

A Cysteine-Rich Receptor-Like Protein Kinase CaCKR5 Contributes to Immune Defense Against *Ralstonia Solanacearum* in Pepper

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Abstract

Background: Cysteine-rich receptor-like kinases (CRKs) represent a large subfamily of receptor-like kinases and have important roles in numerous different physiological processes in plants.

Results: *CaCRK5* transcripts were observed to accumulate after the inoculation of *R. solanacearum* and treatment with salicylic acid. The fusion between *CaCRK5* and the green fluorescence protein was targeted to the plasma membrane. Suppression of *CaCRK5* via virus-induced gene silencing made pepper plants significantly susceptible to *R. solanacearum* infection, which was accompanied by decreased expression of defense related genes *CaPR1*, *CaSAR8.2*, *CaDEF1* and *CaACO1*. Overexpression of *CaCRK5* in tobacco conferred increased resistance against *R. solanacearum*. Furthermore, electrophoretic mobility shift assay and chromatin immunoprecipitation with quantitative real-time PCR analysis verified that a homeodomain zipper I protein *CaHDZ27* can bind to the *CaCRK5* promoter, and directly activate its expression. Yeast two-hybrid and bimolecular fluorescence complementation analyses suggested that *CaCRK5* could heterodimerize with the homologous member *CaCRK6* in the plasma membrane.

Conclusions: Our data indicated that *CaCRK5* played a positive role in pepper resistance against *R. solanacearum* infection.

Background

When plants are attacked by pathogen, plant immune receptors can detect pathogen infection and elicit a battery of defense responses. As the first line of plant defense, pattern recognition receptors (PRRs) can perceive pathogen-associated molecular patterns (PAMPs) that are released during pathogen infection and activate plant immunity [1]. More researches uncovered that most surface-localized PRRs identified so far are receptor-like kinases (RLKs) in plants [2, 3]. Well-characterized PRRs include the *Arabidopsis* leucine-rich repeat (LRR) receptor kinases FLAGELLIN SENSING 2 (FLS2) or EF-TU RECEPTOR (EFR), which can recognize bacterial flagellin [4] or EF-Tu [5], respectively. RLKs comprise a superfamily of transmembrane proteins, playing critical roles in various signal transduction mechanisms. RLKs are large group in higher plants, there are about 610 RLKs in model plants *Arabidopsis* and rice, respectively [6, 7]. A typical RLK contains an extracellular receptor, a transmembrane domain and an intracellular Ser/Thr kinase domain[6]. Although roles for most RLKs are unknown, many RLKs were reported to regulate plant physiological processes, including development, hormone perception and defense responses [8].

Cysteine-rich RLKs (CRKs), a prominent subfamily of RLKs, is predicted to be transmembrane receptor proteins. CRKs are characterized by the presence of one to four copies of domain 26 of unknown function (DUF26), a C-X8-C-X2-C motif in the extracellular receptor region in the N-terminus[9]. The conserved Cys residues may participate in the formation of the three-dimensional structure of the protein through disulfide bonds[10], which are known to mediate protein-protein interactions[11]. CRKs have been suggested to play important roles in the regulation of immune responses. Most of the CRKs in *Arabidopsis* are induced by pathogen attack and application of SA at the transcriptional level [12, 13].

Some CRKs has been functionally characterized in the response to pathogen infection. For example, CRK45 acted as a positive regulator in *Arabidopsis* disease resistance. *CRK45* overexpression enhanced resistance to *Pseudomonas syringae*, whereas the *crk45* mutant was more sensitive to *P. syringae*[13]. Overexpression of *CRK4*, *CRK6* and *CRK36* in *Arabidopsis* increased disease resistance to the bacterial pathogen *Pst* DC3000 through a positive role in the PTI response by association with the FLS2 [14]. Intriguingly, recent studies revealed that some CRKs form a receptor complex with RLKs and play roles in transducing signals in the plant immune. For example, increased expression of *CRK28* in *Arabidopsis* enhanced ROS burst and disease resistance toward *P. syringae*. CRK28 associated with FLS2/BAK1 immune complex as well as associated with the closely related CRK29 [15]. However, whether members of this large CRK subfamily in pepper regulate defense responses are unknown.

Here, we report the identification of a CRK coding genes *CaCRK5* that function as a positive regulator in the pepper response to *R. solanacearum* infection. Suppression of *CaCRK5* in pepper plants via virus-induced gene silencing (VIGS) increased susceptibility to pathogen. Overexpression of *CaCRK5* in tobacco enhanced resistance to pathogen. In addition, *CaCRK5* was targeted and up-regulated by CaHDZ27. Lastly, we demonstrate that CaCRK5 could interact with its homologous member CaCRK6 in the plasma membrane.

Methods

Plant materials and Pathogen inoculation

Pepper (*Capsicum annuum* cultivar L. cv. Fj8) was obtained from the pepper breeding group at Fujian Agriculture and Forestry University (FAFU), it exhibited medium-resistance to *R. solanacearum* that was identified by Prof. Shuilin He at FAFU in China. Pepper and tobacco (*Nicotiana benthamiana*) plants were grown in plastic pots containing steam-sterilized soil at 26°C with a long-day photoperiod (16 h light/8 h dark) with 60% relative humidity.

Virulent strains FJC100301 of *R. solanacearum* was used in this study. The *R. solanacearum* strain was grown in PSA medium. Bacteria cell solution was harvested with sterile 10 mM MgCl₂ and re-suspended to 1.0×10⁸CFU/mL. For pepper inoculation, seedlings were irrigated by cutting the roots and pouring 30 mL bacterial inoculum into each pot. For leaf inoculation, the leaf was infiltrated with 10 μL of the *R. solanacearum* suspension using a syringe without a needle. For each plant, a disease index on a scale of 0 to 4 was calculated as described previously.

Subcellular Localization

For the subcellular localization assay, CaCRK5 were cloned into pMDC83 vector, resulting in 35S:CaCRK5-GFP construct. The plasmids were transformed into *A. tumefaciens* GV1301. The transient expression assays were conducted as described previously [44]. Fluorescence of GFP in the transformed leaves was imaged using a confocal laser scanning microscope (TCSSP8; Leica, Solms, Germany).

Virus-Induced Gene Silencing

The tobacco rattle virus (TRV)-based virus-induced gene silencing system was used for gene silencing in pepper. To achieve CaCRK5 specific silencing, about 200 bp DNA fragment in their 3'-untranslated region were PCR amplified and cloned into TRV2 to generate TRV2:CaCRK5. *A. tumefaciens* GV3101 carrying TRV1 with TRV2:CaCRK5 was co-infiltrated into the fully expanded cotyledons of pepper plants. Then, the plants were incubated at 16°C for 56 h, and grown at 26°C. The experiments were performed with at least 50 plants per treatment and repeated three times. The rate of the disease index was calculated as previously described[44].

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNAs were synthesized using One Step PrimeScript™ cDNA Synthesis Kit (TaKaRa, Shiga, Japan). The real-time RT-PCR analysis was performed using a Bio-Rad real-time PCR system (Foster City, CA, USA.) and the SYBR Premix ExTaq II system (Takara). The relative expression of the CaCRK5 and defense-related genes were calculated with the $2^{-\Delta\Delta Ct}$ method. *CaActin* or *NtActin* served as the reference gene to normalize the total amount of cDNA in each reaction. The primers were listed in Additional file 3.

Yeast two-hybrid analysis

The pGADT7 vector was used for GAL4 AD, and the pGBKT7 vector was used for GAL4 BD. ORFs of CaCRK5 and CaCRK6 were amplified and cloned into pGADT7 to generate constructs designated AD-CaCRK5 and AD-CaCRK6. The cDNAs of CaCRK5 and CaCRK6 were amplified and cloned into pGBKT7 to generate constructs designated BD-CaCRK5 and BD-CaCRK6. The corresponding AD and BD plasmids were co-transformed into Y2H Gold yeast strain. Their interaction was identified by spot assay. The transformants were tested on SD (Synthetic Dextrose) screening medium. The plates were incubated at 30°C till the formation of colonies.

