CaLRR-RLK1, a novel RD receptor-like kinase from Capsicum annuum and transcriptionally activated by CaHDZ27, act as positive regulator in Ralstonia solanacearum resistance

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Abstract

Background: Bacterial wilt caused by Ralstonia solanacearum is one of the most important diseases in pepper worldwide, however, the molecular mechanism underlying pepper resistance to bacterial wilt remains poorly understood.

Results: Herein, a novel RD leucine-rich repeat receptor-like kinase, CaLRR-RLK1, was functionally characterized in immunity against R. solanacearum. CaLRR-RLK1 was targeted exclusively to plasma membrane and was up-regulated by R. solanacearum inoculation (RSI) as well as by the exogenous application of salicylic acid (SA), methyl jasmonate (MeJA) or ethephon (ETH). The silencing of CaLRR-RLK1 led to enhanced susceptibility of pepper plants to RSI, accompanied by down-regulation of immunity-related genes including CaACO1, CaHIR1, CaPR4 and CaPO2. In contrast, transient overexpression of CaLRR-RLK1 triggered hypersensitive response (HR)-like cell death and H2O2 accumulation in pepper leaves, manifested by darker trypan blue and DAB staining respectively. In addition, the ectopic overexpression of CaLRR-RLK1 in tobacco plants enhanced resistance to R. solanacearum, accompanied with the immunity associated marker genes including NtPR2, NtPR2, NtHSR203 and NtHSR515. Furthermore, it was found that CaHDZ27, a positive regulator in pepper response to RSI in our previous study, transcriptionally activated CaLRR-RLK1 by direct targeting its promoter probably in a CAATTATTG dependent manner.

Conclusion: The study revealed that CaLRR-RLK1 confers pepper resistance to R. solanacearum as the direct targeting of CaHDZ27.

Keywords: CaLRR-RLK1, CaHDZ27, Pepper, Ralstonia solanacearum, Plant immunity

Background

Bacterial wilt, caused by Ralstonia solanacearum [1], is a highly destructive vascular disease in pepper (Capsicum annuum), a solanaceous vegetable of great agricultural importance. It is believed that the most efficient way to cope with such kind of disease is to develop crop variety with high level of disease resistance, a better understanding of molecular basis of pepper resistance to R. solanacearum will benefit its genetic improvement.

Upon challenge by pathogens, plant employ two interconnected layers of immunity including pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) to protect them [2, 3]. Plant perceive exogenous PAMPs derived from microbial pathogens by cell surface-localized proteins, known as pattern recognition receptors (PRRs), which results in PTI. In addition, PRRs can also activate...
immune response by recognizing the endogenous damage-associated molecular patterns (DAMPs), which are produced as a consequence of pathogen infection and perception [4]. The adapted pathogen develop effectors, which are secreted and injected into host cells where they repress PTI by targeting specific signaling components, leading effectors triggered susceptibility (ETS) [5, 6]. PTI, also called basal defense, is required but not sufficient for disease resistance. Under the selection pressure of ETS, plant might evolve specific receptors, typically resistance (R) proteins, to detect the effectors and result in ETI, which is often accompanied with hypersensitive reaction (HR) and systematic acquired resistance (SAR). Although ETI is more robust, intensive and more directly associated with transcriptional regulation of defense gene expression than PTI, overlapping downstream molecular events have been found between PTI and ETI [7].

