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Ectopic expression of the cowpea (*Vigna unguiculata*) *VuCERK1* gene confers enhanced resistance to *Pto* DC3000 *hrcC*⁻ in *Arabidopsis*

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Abstract: Pattern recognition receptors (PRRs) play multiple roles in plants. As a kind of PRRs, chitin elicitor receptor kinase 1 (CERK1) proteins were reported to function in plant resistance to fungal and bacterial pathogens, and tolerance to salt stress. In this study, a predicted cowpea *CERK1* homologous gene, designated as *VuCERK1*, was identified by database search. VuCERK1 protein contains 618 amino acid residues, with a predicted molecular mass of 67.5 kDa and a predicted isoelectric point of 5.04. VuCERK1 shows 58% and 60% sequence identity with AtCERK1 and OsCERK1, respectively. VuCERK1 also shows similar subcellular pattern with AtCERK1 and OsCERK1, suggesting VuCERK1 may function in cowpea immune responses. Gene expression assay indicated, that *VuCERK1* was expressed in four different seedling tissues tested, comprising first leave, epicotyl, hypocotyl and root, and it could be induced by salt stress. Furthermore, transient expression of *VuCERK1* in *Nicotiana benthamiana* induced obvious cell death. In addition, heterologous overexpression of *VuCERK1* in *Arabidopsis thaliana* conferred enhanced disease resistance to *Pseudomonas syringae* pv. tomato strain DC3000 *hrcC*⁻ (*Pto* DC3000 *hrcC*⁻).

Keywords: chitin elicitor receptor kinase 1; cowpea; pattern recognition receptors; *Pseudomonas syringae* pv. tomato strain DC3000 *hrcC*⁻

Pathogen-associated molecular patterns (PAMPs) are unique molecular structures from pathogens. Normally, the recognition of PAMPs by special pattern recognition receptors (PRRs) activates a set of defence responses, known as PAMP-triggered immunity (PTI) responses (Wu & Zhou 2013; Macho & Zipfel 2014; Tang et al. 2017). So far, dozens of PAMPs and their corresponding PRRs have been identified, including bacterial flagellin and FLAGELLIN SENSING 2 (FLS2) (Gómez-Gómez & Boller 2000), ELONGATION FACTOR-TU (EF-tu) and its receptor ELONGATION FACTOR-TU RECEPTOR (EFR) (Kunze et al. 2004; Zipfel et al. 2006), PEP1 RECEPTOR 1/2 (PEPR1/2) and plant elicitor peptides

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(Huffaker & Ryan 2007; Krol et al. 2010; Yamaguchi et al. 2010), CHITIN ELICITOR RECEPTOR KINASE 1and CHITIN ELICITOR BINDING PROTEIN (CEBiP), and their ligand chitin (Miya et al. 2007; Wan et al. 2008; Shimizu et al. 2010), LYM1/3 and bacterial peptidoglycan (PGN) (Willmann et al. 2011).

Lysin motif (LysM)-containing proteins play important roles in perception and signalling when invaded by fungal and bacterial pathogens in plants (Gust et al. 2012; Desaki et al. 2018, 2019; Yang et al. 2022). So far, many LysM-containing proteins have been identified to function in chitin and PGN triggered immunity in plants. LysM-containing proteins, OsCEBiP and AtCERK1, were first found to recognize chitin in rice and *Arabidopsis*, respectively. In rice, OsCERK1 was demonstrated to collaborate with OsCEBiP in the transduction of chitin-induced defence signals (Kaku et al. 2006; Shimizu et al. 2010; Hayafune et al. 2014). In *Arabidopsis*, AtCERK1 was found to function in both chitin recognition and signalling (Miya et al. 2007; Iizasa et al. 2010; Petutschnig et al. 2010). Meanwhile, AtLYK4 and AtLYK5, two LysM-containing proteins, were further identified to play an essential role in chitin signalling (Wan et al. 2008; Petutschnig et al. 2010; Cao et al. 2014). AtCERK1 and AtLYK5 were both proved to own chitin-binding activity. However, AtLYK5 exhibited much stronger activity (Iizasa et al. 2010; Liu et al. 2012; Cao et al. 2014). Additionally, AtLYK4 and AtLYK5 demonstrated partial function redundancy in chitin signalling (Cao et al. 2014).