Bimolecular fluorescence complementation (BiFC) assay

To generate BiFC constructs, CaCRK5 and CaCRK6 full length cDNAs were cloned into puc-SPYNE^{GW} and puc-SPYCE^{GW} vectors, which contain the N-terminal or C-terminal fragment of YFP (nYFP or cYFP, respectively). For transient expression, *A. tumefaciens* strain GV3101 carrying each construct was used to infiltrate leaves of 5-week-old *N. benthamiana* plants. For microscopic analysis, lower epidermis cells were observed using a confocal laser scanning microscope

Tobacco transformation

Transgenic tobacco plants were generated by using *A. tumefaciens* mediated tobacco leaf disc transformation [45]. The CaCRK5 coding region was cloned into pK7WG2 vector to get 35S:CRK5 constructs. Two constructs were introduced into *A. tumefaciens* GV3101 and used to transform *N.*

benthamiana. Transformed seed stock was selected for kanamycin resistance by planting seeds on Murashige and Skoog agar plates containing 50 µg of kanamycin per milliliter.

Histochemical staining

Histochemical staining was performed as previously described [44]. The infiltrated leaves were stained with trypan blue to visualize the cell death response and DAB solution for H₂O₂ detection.

Electrophoretic mobility shift assay (EMSA)

Expression of the CaHDZ27-GST fusion protein was induced in transformed *E. coli* BL21 cells, by adding IPTG (isopropyl β-D-1-thiogalactopyranoside, 0.05 mM) at 37°C for 4 h. The recombinant proteins were extracted from the cells and purified using BeaverBeads™ GSH protein purified kit according to manufacturer's instructions.

The EMSA was performed as described previously[46]. Cy5-labelled DNA fragments were synthesized and used as probes, while unlabeled DNA of the same sequence was used as a competitor.

Chromatin immunoprecipitation (ChIP)-qPCR analysis

ChIP assay was performed using the method as described previously[44]. Chromatin was extracted from pepper leaves infiltrated with 35S:HA-CaHDZ27 and cross-linked with 1% formaldehyde (v/v). The chromatin was broken into fragments by probe sonicator to an average length of 300 to 500 bps. The fragmented DNA was immunoprecipitated with anti-HA antibody. Enrichment of DNA samples was analyzed by quantitative real-time PCR.

Results

CaCRK5 encodes a pathogen induced receptor-like kinase of the arginine aspartate (RD) family

In cDNA-AFLP experiments designed to isolate genes involved in the pepper resistance to the infection of *R. solanacearum*, a partial cDNA fragment of *CaCRK5* was obtained. As its expression was significantly upregulated after inoculation of *R. solanacearum*, we decided to study the function of this gene further. *CaCRK5* cDNA clones were isolated from a cDNA library made from *R. solanacearum* inoculated leaves of pepper inbred line CM334. The proteins deduced from the cDNA clones of *CaCRK5* contained 669 residues. SMART (<http://smart.embl-heidelberg.de/>) analysis of the domain architecture showed that their primary structures were composed of two cysteine-rich DUF26 domains, a transmembrane region and a serine/threonine kinase domain, and therefore *CaCRK5* belongs to the family of cysteine-rich kinases [10]. In addition, *CaCRK5* contains a conserved arginine-aspartic (RD) motif in the protein kinase domain (Fig.1). For most RD kinases, the phosphorylation in the activation loop is crucial for triggering kinase activity which usually displays phosphorylation/autophosphorylation ability. Non-RD kinases usually exhibit lower kinase activities because of the lack of activation loop autophosphorylation. RD and non-RD kinases often cooperate to control innate immune signaling in plants [16-19].

Genome-wide identification of CRK family in pepper

In the start of this work, we evaluate the CRK gene family in the pepper genome, Hidden Markov Model (HMM) profile of the Stress-antifung domain (PF01657) was used to search the pepper genome database PGP (<http://peppergenome.snu.ac.kr/>). The *Arabidopsis* CRK gene family sequences [10] were also used as query sequences to search against the PGP and the NCBI database. All of them were further subjected to domain analysis using NCBI-CDD (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and SMART, to ensure the presence of three important domains for typical CRK proteins including stress-antifung domains, transmembrane domain, and kinase domain. A total of 27 CRK genes (CaCRKs) were identified in the pepper cultivar CM334 genome, which were numbered from CaCRK1 to CaCRK27 according to their localization on chromosomes. Except for CaCRK13/24/25 with only one DUF26 domain, most CRKs contained two DUF26 domains. The length of CaCRK proteins varied from 332 to 1120aa, and molecular weights from 36.76 kDa to 128.25 kDa. The predicted isoelectric points of CaCRK ranged from 5.53 to 9.37(Additional file 1).

Chromosome physical localization analysis revealed that 27 CaCRKs were distributed on 7 of the 12 chromosomes in pepper. Chromosomes 2 contained the largest number of CRKs with 9 genes (34.6%), chromosomes 12 had only 1 gene. Gene clustering was the most common feature of the CRK genes distribution (Fig. 2a). Phylogenetic analysis was performed using the full length protein sequences of CRK from pepper, and an unrooted phylogenetic tree constructed by the neighbor-joining (NJ) method. Multiple CRKs were present in tandem repeat on the same chromosome (e.g. CaCRK1-7, CaCRK8/9, CaCRK10/11, CaCRK13-15 and CaCRK24/25) (Fig. 2b). These results suggested that the expansion of CRKs in pepper may be due to tandem duplication. This distribution and physical clustering pattern is consistent with CRKs in *Arabidopsis*[15] and soybean[20].

Expression of *CaCRK5* response to infection of *R. solanacearum* and treatment with exogenous SA, MeJA and ETH

To characterize the expression pattern of *CaCRK5* in detail, quantitative real-time PCR (qRT-PCR) analyses were performed. 6-week-old pepper plants were leaf-inoculated with *R. solanacearum*. *CaCRK5* was rapidly up-regulated over time after inoculation of *R. solanacearum*, and the highest level of expression was observed at 12 h post inoculation (hpi) with about 12.4-fold of that in control plant (Fig. 3a). Next, we investigated the expression of *CaCRK5* treated with the defense-related signaling molecules salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). The analysis showed the expression of *CaCRK5* was increased after SA treatment and reached a peak at 48h, and no significant change was observed after methyl jasmonate (MeJA) or ethephon (ETH) treatment (Fig. 3b). These results supported that *CaCRK5* was involved in pepper defense against *R. solanacearum* invasion.

Subcellular localization of CRK5

Since CaCRK5 encoded a potential transmembrane domain, it was predicted to localize to plasma membrane. To test the hypothesis, CaCRK5 was fused with green fluorescent protein (GFP) under the

control of the CaMV 35S promoter. CBL1n protein, which is known to be localized to the plasma membrane [21], was fused with red fluorescent protein (RFP). 35S:GFP, 35S:CaCRK5-GFP were transiently co-expressed with 35S:CBL1n-RFP in leaf epidermal cells of *N. benthamiana*. As shown in Fig.4, 35S:GFP construct served as a negative control, and green fluorescence was ubiquitously distributed throughout the cell. CaCRK5-GFP green fluorescence overlapped very closely with the red fluorescence in the plasma membrane suggesting that they are localized on the plasma membrane in the plant cell.

Silencing of *CaCRK5* in pepper plants increases susceptibility to *R. solanacearum* infection

To assess the role of *CaCRK5* in the interaction between pepper and *R. solanacearum*, loss-of-function experiments in pepper seedlings by virus-induced gene silencing (VIGS) were performed [22]. As shown in Additional file 2, photobleaching was observed in newly emerged true leaves of plants infiltrated with *Agrobacterium* carrying *CaPDS*, indicating that the VIGS system worked efficiently. qRT-PCR analysis showed that expression of *CaCRK5* in pepper leaves was significantly down-regulated during *R. solanacearum* infection in VIGS plants (Fig. 5a), indicating that *CaCRK5* were efficiently silenced.

CaCRK5 silenced and control (TRV:00) pepper plants were subjected to *R. solanacearum* challenge. Phenotypic analysis indicated that *CaCRK5* silenced pepper plants showed more severe disease symptoms than control plants after *R. solanacearum* infection (Fig. 5b). From 6 days post inoculation with *R. solanacearum*, *CRK5* silenced pepper plants exhibited significant delay of wilt symptom. The disease index of *CaCRK5* silenced plants was significantly increased, compared to the control plants (Fig. 5c). To address whether silencing of *CaCRK5* affect growth of *R. solanacearum*, the bacterial population was determined. As shown in Fig. 5d, growth of *R. solanacearum* was significantly enhanced in *CaCRK5* silenced plants 3 day post leaf-inoculation, compared with control plants. Based on these observations, we believed that *CaCRK5* participate defense responses in pepper.