Leucine-rich repeat receptor-like protein kinases (LRR-RLKs) are typical plant cell surface-localized PRRs, they contain an extracellular domain with up to 30 leucine-rich repeat (LRRs) to perceive ligands including small molecules, peptides, and entire proteins [8, 9], a TMD (transmembrane domain) for its cell membrane targeting and an intracellular serine/threonine (Ser/Thr) kinase domain to phosphorylate specific substrates for the signal transduction. Approximately 223 LRR-RLKs in Arabidopsis [10, 11], 226 in Zea mays [12], 234 in tomato [13], 379 in populous [14], 303 in Brassica rapa [15], 188 in pepper [16] have been identified. Some LRR-RLKs have been implicated to regulate plant innate immunity [17–19]. For example, a well-studied PRR LAGELLIN SENSITIVE 2 (FLS2) recognizes and binds a 22-amino acid epitope of bacterial flagellin called flg22 [20, 21]. Upon perception of flg22, FLS2 interacts with another LRR-RLK, BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) [22, 23], and phosphorylates the NADPH oxidase RbohD by association with a receptor-like cytoplasmic kinase (RLCK), BOTRYTIS-INDUCED KINASE 1 (BIK1) [24, 25], leading to a calcium burst and reactive oxygen species (ROS) production, and thereby mounts PAMP-triggered immunity (PTI). By sensing and binding Ax21 derived from pathogen, XA21 confers broad-spectrum resistance to the bacterial (PTI). By sensing and binding Ax21 derived from pathogen, XA21 confers broad-spectrum resistance to the bacterial (PTI). By sensing and binding Ax21 derived from pathogen, XA21 confers broad-spectrum resistance to the bacterial (PTI). By sensing and binding Ax21 derived from pathogen, XA21 confers broad-spectrum resistance to the bacterial (PTI). By sensing and binding Ax21 derived from pathogen, XA21 confers broad-spectrum resistance to the bacterial (PTI). By sensing and binding Ax21 derived from pathogen, XA21 confers broad-spectrum resistance to the bacterial (PTI). By sensing and binding Ax21 derived from pathogen, XA21 confers broad-spectrum resistance to the bacterial (PTI). By sensing and binding Ax21 derived from pathogen, XA21 confers broad-spectrum resistance to the bacterial A key step in both PTI and ETI is the massive transcriptional reprogramming dictated by various transcription factors. Through integrating the signal initiation and along the signaling pathways transmitting them into appropriate transcription of many defense associated genes including PRRs, R proteins and other signaling components [31–33], transcription factors play important roles in the regulation of plant immunity [34–38]. The HD-Zip transcription factors, characterized by two conserved domains, the DNA-binding homeodomain (HD) and an adjacent leucine zipper (LZ) motif, are unique to plants [39]. HD-Zip proteins are classified into four subfamilies (I–IV) based on their conserved HD-Zip domain, gene structure and additional conserved motifs [40]. HD-Zip I members were reported to play essential roles in response to environmental conditions [41, 42]. A HD-Zip I protein in pepper, CaHDZ27, was previously found to function as a positive regulator in plant immunity toward R. solanacearum by forming homodimers [42]. However, how CaHDZ27 linked with the upstream signaling components remains to be elucidated.

In the present study, a pepper putative LRR-RLK gene CalLRR-RLK1 which is up-regulated by the inoculation of R. solanacearum was functionally characterized in immunity, and its transcriptional regulation by CaHDZ27 was identified. The results showed that CalLRR-RLK1 act as a positive regulator in pepper response to RSI, the promoter of CalLRR-RLK1 is targeted by CaHDZ27 in a pseudopalindromic DNA (CAATTATTG) dependent manner, and it is up-regulated by CaHDZ27 during pepper response to RSI.

Results

Isolation and sequence analysis of CalLRR-RLK1

A putative LRR-RLK gene in pepper was originally identified as up-regulation in pepper plants challenged by R. solanacearum in our previous gene differential expression display by cDNA-AFLP (cDNA-amplified fragment length polymorphism). For cDNA-AFLP analysis, 64 primer pairs were used for selective PCR amplification and total 400 transcript derived fragments (TDFs) obtained. Among them, 114 displayed altered expression patterns after inoculation of R. solanacearum, of which 35 up-regulated and 79 down-regulated. The corresponding gene sequence including the promoter, 5’UTR, 3’UTR and the CDS were identified using pepper genome sequence (http://peppersequence-genomics.cn/page/species/index.jsp). The gene product was designated CalLRR-RLK1 (GenBank accession number: XP_016560889) as it represents the first report of a LRR-RLK from pepper. The full-length complementary DNA (cDNA) sequence of CalLRR-RLK1 was cloned by PCR with a specific pair of primers. By sequence analysis,
the cDNA contains an open reading frame (ORF) of 2955 bps in length, its deduced amino acid sequence are 984 aa in length, containing a predicted extracellular region with a N-terminal signal peptide (SP, residues 1–25), a LRR_NT2 (a leucine-rich repeat N-terminal domain (residues 24–66), 21 extracellular leucine-rich repeats (LRRs, residues 96–596), a trans-membrane domain (TMD, residues 626–648) and a kinase domain (residues 684–946) (Fig. 1a).