LysM-containing proteins have also been reported to participate in plant signalling in response to bacterial pathogens (Willmann et al. 2011; Ao et al. 2014). In *Arabidopsis*, three LysM-containing proteins, AtLYM1, AtLYM3, and AtCERK1 form a system to recognize PGN, while AtLYM1 and AtLYM3 physically interact with PGNs (Willmann et al. 2011). The *Arabidopsis cerk1* mutant showed enhanced susceptibility to the bacteria pathogen *Pto* DC3000 and *Pto* DC3000 *hrcC*$^-$ (Willmann et al. 2011; Shinya et al. 2014). In rice, OsCERK1 is essential for PGN signalling initiated by two LysM-containing proteins, OsLYP4 and OsLYP6 (Ao et al. 2014). The linkage between chitin and salt signalling mediated by AtCERK1 was studied in *Arabidopsis*. Genes induced by salt stress and chitin were found to be highly correlated. However, this correlation was not observed when treated with other MAMPs or abiotic stresses. The *Arabidopsis cerk1* mutant showed more sensitive to NaCl treatment compared to the wild type (Espinoza et al. 2017).

As an important member of LysM-containing proteins functioning in plant immunity, the CERK1 have been studied in many plant species (Yang et al. 2022). Nevertheless, little information has been reported on its ortholog VuCERK1 in cowpea. We do not know if VuCERK1 shows high sequence identity to OsCERK1 and AtCERK1 or if it functions in the same mechanism in plant immunity with *Arabidopsis* or rice. In this study, a predicted cowpea *CERK1* mRNA sequence (LOC114188082), which encodes a protein with 618 amino acid residues, was identified from the NCBI database. The candidate gene is designated as *VuCERK1*. VuCERK1 shows high sequence, as well as similar subcellular localization with AtCERK1 and OsCERK1. Transient expression of *VuCERK1* in *Nicotiana benthamiana* induces cell death, and heterologous expression of *VuCERK1* in *Arabidopsis* increases resistance to *Pto* DC3000 *hrcC*$^-$.

MATERIAL AND METHODS

Plant materials and growth conditions. The seeds of the cowpea cultivar Xiabao2 were collected and preserved by the Hubei Engineering Research Center for Legume Plants. Cowpea seedlings, *Arabidopsis thaliana* Col-0 and mutant *cerk1-2* (Columbia background), and *Nicotiana benthamiana* (wild type) were grown in a mixture of nutrient soil and vermiculite at a 1 : 1 ratio. The plants were grown at a temperature of 22 ± 1 °C with a photoperiod cycle of 12 h of light and 12 h of dark.

Phylogenetic tree analysis. The phylogenetic tree was constructed in MEGA 11 using the Neighbor-Joining method. The numbers for each branch indicate the bootstrap values with 1 000. The protein sequences were collected from a previous study (Chen et al. 2020).

Expression analysis of *VuCERK1*. The expression of *VuCERK1* in cowpea seedlings was tested. Different plant organs from the seedings, including first leave, epicotyl, hypocotyl and root were harvested and quickly kept in liquid nitrogen for expression analysis. For NaCl treatment, one-week-old cowpea seedlings cultured in the pots were treated with 200 mM NaCl solution as previously described (Ravelombola et al. 2019). Briefly, the pots were soaked with 200 mM NaCl solution up to two-thirds of the pot height. In this study, this process lasted for 20 min and then the pots were transferred to normal condition. The roots were harvested three hours post-treatment for gene expression analysis.

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Total RNA was extracted using the TRIZOL method. The TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech, Beijing, China) was used for first-strand cDNA synthesis, following the instructions. Before the quantitative real-time PCR (Q-RT PCR), the first-strand cDNA was diluted five times with RNase-free water.

The Q-RT PCR was carried out to analyse the expression of *VuCERK1* using SYBR Green PCR master mix II (Takara, Dalian, China) and system. The experiment was performed as previously described (Shi et al. 2013a). Gene specific primers of *VuCERK1* for Q-RT PCR (VuCERK1_qPCR_F/R) were designed using Primer-Blast of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The *UBQ1* homolog gene in cowpea was amplified as an internal control, and the primers (VuUbq_qPCR_F/R) were designed as previously described (Sadhukhan et al. 2014). In gene expression analysis in *Arabidopsis*, *ACTIN2* was used as internal control, and the primers (AtACTIN2_qPCR_F/R) were obtained from previously reported (Shi et al. 2013a). The primers are listed in Table S1 in Electronic Supplementary Material (ESM).