Next, we investigated the cell death and oxidative burst in *CaCRK5* silenced and the control leaves. Trypan blue and DAB staining confirmed that hypersensitive cell death and H₂O₂ accumulation were significantly reduced in *CaCRK5* silenced leaves 48 h after inoculation with *R. solanacearum* (Fig. 5e), indicating that *CaCRK5* play pivotal roles in early defense response associated with cell death during *R. solanacearum* infection. We further determined the effects of *CaCRK5* silencing on the expression of defense related genes in pepper during *R. solanacearum* infection. qRT-PCR analyses showed that *CaCRK5* silencing in pepper leaves significantly attenuated expression of defense related genes, including *CaNPR1*[23], *CaSAR8.2*[24], *CaDEF1*[25] and *CaACO1*[26], during *R. solanacearum* infection (Fig. 5f).

Overexpression of *CaCRK5* in tobacco reduces susceptibility to *R. solanacearum* infection

A gain-of-function approach was also employed to study the function of *CaCRK5* in the defense response. Due to technical difficulties, we used the tobacco plant (*Nicotiana benthamiana*), which is also a host for *R. solanacearum*. At least 10 transgenic tobacco lines were obtained and confirmed by kanamycin resistance analysis. Two T₃ *CaCRK5* overexpressed lines exhibited constitutive expression (Fig. 6a), and were used in subsequent experiments.

Wild-type and transgenic plants (L3 and L7) at 4 weeks old were root-inoculated with *R. solanacearum*. The effect of *CaCRK5* overexpression on development of bacterial wilt disease was observed. As shown in Fig. 6b, the *CaCRK5* overexpressed plants showed much weaker symptoms at 12 d post-inoculation, compared with the wild-type. The wild-type plants showed wilt symptom from 6 day after inoculation and completely died 14 d after inoculation with *R. solanacearum*. The *CaCRK5* overexpressing plants were observed significant delay of wilt symptom compared to wild-type plants (Fig. 6c), showing that overexpression of *CaCRK5* conferred increased disease tolerance to *R. solanacearum* in tobacco. In addition, we also checked the expression of defense related genes, including *NtPR2*, *NtPR3*, *NtHSR201* and *NtHSR505*. qRT-PCR analysis indicated that expressions of these tested defense related genes were increased in *CaCRK5* overexpressed plants compared with wild-type plants, during the inoculation of *R. solanacearum* (Fig. 6d). Collectively, these data suggested that *CaCRK5* overexpression enhanced defense responses against *R. solanacearum* infection in tobacco.

CaCRK5 is directly regulated by transcription factor CaHDZ27

To better understand the regulatory mechanism of *CaCRK5*, a 2000-bp promoter region upstream of the *CaCRK5* coding sequence was identified. Sequence analysis using the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) suggested that the *cis*-elements in the promoter of *CaCRK5* included two TCA-element involved in salicylic acid responsiveness, five binding sites for MYB transcription factor, and one binding sites for MYC. In addition, a known binding site (CAATTATTG) for the HD-Zip subfamily I member CaHDZ27 was located between positions -625 and -617. As CaHDZ27 also regulate the defense in pepper against *R. solanacearum* infection [27], we speculated that *CaCRK5* might be targeted by CaHDZ27.

An electrophoretic mobility shift assay (EMSA) was conducted to access the interaction of CaHDZ27 with the *CaCRK5* promoter, and CaHDZ27 was found to bind to the Cy5-labeled *CaCRK5* promoter probe. Moreover, binding was gradually attenuated by increasing concentrations of unlabeled probe, indicating that CaHDZ27 binds specifically to the CAATTATTG in the *CaCRK5* promoter in vivo (Fig. 7a). Chromatin immunoprecipitation (ChIP)-qPCR was conducted to confirm interaction of CaHDZ27 and the *CaCRK5* promoter in vitro. The pepper leaves infiltrated with *Agrobacterium* strain GV3101 containing 35S:HA-CaHDZ27 or 35S:HA were harvested for ChIP assay. Chromatin from these pepper leaves was immunoprecipitated using anti-HA antibodies and enrichment of DNA sample was determined by qRT-PCR. The result showed that CaHDZ27 was significantly enriched in the *CaCRK5* promoter, and the enrichment was significantly enhanced by the inoculation of *R. solanacearum* (Fig. 7b and c), suggesting that CaHDZ27 could bind to the CAATTATTG in the *CaCRK5* promoter in vitro, and binding was increased by the *R. solanacearum* infection.

To further investigate the *CaCRK5* regulated by CaHDZ27 in the transcriptional level, we transiently expressed 35S:CaHDZ27-GFP in pepper leaves, and 35S:GFP infiltrated leaves were used as a negative control. qRT-PCR analysis demonstrated that the transcript level of *CaCRK5* was increased in 35S:CaHDZ27-GFP infiltrated leaves compared with the control (Fig. 8a). Using anti-GFP immune-blotting,

CaHDZ27 was confirmed to be expressed in pepper leaves (Fig. 8b). We used a VIGS approach to silence *CaHDZ27*. The transcript level of *CaCKR5* is reduced significantly in *CaHDZ27*-silenced plants compared with TRV:00 infiltrated plants, 48 h post the inoculation of *R. solanacearum* (Fig. 8c and d). These data suggested that the transcriptional expression of *CaCKR5* was positively regulated by CaHDZ27.

CaCRK5 interacts with CaCRK6

Previous studies indicated that plants employ immune receptor complex for sensing microbe-derived molecular patterns and effectors to trigger inducible immune defenses[28-31], and a few CRKs have been known to function in association with each other, such as AtCRK28/AtCRK29[15] and AtCRK39/AtCRK40[32]. Thus, we investigated the ability of CaCRK5 to heterodimerize with the closely related CaCRK6, which has 77.4% amino acid sequence identity to CaCRK5.

The cDNAs of CaCRK5 and CaCRK6 were cloned into the pGBKT7 and pGADT7 vectors separately, and generated DNA-binding domain (BD) and activation domain (AD) fusions. BD-CaCRK5/AD-CaCRK6 and BD-CaCRK6/AD-CaCRK5 transformant yeast cells can grow on SD/-Trp-Leu-His-Ade medium, as did positive control (pGBKT7-53/pGADT7-T). In contrast, no growth was observed in the negative controls. The result indicated that CaCRK5 interacts with CaCRK6 each other in yeast cells (Fig. 9a). To provide more evidence for the interaction between CaCRK5 and CaCRK6, we performed bimolecular fluorescence complementation (BiFC) assays. CaCRK5 and CaCRK6 were fused to the N- and C-terminal ends of yellow fluorescent protein (YFP) to generate CaCRK5-nYFP/CaCRK6-cYFP and CaCRK5-cYFP/CaCRK6-nYFP, respectively. Then, these constructs were transiently co-expressed in *Nicotiana benthamiana* leaves. At 48h post infiltration, the YFP fluorescence signals were checked by confocal microscopy. As showed in Fig. 9b, YFP fluorescence was observed at the plasma membrane in *N. benthamiana* leaves. These results indicate that CaCRK5 heterodimerizes with CaCRK6 at the plasma membrane of plant cells.

Discussion

The tandem repeats contributed to the expansion of the CaCRK family in pepper

CRKs are a subfamily of plant RLKs. In this study, 27 CaCRKs were identified in pepper genome; many CRKs were present in tandem repeat which made physical clusters at specific positions in the chromosome. Previous studies showed that a large proportion of the RLK gene family is found in tandem repeats [33-35]. Tandem repeats are enriched in stress-responsive genes [36-38]. The expansion and degree of tandem repeats are significantly correlated with the stress responsiveness of RLK genes[36]. The tandem repeats of CaCRKs in pepper may facilitate the stress adaptive evolution.

CaCRK5 played a positive role in the pepper resistance against *R. solanacearum*

Previous studies have shown that some CRK members including AtCRK29 in *Arabidopsis* were induced in response to treatment with pathogens, SA and flg22 [15, 39, 40], suggesting a role for these CRKs in plant immunity. The CaCRK5 closest homologue in *Arabidopsis* is AtCRK29 protein with 41.6% identity to

CaCRK5. In the study, expressions of *CaCRK5* were increased over time after inoculation of *R. solanacearum*, with high levels of gene expression at the early stage of infection. *CaCRK5* was strongly up-regulated after treatment with SA, and no significant change of *CRK5* was observed treated with MeJA and ETH.

