survival rates were recorded 28 days post inoculation (dpi).

2.4. Chitin treatment of apple root tissues

Shrimp shell chitin (Sigma, St. Louis, MO) was ground with mortar and pestle to a very fine powder, and the stock slurry was prepared at the concentration of 10 mg/mL in water [38]. The chitin solution was autoclaved twice before using for apple root tissue treatment. After 4 weeks of root elongation, 'Gala' plants were transferred to autoclaved 1/2 strength Murashige and Skoog (MS) liquid medium at pH 5.6 in Magenta™ boxes. After remaining in the liquid medium for three days, chitin stock solution was added to the boxes to obtain a final concentration of 1 μM [39]. The same amount of water was added to control plants. The roots of all treated plants from two biological replicates were collected at five timepoints of 0, 0.5, 1, 1.5 and 2 h after treatment.

2.5. Subcellular localization for MdCERK1

The full-length cDNAs of *MdCERK1* was constructed into the vectors *pRI101-GFP*. The *PRI101-MdCERK1* recombinant plasmid and the *PRI101-GFP* vector were transformed into *Agrobacterium tumefaciens* EHA105, respectively. The *Agrobacterium* strains containing different constructs were incubated, and resuspended with infiltration buffer (0.2 mM acetosyringone, 10 mM MES and 10 mM $MgCl_2$) to a final concentration of OD600 = 0.6. Equal volumes of different combinations of *Agrobacterium* strains were mixed and infiltrated into onion epidermal cells through *Agrobacterium* infection. The onion cells were cultured at 23 °C for 48 h. Using a Zeiss LSM 5 Pa Confocal Microscope (excitation wavelength 488 nm by argon laser, emission wavelength 550 nm) to observe MdCERK1's subcellular localization.

2.6. Primer design

The nucleotide sequences of MD17G1102100, MD09G1111800 and