For each expression analysis, three independent samples from each plant material were used for RNA extraction and first-strand cDNA synthesis. For Q-RT PCR, three technical replicates were conducted for both internal control and target gene for each sample. All the experiments were repeated three times with similar results.

Subcellular localization of VuCERK1. The transient expression vector pSAT6-EYFP-N1(pE3225) was utilized to examine the subcellular localization pattern of VuCERK1. The coding region of *VuCERK1* was amplified from the cDNA of cowpea leaves using gene-specific primers. The primers (VuCERK1_pSAT6_F/R) are listed in Table S1 in ESM. The empty pSAT6-EYFP-N1 was digested with *Hind* III and *Kpn* I. Subsequently, the fragments containing the coding region of *VuCERK1* were inserted into the linear pSAT6-EYFP-N1 using ClonExpress® II One Step Cloning Kit (Vazyme, Nanjing, China), following the provided instructions. The recombinant vector was then transiently expressed in onion epidermic cell using the GJ-1000 High-Pressure Gene Gun system (Scientz, Ningbo, China), following the manufacturer's instruction. A fluorescent signal was detected using a Leica SP8 (Leica, Wetzlar, Germany) confocal microscope. The experiment of subcellular localization was repeated three times with similar results.

Construction of overexpression vector. The Gateway™ BP Clonase™ and Gateway™ LR Clonase™ kits from Invitrogen company (Carlsbad, USA) were used for vector construction. *VuCERK1* fragments were amplified from total cDNA template derived from cowpea leaves and subsequently extracted using an agarose gel extraction kit. The primers (VuCERK1_OE_F/R) are listed in Table S1 in ESM. The fragments were then transferred into entry vector pDonor207 via the BP reaction. The right clone was used for cloning *VuCERK1* into pEarleyGate103 vector via the LR reaction. The constructed pEarleyGate103-VuCERK1 plasmids were sequenced, and those without mutations were transformed into *Agrobacterium tumefaciens* strain GV3101 competent cells to obtain the strain for transformation.

Transient expression of *VuCERK1* in *Nicotiana benthamiana*. The GV3101 strain harbouring pEarleyGate103-VuCERK1 was used for transient expressing, following the method previously described (Liu et al. 2010). The experiment of cell death observation was repeated three times with similar results.

Pathogen inoculation. Inoculation of *Pto* DC3000 *hrcC*$^-$ was performed as previously described (Mengiste et al. 2003). The experiment was repeated three times with similar results.

RESULTS

Identification of *VuCERK1* gene and sequence analysis. A cowpea mRNA sequence (LOC114188082), predicted to encode a CERK1 homolog, was identified from the NCBI database. To verify the accuracy of this sequence, specific primers were designed to amplify the CDS region using cDNA from the cowpea Xiabao2 cultivar left as the template. Sequencing results confirmed the amplified fragments were 100% identical to its CDS region. This gene was then designated as *VuCERK1* and encodes a protein with 618 amino acid residues, having a molecular mass of 67.5 kDa and a predicted isoelectric point of 5.04. Bioinformatics analysis revealed that VuCERK1 contains a very conserved trans-membrane domain, similar to its orthologs AtCERK1 and OsCERK1. Each of these sequences contains a signal peptide, three LysM domains, a trans-membrane helix, and a protein kinase domain (Figure 1A). VuCERK1 shares sequence identity of 58% and 60% with AtCERK1 and OsCERK1, respectively. Phylogenetic tree analysis based on protein sequences indicated that VuCERK1 is closely related to OsCERK1, and VuCERK1 shares higher sequence identity with CERK1 than other LYK proteins from *Arabidopsis* and rice (Figure 1B).