The kinase domain shares high sequence homology with that of other RLK proteins (Fig. 1b). The conserved active site signature motif (VHRDVKSNNILLD residues) and the G-T/S-XX-Y/F-X-APE motif [43] indicate that CaLRR-RLK1 is a Ser/Thr kinase rather than a Tyr kinase. In addition, CaLRR-RLK1 appear to be a RD type protein kinase since it contains a conserved arginine-aspartic acid motif in its kinase subdomain [4].

Phylogenetic tree analysis was performed based on the amino acid sequence of CaLRR-RLK1 and its close homologs in other plant species. The result showed that CalRR-RLK1 falls into a clade with Solanum tuberosum LRR-RLK (XP_006357742), Solanum lycopersicum LRR-RLK (XP_004231961) and Solanum pennellii LRR-RLK (XP_015067035) (Additional file 1: Figure S1).

Expression of CalRR-RLK1 induced by R. solanacearum or exogenous hormones

To confirm the inducible expression of CalRR-RLK1 in pepper plants against R. solanacearum inoculation, we detected its transcript levels in pepper leaves by qRT-PCR analysis. The result showed that the transcript levels of CalRR-RLK1 were increased at 12 to 48 h post inoculation of R. solanacearum compared to the mock treatment (Fig. 2a).

SA, JA and ET are signaling molecules that participate in the regulation of plant immunity [44–47]. To confirm the result that CalRR-RLK1 was induced by RSI, the
transcript levels of *CaLRR-RLK1* in pepper plants treated with exogenous applications of SA, MeJA or ETH were measured by qRT-PCR. The result showed that the transcript levels of *CaLRR-RLK1* were up-regulated dramatically and peaked at 6 h post application (hpa) of exogenous application of MeJA (Fig. 2b), followed by a gradual decrease during 12–48 hpa. Upon application of ETH, the *CaLRR-RLK1* transcript abundance was induced as early as 3 hpa (Fig. 2c). Similarly, the maximum transcripts occurred at 3 hpa of SA and then decreased to basal levels at 12 hpa (Fig. 2d). These results imply that *CaLRR-RLK1* is involved in pepper defense against *R. solanacearum* regulated by a signaling mediated by SA, JA and ET.

**Subcellular localization of CaLRR-RLK1**

To study the subcellular localization of *CaLRR-RLK1*, we fused the full length ORF of *CaLRR-RLK1* without the termination codon to 5' terminal of the green fluorescent protein (GFP), resulting 35S:*CaLRR-RLK1*-GFP construct, using 35S:GFP and 35S:CBL1n-RFP (specifically targeting to the plasma membrane) [48] as controls. By agro-infiltration, *CaLRR-RLK1*-GFP and CBL1n-RFP or GFP and CBL1n-RFP were transiently co-overexpressed in *Nicotiana benthamiana* leaves. The results showed that green fluorescent signal of *CaLRR-RLK1*-GFP completely overlapped with the red fluorescent signal in the plasma membrane, while the control GFP was ubiquitously distributed throughout the cell (Fig. 3), indicating the localization of *CaLRR-RLK1* in the plasma membrane.

**CaLRR-RLK1 silencing in pepper results in enhanced susceptibility to R. solanacearum**

To further analyze the possible role of *CaLRR-RLK1* in pepper response to RSI, virus-induced gene silencing (VIGS) approach was used to investigate the effect of silencing of *CaLRR-RLK1* on pepper resistance to RSI. A fragment 182 bps in length within the 3'-UTR region of *CaLRR-RLK1* was cloned into pTRV2 vector resulting in TRV:*CaLRR-RLK1* (using TRV:00 as a control). The *Agrobacterium* GV3101 cells harboring TRV:*CaLRR-RLK1* were infiltrated onto the cotyledon of the 12–14 days old pepper plants, and the TRV:*CaPDS* pepper plants were used to monitor the gene silencing process. When the seedlings were grown for about 25 days, plants infiltrated with TRV:*CaPDS* exhibited bleaching phenotype in their newly emerging leaves, the TRV:00 and TRV:*CaLRR-RLK1* pepper plants were inoculated...
with *R. solanacearum*, and their transcript levels of *CaLRR-RLK1* were measured by qRT-PCR using randomly selected 6 TRV:*CaLRR-RLK1* pepper plants to detect the silencing efficiency of *CaLRR-RLK1*. The result showed that transcript levels of TRV:*CaLRR-RLK1* in *R. solanacearum* inoculated or mock treated plants were about 20–30% of that in the control (TRV:00) plants (Fig. 4a), indicating the success of *CaLRR-RLK1* silencing in pepper plants by VIGS.