Based on the subcellular localizations of GFP fusions, CaCRK5 are probably targeted to the plasma membrane. Several CRK proteins also have been reported to be localized in the plasma membrane, including CRK4, CRK6, and CRK36 in *Arabidopsis* [14] and GbCRK18 in cotton[41]. The TRV-based VIGS system was used to investigate the function of *CaCRK5* in the response to *R. solanacearum* infection. Expression of *CaCRK5* was significantly down-regulated during *R. solanacearum* infection in pepper leaves in which *CaCRK5* was silenced. CaCRK5 silenced pepper plants showed an increased susceptibility to infection by *R. solanacearum*, pepper leaves significantly compromised H₂O₂ accumulation and cell death. These findings suggested that *CaCRK5* may function as a positive regulator in pepper resistance against *R. solanacearum* infection. Furthermore, transient expression of *CaCRK5* in pepper leaves resulted in HR-like cell death, which was accompanied by accumulation of ROS. Consistently, *CaCRK5* overexpression tobacco plants exhibited enhanced resistance against infection by *R. solanacearum*.

***CaCRK5* was transcriptionally regulated directly by CaHDZ27**

HD-Zip transcription factors can be divided into four subfamilies (HD-Zip I–IV) based on their sequence similarities[42], and HD-Zip I proteins were reported to play roles in biotic and abiotic stress responses[43]. CaHDZ27, a HD-Zip I member, acts a positive regulator in pepper response to *R. solanacearum* infection[27]. Here we showed that CaHDZ27 acts as a positive regulator of *CaCRK5*, CaHDZ27 binds to a 9-bp CAATTATTG motif of the *CaCRK5* promoter revealed by EMSA and CHIP-qPCR. Expression of *CaCRK5* is increased in CaHDZ27 transit expressed pepper leaves, and decreased in CaHDZ27 silenced pepper plants with the inoculation of *R. solanacearum*. Moreover, *CaCRK5* play a positive role in pepper resistance against *R. solanacearum* infection, consistent with the role of CaHDZ27. These results suggest that *CaCRK5* and CaHDZ27 coordinate to regulate the pepper defense against *R. solanacearum* infection.

CaCRK5 heterodimerizes with CaCRK6

Yeast two-hybrid and bimolecular fluorescence complementation analyses indicated that *CaCRK5* heterodimerizes with *CaCRK6* in vivo in both yeast and plant cells. Recent evidence suggest that pattern recognition receptors (PRRs) enable the rapid recruitment of other RLKs, including CRKs, to enhance PRR signaling intensity [15, 39]. *CaCRK5* might act in concert with *CaCRK6*, and other RLKs in the pepper defense against *R. solanacearum* infection.

Conclusions

In summary, our data suggest that *CaCRK5* may significantly contribute to immune defense against *R. solanacearum* in pepper and tobacco. VIGS in pepper plants and gain-of-function analyses of *CaCRK5* in tobacco, revealed that *CaCRK5* positively regulates plant immune responses. In addition, CaHDZ27 promotes *CaCRK5* expression by directly binding to its promoter. We propose that the pepper defense response mediated by *CaCRK5* could be enhanced by CaHDZ27.

Abbreviations

CRK: Cysteine-rich receptor-like kinases; VIGS: Virus-induced gene silencing; PRR: pattern recognition receptor; PAMP: pathogen-associated molecular patterns; PTI: PAMP-triggered immunity; HMM: Hidden Markov Model; *R. solanacearum*: *Ralstonia solanacearum*; hpi: hours post inoculation; SA: Salicylic acid; JA: Jasmonic acid; ET: ethylene.

Declarations

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Author contributions

SM and SH conceived and designed the research. SM, FG, TZ, WH and QM organized and performed the experiments. SM and FG analyzed the data. SM and SH wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

Not applicable.

Consent for publish

Not applicable.

Competing interests

The authors have no conflicts of interest to declare.

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Figures

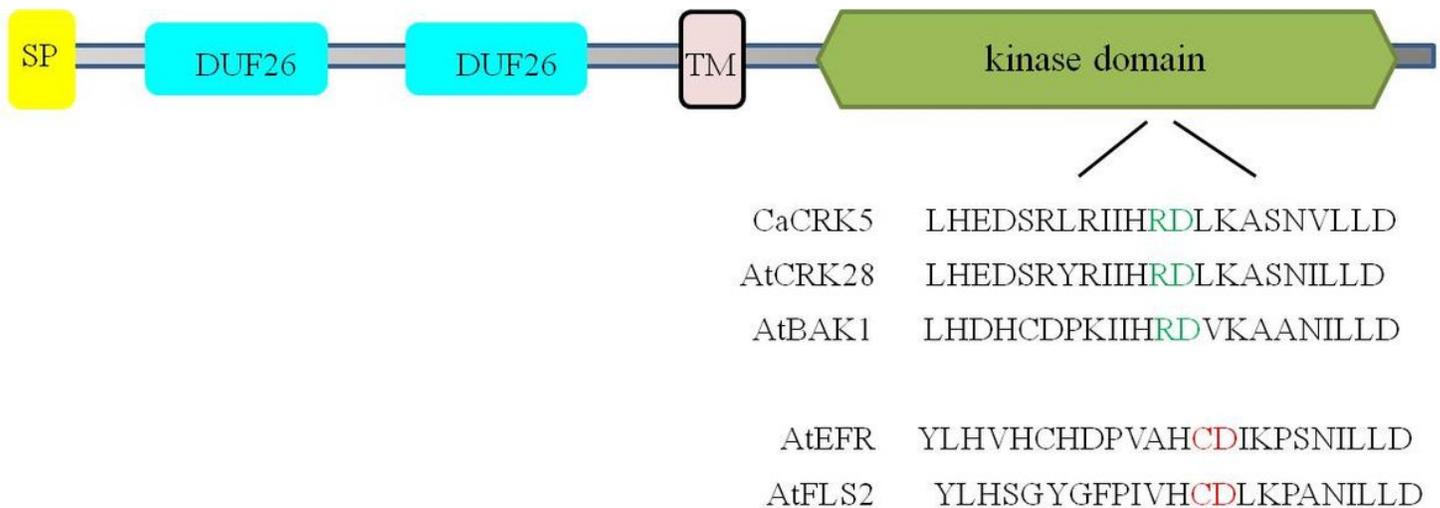


Figure 1

Schematic diagram of CaCRK5 protein domain architecture. It contains the signal peptide (SP), two domains 26 of unknown function (DUF26), transmembrane domain and kinase domain. The protein kinase domain harbors a RD motif represented by a green R and D. non-RD motif, which is often found in association with pattern recognition receptors such as AtEFR and AtFLS2, was represented by red C and D.