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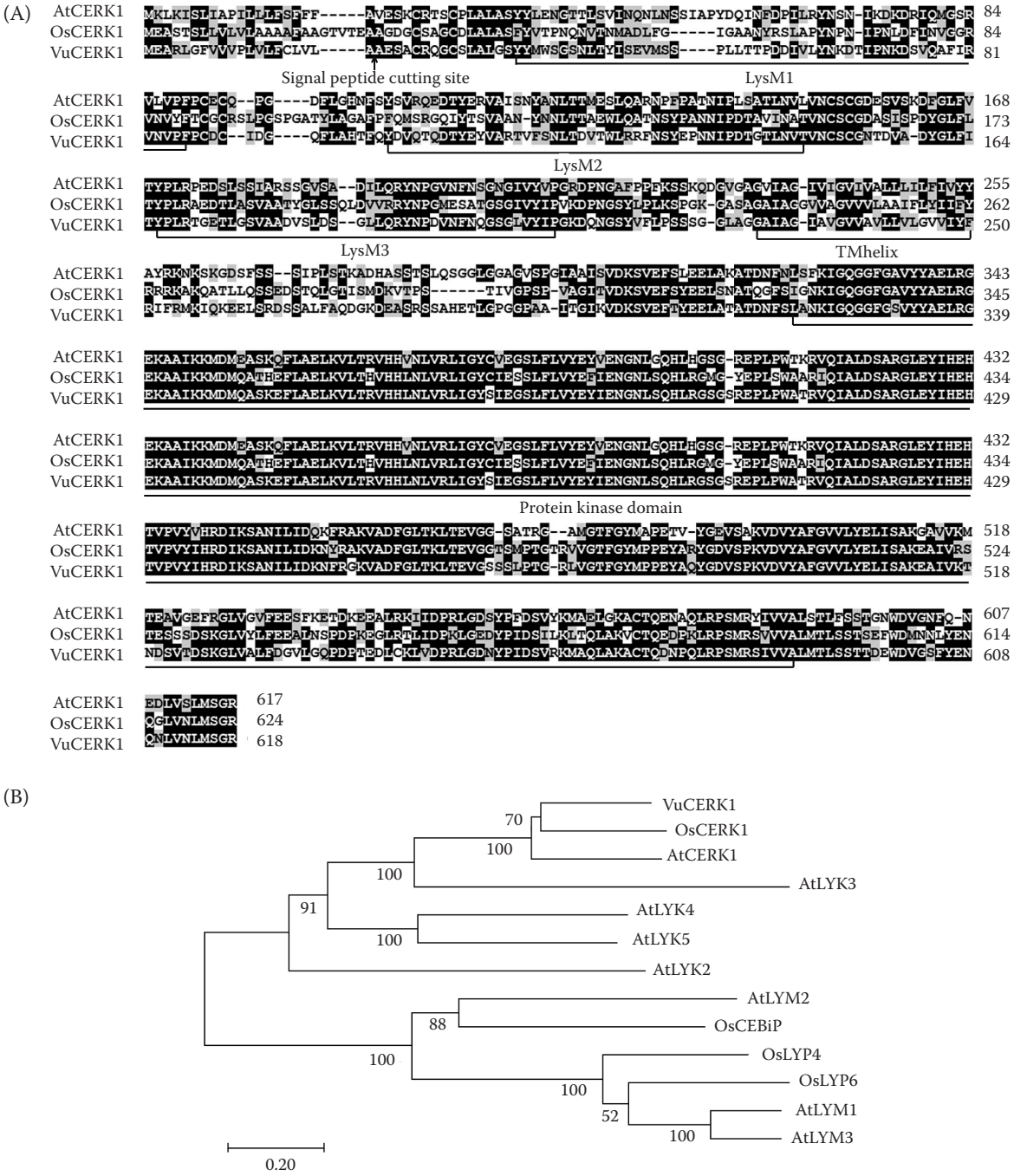

Figure 1. Sequence analysis of VuCERK1 protein: Alignment of protein sequences of AtCERK1, OsCERK1, and VuCERK1 (A); phylogenetic tree of LYK proteins from *Arabidopsis*, rice, and cowpea (B)

For the phylogenetic tree, the sequences were analysed by MEGA11; the tree was generated by using the Neighbor-Joining method; the numbers for each branch indicate the bootstrap values with 1 000 replications; the scale bar represents substitution rate per site

Expression analysis of *VuCERK1*. We then checked the expression levels of *VuCERK1* in various tissues (Figure 2A). The results showed *VuCERK1* was expressed in all tested tissues, with higher levels observed in the root and lower levels in the epicotyl. In *Arabidopsis*, *AtCERK1* is induced by salt stress, and the *cerk1* mutant shows enhanced sensitivity to NaCl (Espinoza et al. 2017). We also test the effect