With the *CaLRR-RLK1* silencing pepper plants, which were confirmed by qRT-PCR individually, the effect of *CaLRR-RLK1* silencing on pepper immunity against RSI was assayed. All of *CaLRR-RLK1* silenced pepper plants did not exhibit any morphological changes compared to
the control plants. Inoculated with *R. solanacearum* by root irrigation, *TRV*:CaLRR-RLK1 plants exhibited significantly enhanced susceptibility to RSI compared to the control plants (Fig. 4b). After 6 days post inoculation (dpi), *TRV*:CaLRR-RLK1 plants displayed a higher disease index than the control. *TRV*:CaLRR-RLK1 plants began to display the bacterial wilt symptom at 6 dpi and complete wilt phenotype at 12 dpi, while the control plants did not begin to display the bacterial wilt symptom until 7 dpi and complete wilt until 14 dpi (Fig. 4c). Consistently, a higher population density of *R. solanacearum* was detected in stems of *TRV*:CaLRR-RLK1 plants at 9 dpi by root irrigation (Fig. 4d) and also in *R. solanacearum* inoculated leaves at 3 dpi (Fig. 4e), compared to that in the control plants.

To further investigate whether silencing of CaLRR-RLK1 affect the expression of immunity-related genes in pepper, qRT–PCR analysis was performed. The results showed that expressions of HR related CaHIR1 [49], ROS detoxification-associated CaPO2 [50], ET-responsive ACC oxidase gene CaACO1 [51], JA/ET signaling associated CaPR4 [52] were significantly down-regulated in CaLRR-RLK1 silenced leaves, compared with that in the control plants challenged with RSI. However, there was no significant difference for the transcript levels of SA signaling associated *CaPR1* [53] and *CaNPR1* [54] (Fig. 4f). These results indicate that silencing of CaLRR-RLK1 increased susceptibility of pepper plants to RSI.

Transit expression of CaLRR-RLK1 triggered HR cell death and induced the transcription of immunity associated genes

To confirm the results in the gene silencing by VIGS, CaLRR-RLK1 was transiently overexpressed via agro-infiltration. GV3101 cells containing 35S:HA-CaLRR-RLK1 or 35S:HA were infiltrated into pepper leaves, the success of the transient overexpression was confirmed by qRT-PCR (Fig. 5a) or immunoblot with antibodies of HA (Additional file 2: Figure S2). Darker staining of trypan blue and DAB were detected in CaLRR-RLK1 transiently overexpressing pepper leaves around the infiltration sites, while no such darker staining was detected in the control plant leaves (35S:HA), indicating that HR cell death and H2O2 accumulation were triggered by transient overexpression of CaLRR-RLK1 (Fig. 5b). Consistently, a significant enhanced electrolyte leakage was also detected in CaLRR-RLK1 transiently overexpressing pepper leaves at 48 and 72 h post infiltration (hpi) compared with that of the control (Fig. 5c).

Quantitative RT-PCR analysis was used to investigate whether transient expression of CaLRR-RLK1 affect defense associated genes including CaACO1, CaHIR1, CaPR4, CaPO2, CaNPR1 and CaPR1 in pepper leaves. The results showed that CaACO1, CaHIR1, CaPR4 and CaPO2 exhibited enhanced transcript levels in CaLRR-RLK1 transiently overexpressing pepper leaves at 48 hpi compared to that in the control, transcript levels of *CaNPR1* and *CaPR1* did not alter (Fig. 5d).

Ectopic overexpression of CaLRR-RLK1 enhanced resistance of tobacco plants to RSI

To further confirm that result that CaLRR-RLK1 act as positive regulator in plant immunity, we investigated the effect of its ectopic overexpression on the resistance of tobacco plants to RSI by developing the transgenic tobacco lines via *Agrobacterium* mediated method. Six transgenic lines (T3) each with at least 10 seedlings were confirmed by kanamycin resistance analysis. Two lines were employed to assay the transcriptional expression of CaLRR-RLK1, the seedlings were inoculated with *R. solanacearum* cells. RT-PCR result showed that CaLRR-RLK1 constitutively expressed in leaves of the two lines (#2 and #8) (Additional file 3: Figure S3).