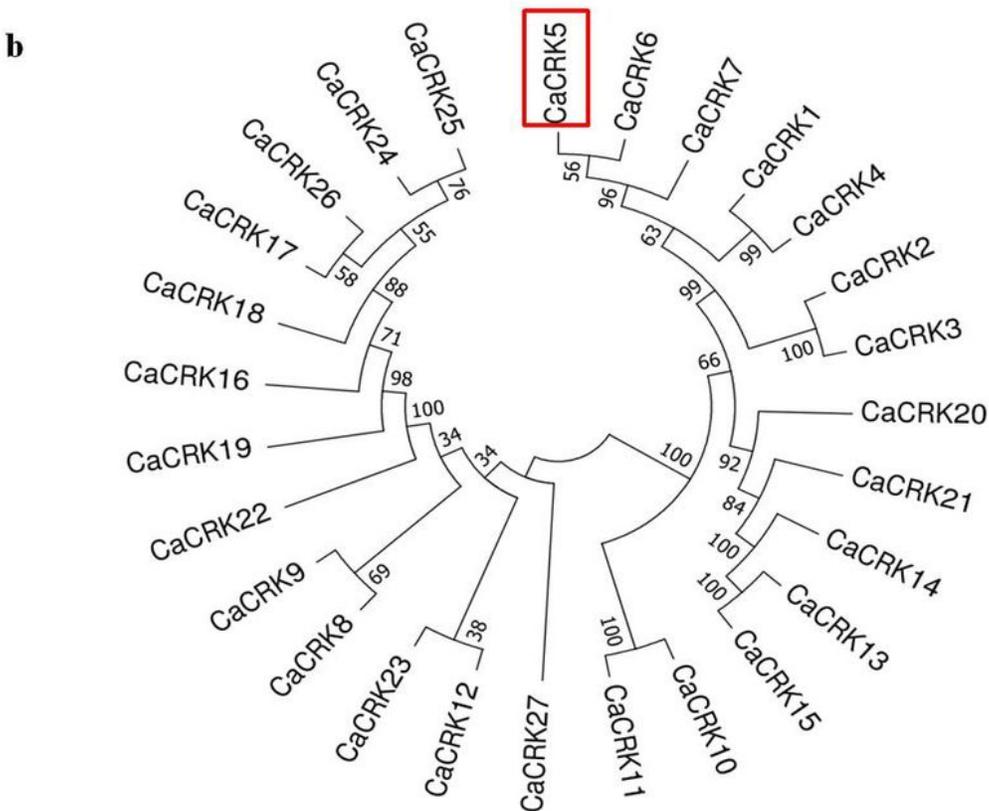
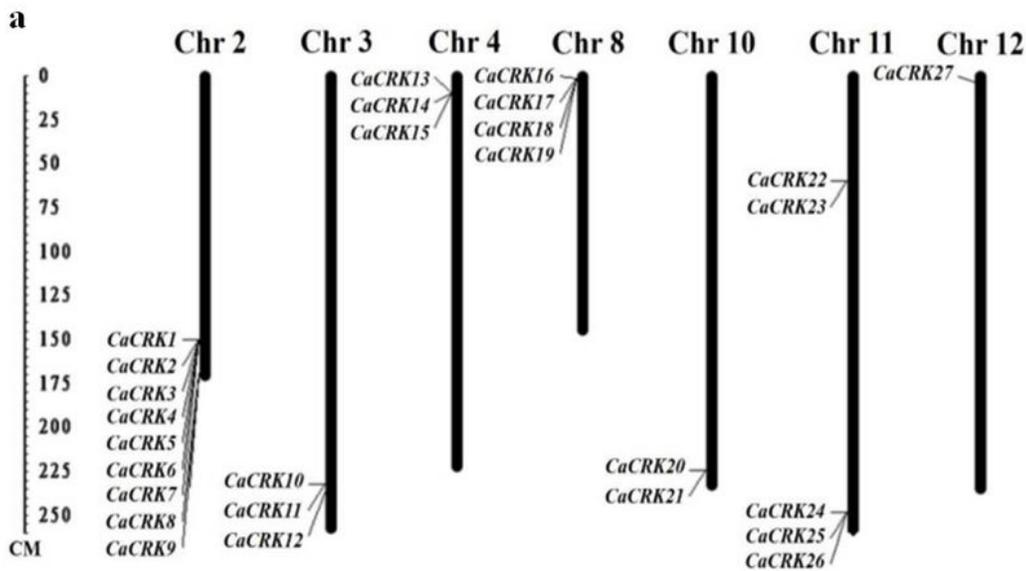


Figure 2

Chromosomal location and phylogenetic analysis of CRK gene family. a Physical genome distribution of CRK gene family in pepper. b Phylogenetic tree was generated based on the amino acid sequences of CRK genes in pepper using the neighbor-joining method in MEGA version 5.1.

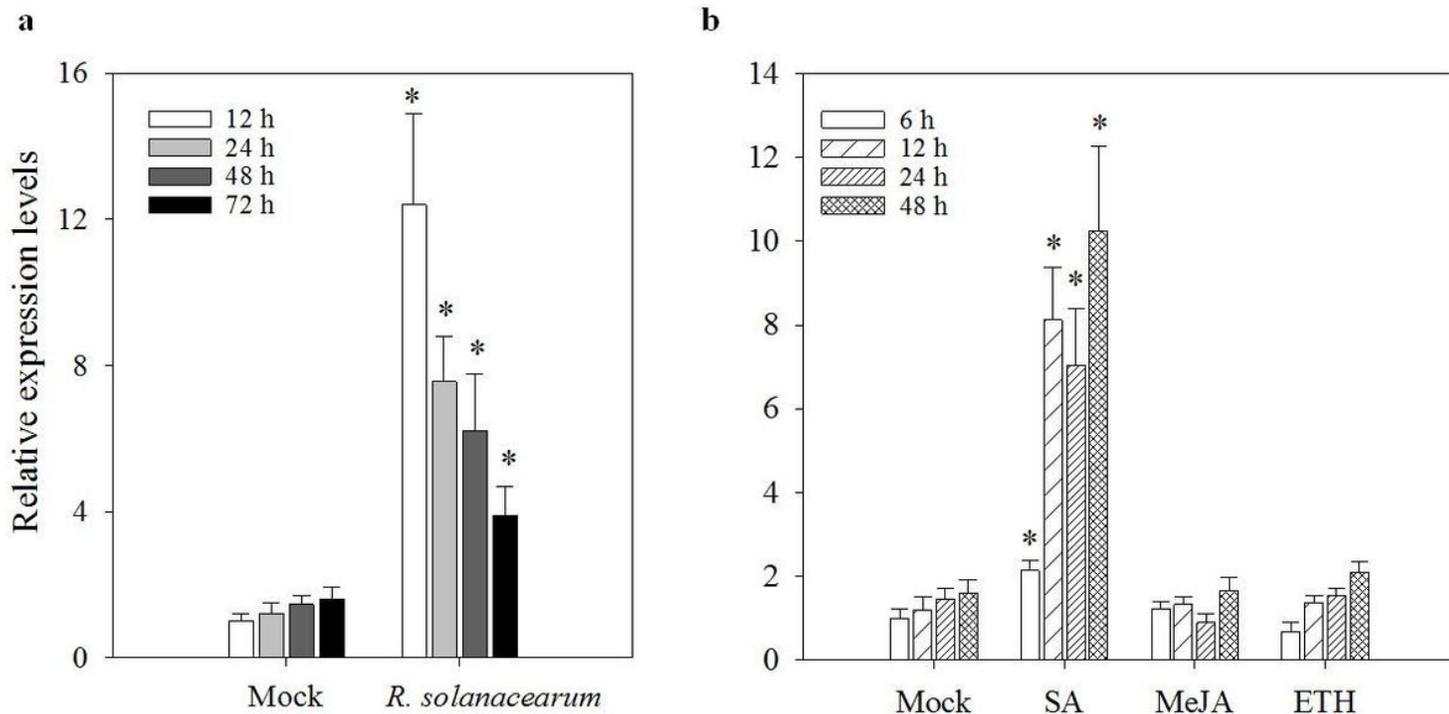


Figure 3

Expression patterns of CaCRK5 under different treatments. a Expression analysis of CaCRK5 in pepper after inoculation with *R. solanacearum* by qRT-PCR. b Expression analysis of CaCRK5 in pepper responding to SA, MeJA and ETH. Error bars represent the mean \pm SD of three independent replicates. Asterisks indicate significant differences as determined by Student's t-test ($P < 0.05$).