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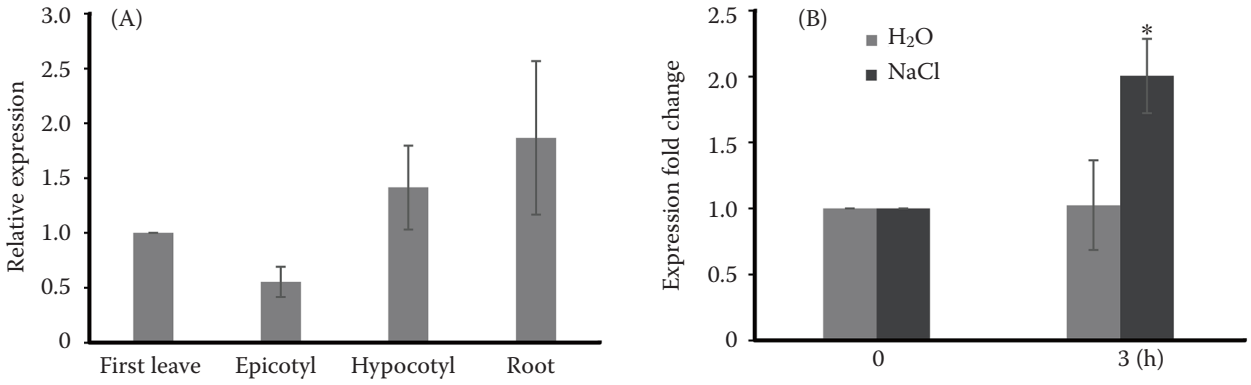

Figure 2. Expression analysis of *VuCERK1*: Expression of *VuCERK1* in different tissues of cowpea seedling (A); induced expression of *VuCERK1* by NaCl treatment in cowpea root (B)

The data are presented as the mean ± SE (n = 3); a statistically significant difference is indicated by one asterisk ($P < 0.05$, Student's t-test)

of salt stress on *VuCERK1* expression level in cowpea young roots. After being treated with NaCl solution for 3 hours, the transcriptional level of VuCERK1 was significantly upregulated (Figure 2B), suggesting that cowpea VuCERK1 may function in a similar model to *AtCERK1*.

Subcellular localization of VuCERK1. To determine the subcellular localization of VuCERK1, a transient overexpression vector was used to construct a fusion of VuCERK1 with YFP (VuCERK1-YFP), and the empty vector was used as the control. After transforming into the onion epidermic cell, yellow fluorescent emanating from the VuCERK1-YFP was observed exclusively at the cell periphery (Figure 3). To confirm the plasma membrane localization, a plasmolysis experiment was conducted. After the plasmolysis, the yellow fluorescent contracted with the protoplast (Figure 3), indicating VuCERK1 is localized in the plasma membrane of plant cells.

Transient expression of *VuCERK1* in *Nicotiana benthamiana*. In prior studies, transient expression of *CERK1*, even in the absence of pathogen invasion

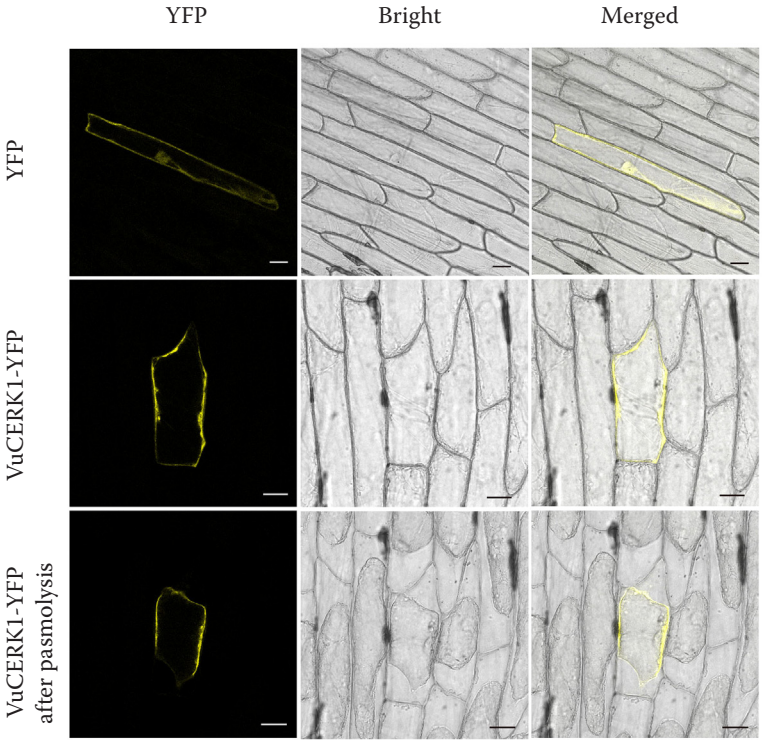

Figure 3. VuCERK1-YFP fusion protein localizes in the plasma membrane in onion epidermic cell