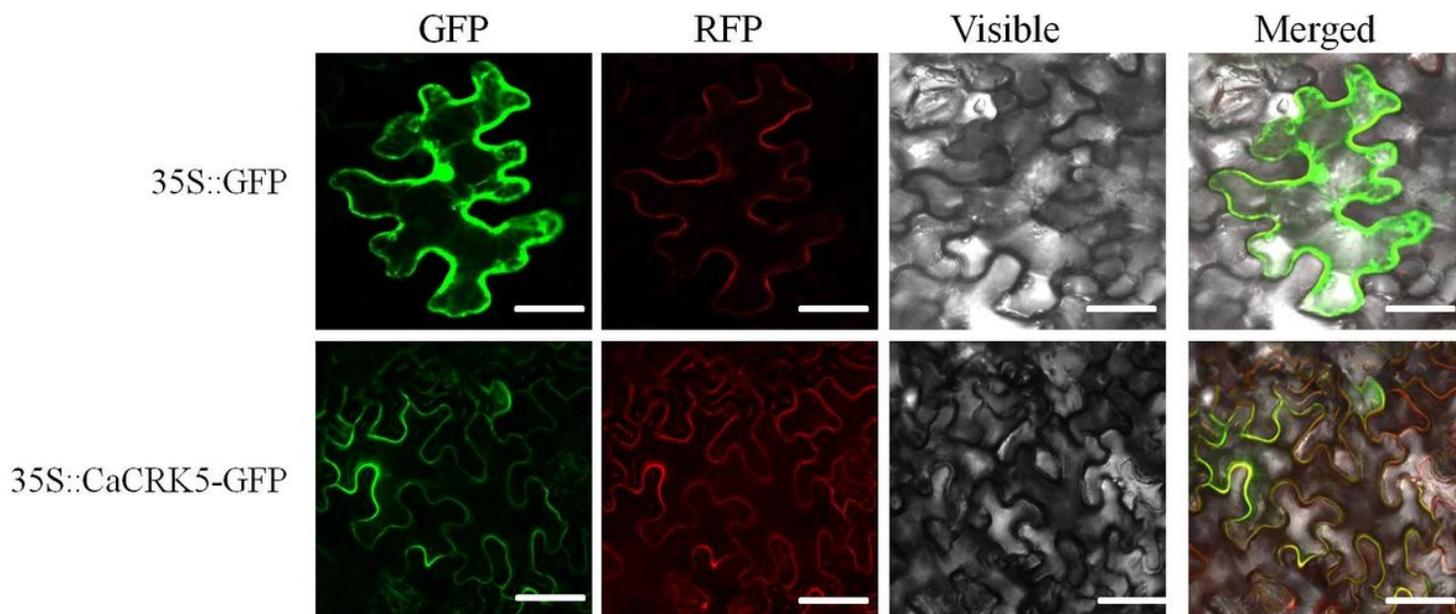


Figure 4

Subcellular localization of CaCRK5. Six-week-old tobacco leaves were infiltrated with *Agrobacterium* carrying 35S:GFP, 35S:CaCRK5-GFP and 35S:CBL1n-RFP. Samples were collected 48 h post inoculation.

Fluorescence was examined using a confocal microscope. GFP, green fluorescent protein; RFP, red fluorescent protein. Bars, 50µm.

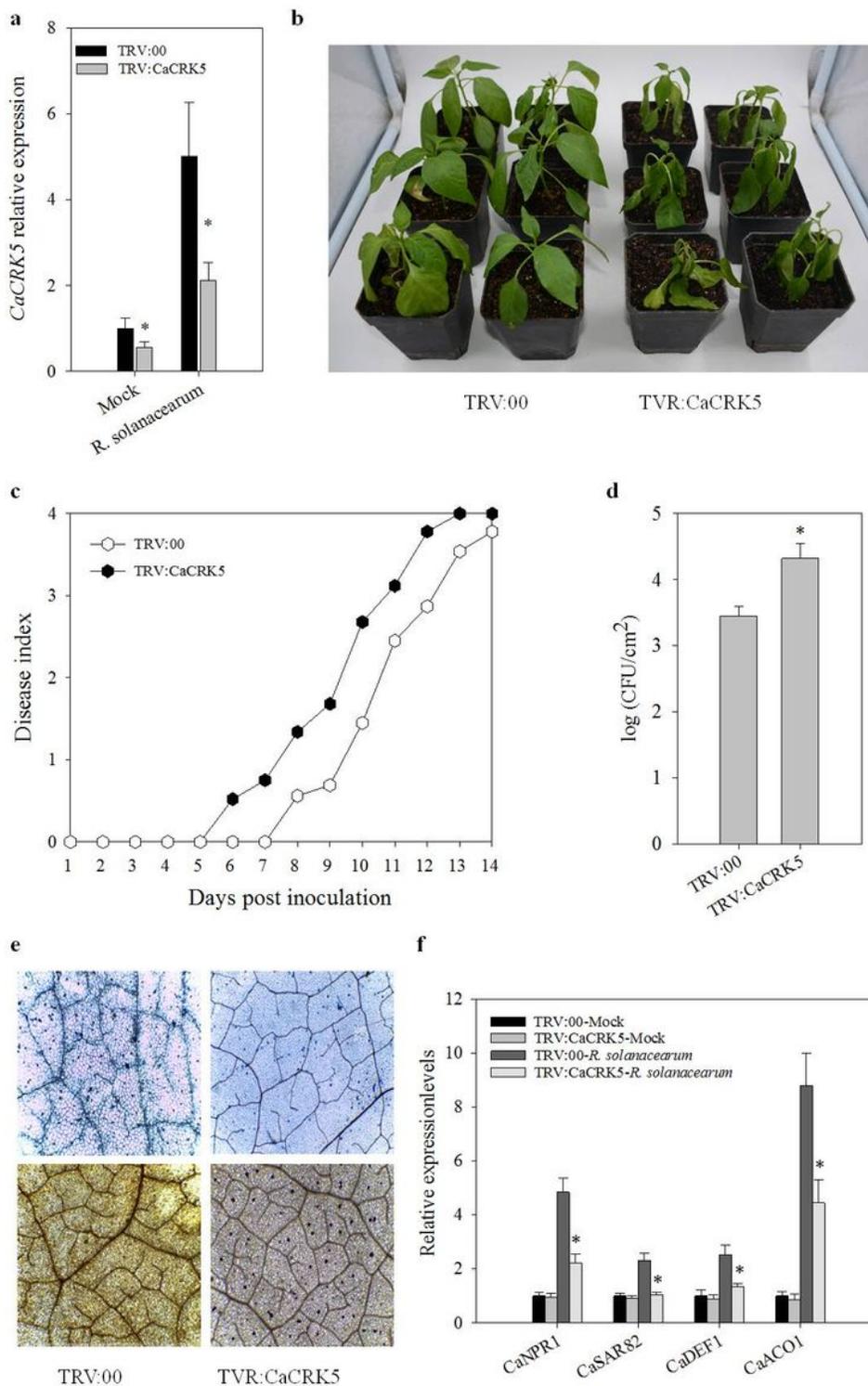


Figure 5

Silencing of CaCRK5 enhanced susceptibility to infection of *R. solanacearum* in pepper. a CaCRK5 was silenced by VIGS. The leaf samples from CaCRK5 VIGS pepper seedlings were collected to detect the expression of CaCRK5 by qRT-PCR. Error bars represent the mean \pm SD of three independent replicates.

The experiments were repeated three times with similar results. b Phenotypes of control and VIGS pepper plants. After the VIGS was established, 4 week old plants were inoculated with *R. solanacearum*. Photographs were taken at 10 d after *R. solanacearum* infection. c Rate of disease index on a scale of 0 to 4 was calculated in CaCRK5 silenced and control plants. d Growth of *R. solanacearum* in leaves of TRV:CaCRK5 and plants at 3 d post leaf inoculation of *R. solanacearum*. The data are shown as means \pm SD from three independent repeats (n=3, *, P<0.05, Student's t-test). e Visualization of diaminobenzidine (DAB) and trypan blue staining 48 h post leaf inoculation with *R. solanacearum*. f The expression levels of defense related genes in control (TRV:00) and CaCRK5-silenced (TRV:CaCRK5) plants. Asterisks indicate statistically significant differences (Student's t test,*P < 0.05).

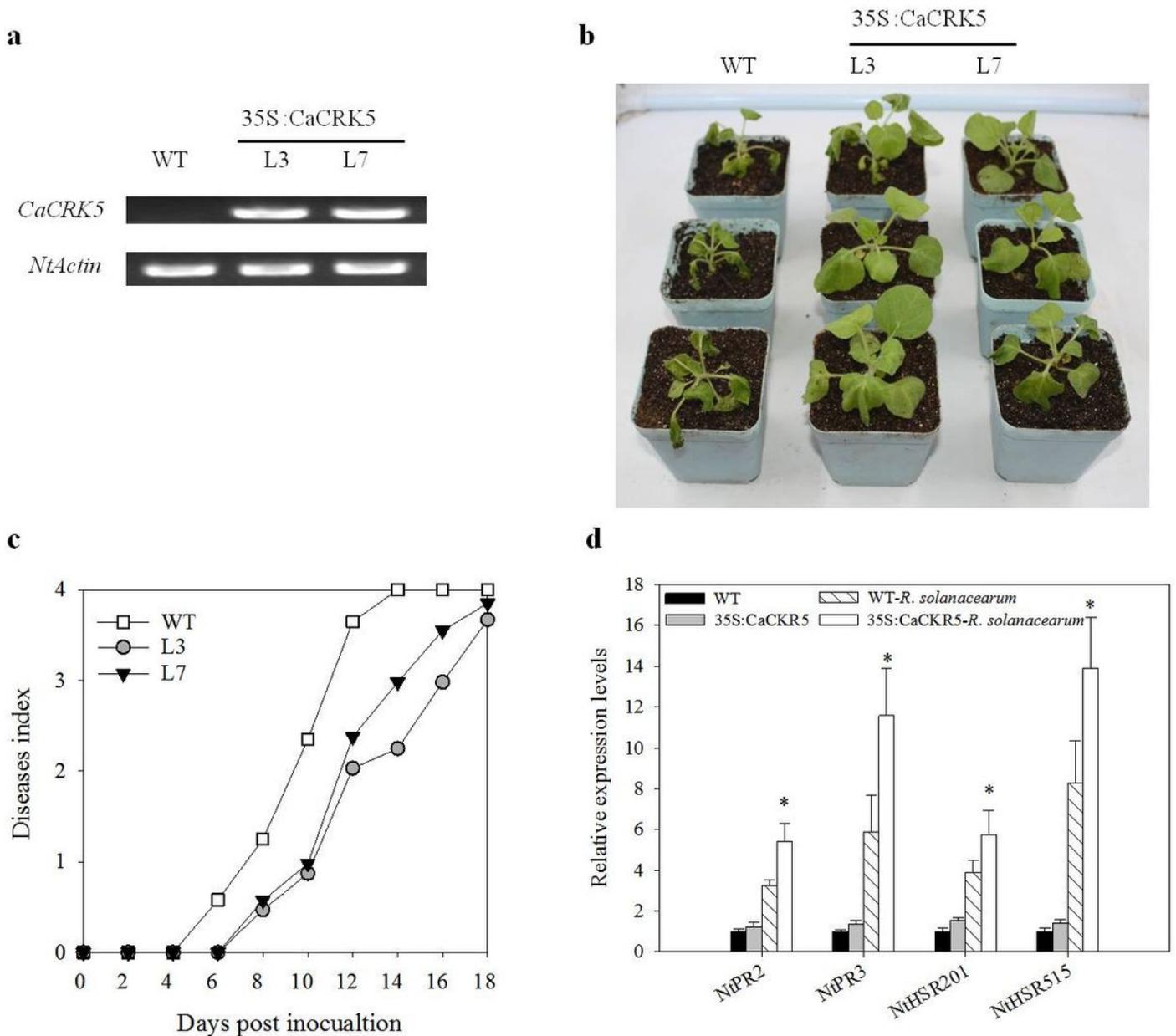


Figure 6

Effect of CaCRK5 overexpression in tobacco on bacterial wilt disease by inoculation with *R. solanacearum*. a Expression levels of CaCRK5 in WT (wild-type) and transgenic tobacco lines (L3 and L7). b Symptoms in wild-type and overexpressed plants. Photograph was taken 12 d after root-inoculation with *R. solanacearum*. c Disease development of bacterial wilt was rated daily on a 0–4 disease index in wild-type and overexpressed plants. Each data point represents the mean disease index from three independent experiments containing 30 plants in total for each treatment. d Expression of four defense related genes (NtPR2, NtPR3, NtHSR201 and NtHSR505) in the CaCRK5 overexpressing line (L3) and wild-type. Four-week-old wild-type and transgenic tobacco seedlings were leaf-inoculated with *R. solanacearum*. After 48 h, total RNAs were extracted for qRT-PCR assays. Data represent means \pm SD (n = 3). Asterisks indicate significant differences according to Student's t test, *P < 0.05.

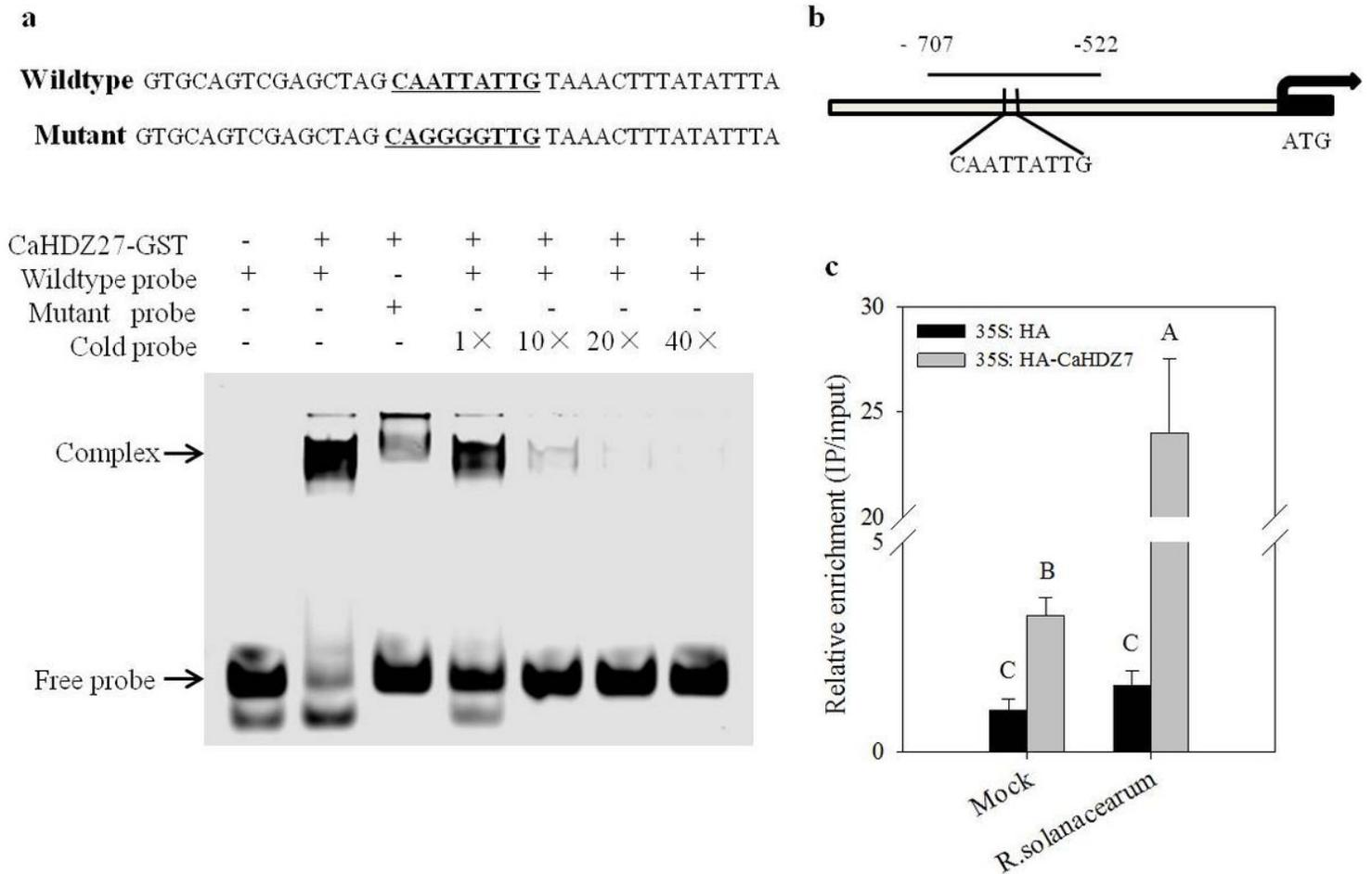


Figure 7

CaHDZ27 binds to the CAATTATTG motif in the promoter of CaCRK5. a EMSA was used to analyze the interaction of CaHDZ27-GST and a Cy5-labeled probe in vitro. The probe sequences containing the CAATTATTG motif in the CaCRK5 promoter are shown. CaHDZ27-GST purified protein was incubated with Cy5 fluorochrome labeled wild-type probe. Non labeled probes at 10- and 100- fold concentrations were added for the competition test. b Schematic representation of the CaCRK5 promoter used for ChIP-qPCR analysis. the blue lines indicate the sequence regions that were used for ChIP-qPCR. c, ChIP-qPCR

assays showed the binding of CaHDZ27 to CaCRK5 promoter in vitro. DNA samples were co-immunoprecipitated with anti-HA antibody. The ChIP value was normalized to its respective input DNA value. Different letters indicate significant differences, as determined by LSD test ($P < 0.01$).