The P_{35S}:*VuCERK1-YFP* and the control vector (pSAT-EYFP-N1) were transiently expressed in onion epidermic cell; for the plasmolysis assay, the transformed tissue was treated with 0.3 g/mL sucrose solution for 5 min; the YFP signal was detected using a Leica SP8 confocal microscopy; bars represent 50 µm

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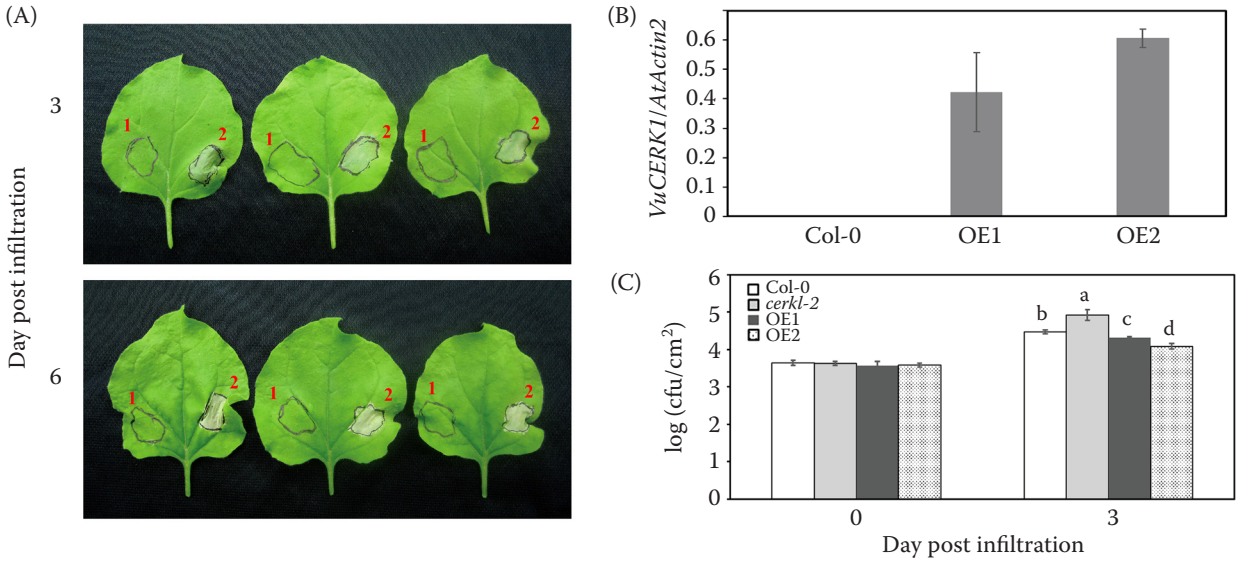

Figure 4. Function analysis of VuCERK1: Transient overexpression of *VuCERK1* induced cell death in *Nicotiana benthamiana* (A); expression analysis of *VuCERK1* in transgenic *Arabidopsis* plants (B); *VuCERK1* overexpression lines OE1 and OE2 displayed enhanced resistance to *Pto* DC3000 *hrcC*⁻ in *Arabidopsis* (C)

The data are presented as the mean ± SE (n = 3); lowercase letters indicate statistically significant differences (P < 0.05, one-way ANOVA Turkey' test)

or chitin treatment, was able to trigger cell death in plants (Pietraszewska-Bogiel et al. 2013; Yamaguchi et al. 2017). To verify if *VuCERK1* shows a similar function, a *VuCERK1* overexpression vector was constructed and then transiently transformed into *Nicotiana benthamiana* leaves via *Agrobacterium*-mediated transformation. The empty pEGAD vector serves as a control. Leaves examined 3 and 6 days post-infiltration were observed. After infiltration for 3 days, cell death was observed in regions where VuCERK1 was transiently overexpressed (Figure 4A, "2" labelled regions) and it became more noticeable after infiltration for 6 days, whereas no cell death occurred in control areas (Figure 4A, "1" labelled regions), suggesting that VuCERK1 may function analogously to the aforementioned CERK1 proteins.

Disease resistance analysis of *VuCERK1* overexpression lines of *Arabidopsis*. To determine the function of VuCERK1 in plant immunity, we ectopically expressed *VuCERK1* in *Arabidopsis*. Two overexpression (OE) lines were selected and the related expression levels of *VuCERK1* in each line were tested (Figure 4B). The plants of Col-0 wild type, the *cerk1-2* mutant, and two OE lines in the WT background were inoculated with *Pto* DC3000 *hrcC*⁻ by injection. The two OE lines showed enhanced resistance to *Pto* DC3000 *hrcC*⁻ compared to the wild type, while the *cerk1-2* mutant showed compromised resistance three days post-inoculation, indicating VuCERK1 plays a positive role in plant disease resistance.

DISCUSSION

In the plant immune system, PTI responses are triggered after the recognition of PAMPs by plant PRRs. Most PRRs identified to date belong to either receptor-like kinases or receptor-like proteins. LysM-containing proteins, which are widely present in prokaryotes and eukaryotes, have been found to perceive peptidoglycan and chitin, playing essential roles in related signalling pathways. In several invertebrates, LysM-containing proteins have also been reported to play significant roles in innate immunity (Shi et al. 2013b, 2016).

CERK1 proteins are highly conserved in different plant species (Yang et al. 2022). In this study, VuCERK1 was identified, and it contains 618 aa, and the number is very close to 617 aa of AtCERK1 and 624 aa of OsCERK1. Based on sequence alignment, VuCERK1 was found to share high sequence homology with OsCERK1 and AtCERK1, and each contains a signal peptide in N-terminal, three LysM domains, a transmembrane domain, and a protein kinase domain. These data further demonstrate the high sequence homology of CERK1 proteins in plants.

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Both OsCERK1 and AtCERK1 are membrane-localized proteins (Miya et al. 2007; Chen et al. 2010), and data presented here shows that the VuCERK1 also localizes in the plasma membrane in onion epidermal cell. The high sequence homology and the same subcellular localization suggest VuCERK1 may have a similar function to OsCERK1 and AtCERK1. In *Arabidopsis*, *AtCERK1* can be induced by salt stress (Espinoza et al. 2017). In this research, *VuCERK1* was showed to be induced by salt stress in the roots of young seedlings, which was consisted with results in *Arabidopsis*.

In previous studies, transient overexpression of *CERK1* could induce cell death in plant without chitin treatment or pathogen invasion (Pietraszewska-Bogiel et al. 2013; Yamaguchi et al. 2017). In this research, transient overexpression of *VuCERK1* in *Nicotiana benthamiana* leaves induces cell death in the inoculation areas, indicating VuCERK1 possesses a similar function as previously reported (Pietraszewska-Bogiel et al. 2013; Yamaguchi et al. 2017). Due to the multi-level expression regulation in stable overexpression lines, the expression level of *VuCERK1* in stable lines of *Arabidopsis* is lower than transient expression in *Nicotiana benthamiana*, which leads to the cell death in *Nicotiana benthamiana* but not in *Arabidopsis*.

In *Arabidopsis*, AtCERK1 functions in peptidoglycan recognition and resistance to bacterial pathogen, and the *cerk1* mutant shows enhanced disease susceptibility to *Pto* DC3000 and *Pto* DC3000 *hrcC*$^-$ (Willmann et al. 2011; Shinya et al. 2014). Data presented here shows that heterologous overexpression of *VuCERK1* enhances resistance to *Pto* DC3000 *hrcC*$^-$ in *Arabidopsis*, suggesting that VuCERK1 functions in plant resistance to bacterial pathogens. Although the function analysis of CERK1 proteins is much more focused on plant resistance to fungal pathogens, due to the limitation of lab conditions, now we cannot conduct such research. In the field condition, cowpea would be infected by some fungal pathogens, such as *Uromyces vignae* and *Erysiphe polygoni*, and the function of VuCERK1 in plant resistance to fungal pathogens needs to be further studied.

CONCLUSION

In this study, the *VuCERK1* gene was identified from cowpea. VuCERK1 shows high sequence identity with AtCERK1 and OsCERK1, and it localizes in the plasma membrane in onion epidermic cell. Expression analysis indicated *VuCERK1* could be induced by salt stress in the roots of cowpea seedlings. Obvious cell death could be induced in *Nicotiana benthamiana* leaves, in which *VuCERK1* was transiently expressed. Heterologous overexpression of *VuCERK1* conferred improved disease resistance to a bacterial pathogen, *Pto* DC3000 *hrcC*$^-$, in *Arabidopsis thaliana*.

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