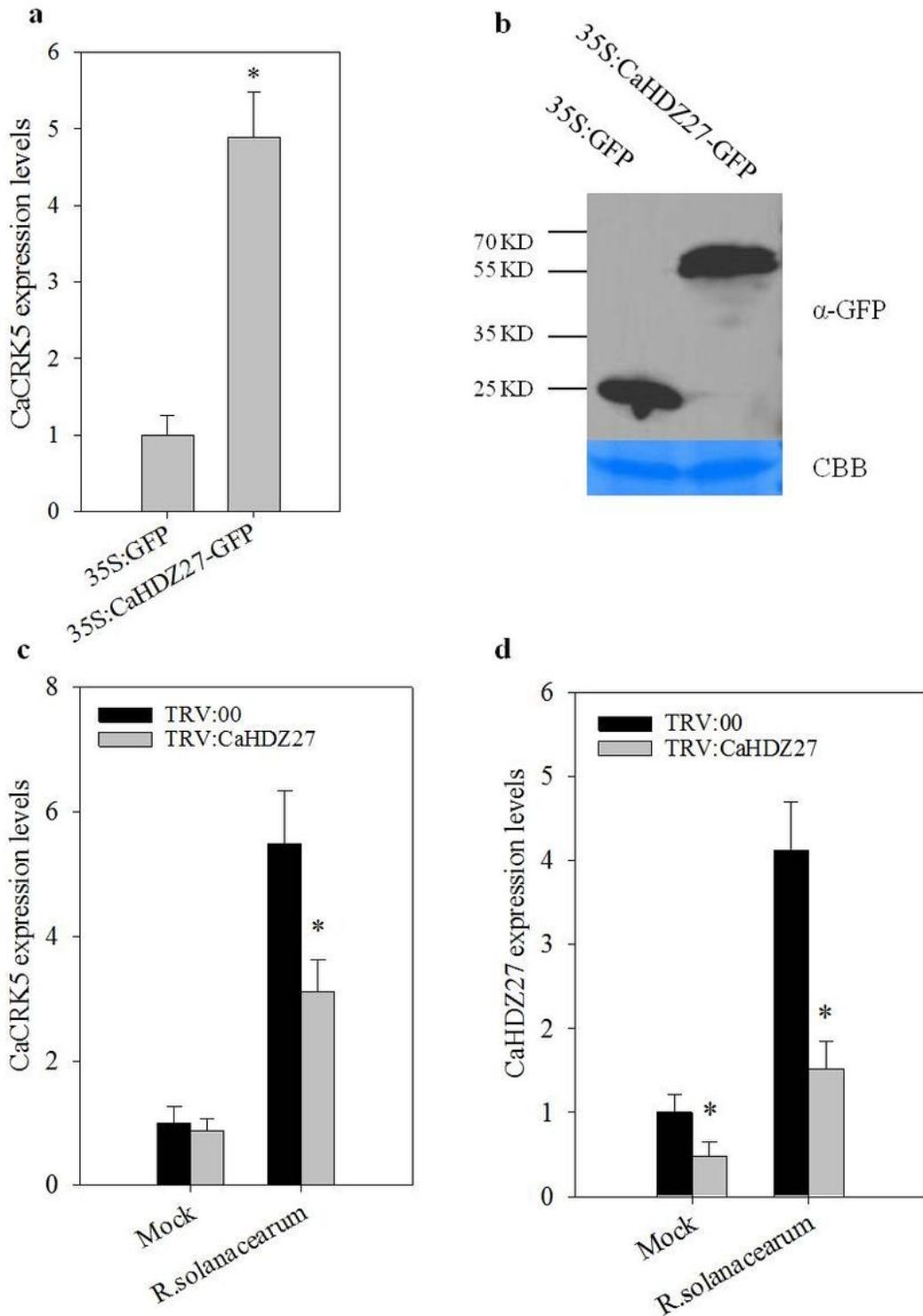


Figure 8

The effect of CaHDZ27 transient expression or silencing on expression of CaCRK5. a Relative transcript levels of CaCRK5 in transiently expressed CaHDZ27 pepper leaves. b The success of CaHDZ27 transient

expression in pepper leaves identified by western blotting 48 h post infiltration. c-d Relative transcript levels of CaCRK5 and CaHDZ27 in CaHDZ27 silenced plants by VIGS. Asterisks indicate significant differences at P values < 0.05.

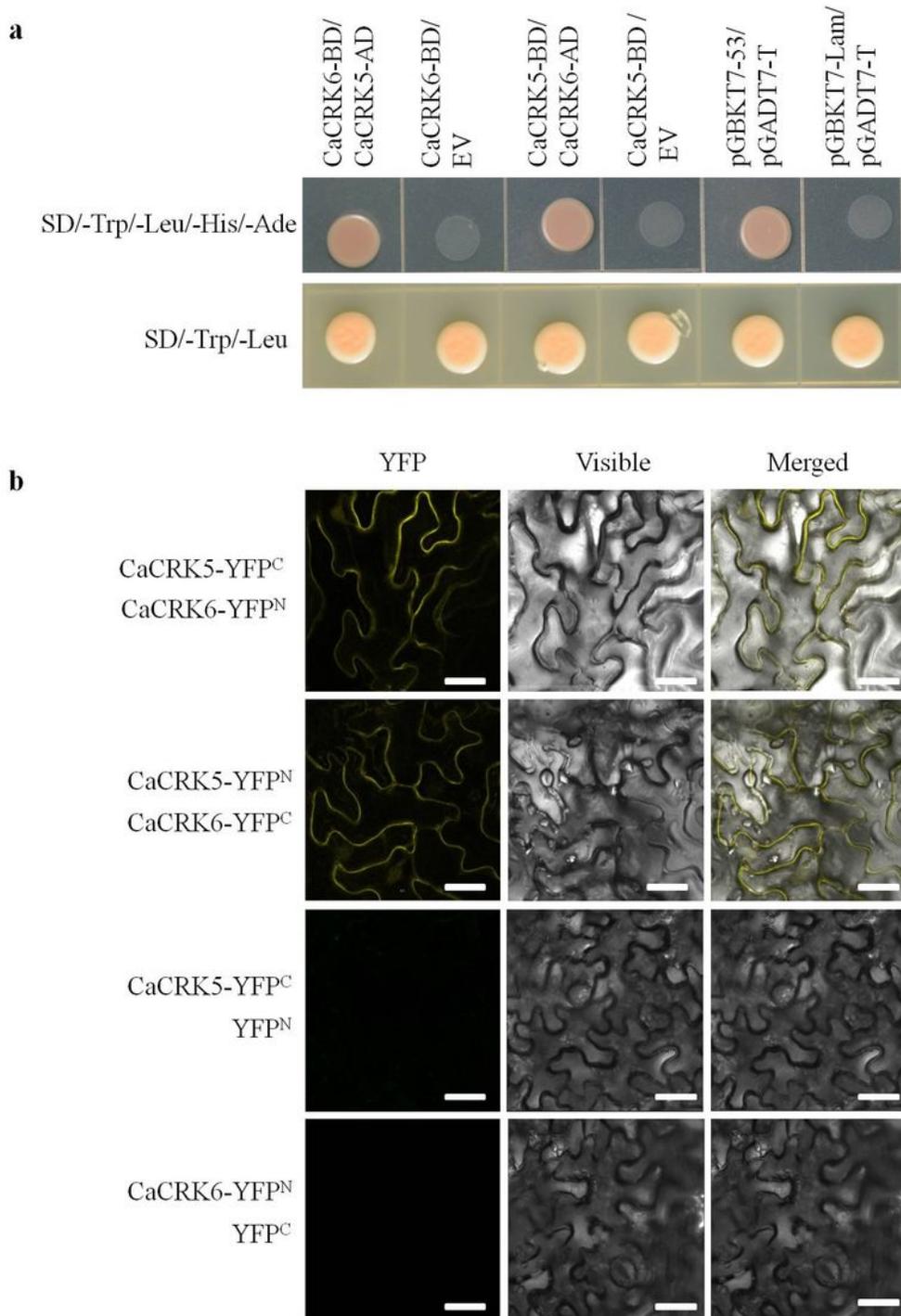


Figure 9

CaCRK5 interacts with CaCRK6. a Y2H assay. CaCRK5-BD and CaCRK6-AD were cotransformed into 'Y2H Gold' yeast cells, which were grown on SD/-Trp-Leu and SD/-Trp-Leu-His-Ade for 3 d. EV indicates the

empty vectors for pGBKT7. b Bimolecular fluorescence complementation (BiFC) assays. YFP fusion constructs were transiently expressed in *N.benthamiana* leaves, and BiFC signals were observed under a fluorescence microscope. Bars=50µm. The experiment was repeated independently three times with similar result.

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