Eight-week-old tobacco plants were inoculated with *R. solanacearum* by root irrigation, the development of bacterial wilt symptoms was monitored. At 7 dpi, wilting and contagion symptoms occurred on the stems of the wild-type plants, while no significant symptom was observed in plants of CaLRR-RLK1 overexpressing lines (Fig. 6a). At 21 dpi, wild-type plants exhibited extremely severe wilting symptoms, but only slight wilt symptoms were observed in plants of CaLRR-RLK1 overexpressing lines (Fig. 6b). At 28 dpi, a significant higher death rate (76.1%) was found in the wild-type plants than that in plants of the two lines, which was 36.5 and 51.1%, respectively (Fig. 6c).

In addition, the transcript levels of immunity associated genes, including SA-induced pathogenesis-related NtPR2 [55] and NtPR3 [56], HR-related NtHSR201 and NtHSR515 [57], were measured by qRT-PCR in tobacco plants of CaLRR-RLK1 overexpressing lines and the wild type, the results showed that the transcript levels of NtPR2, NtPR3, NtHSR201 and NtHSR515 increased in transgenic plants compared to that in wild-type plants in response to *R. solanacearum* infection (Fig. 6d).

The promoter of CaLRR-RLK1 was directly bound and transcriptionally modulated by CaHD227

Silencing of *CaLRR-RLK1* impairs resistance of pepper to RSI, while its overexpression enhances the resistance of pepper or tobacco to RSI. Therefore, its inducible expression under challenge by RSI is crucial for its role as positive regulator. To study the molecular mechanism underlying this inducible expression, the cis-elements in the promoter region of *CaLRR-RLK1* of 1574 bps in length was analyzed (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Noticeably, a 9 bp pseudopalindromic DNA sequence (CAATTATTG), which is potentially bound by HD-Zip transcription factors [42, 58], was found to be present in the promoter (Fig. 7a). Since
our previous study found that CaHDZ27, a HD-Zip I transcription factor, acts a positive regulator in pepper response to RSI, we speculated that CaHDZ27 might act as an upstream regulator of CaLRR-RLK1 by binding the motif CAATTATTG in its promoter. To test if it is the case, we employed chromatin immunoprecipitation (ChIP) assay via HA-CaHDZ27 transient overexpression in pepper leaves, the chromatin was isolated from HA-CaHDZ27 transiently overexpressing pepper leaves after crosslinking with 1% formaldehyde, sheared into fragments of 300-500 bp in length, the DNA fragments bound to CaHDZ27 were immunoprecipitated by antibodies of HA, the de-crosslinked and purified DNA fragments were used as template for qRT-PCR with specific primer pairs based on the sequence flanking the motif (CAATTATTG) in the promoter of CaLRR-RLK1, the result showed that the promoter of CaLRR-RLK1 was significantly enriched by CaHDZ27, and in response to the challenge of R. solanacearum, CaHDZ27 binding enrichment was markedly increased (Fig. 7b), indicating that CaHDZ27 binds to the promoter of CaLRR-RLK1 probably via the motif CAATTATTG.

To assay if CaLRR-RLK1 is transcriptionally modulated by CaHDZ27, we carried out transient overexpression of HA-CaHDZ27 in pepper leaves by infiltration with GV3101 cells containing 35S:HA-CaHDZ27 or 35S:HA, 2 days after agro-infiltration, the transcript levels of CaLRR-RLK1 were checked by qRT-PCR. The result showed that CaLRR-RLK1 was up-regulated in leaves infiltrated with 35S:HA-CaHDZ27, and not down-regulated in leaves infiltrated with 35S:HA-CaHDZ27-SRDX (a repressor version of HA-CaHDZ27), compared with 35S:HA (Fig. 7c). Moreover, we silenced CaHDZ27 in pepper plants by VIGS and checked the transcript level of CaLRR-RLK1 significantly enriched by CaHDZ27, and in response to the challenge of R. solanacearum, CaHDZ27 binding enrichment was markedly increased (Fig. 7b), indicating that CaHDZ27 binds to the promoter of CaLRR-RLK1 probably via the motif CAATTATTG.

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in CaHDZ27-silenced plants by qRT-PCR, the result showed that the transcript level of CaLRR-RLK1 decreased in TRV:CaHDZ27 pepper leaves upon *R. solanacearum* inoculation compared to that in the control (TRV:00) plants (Fig. 7d). To provide additional evidence that the transcript of CaLRR-RLK1 was activated by CaHDZ27, the promoter of CaLRR-RLK1 was cloned and inserted into pMDC163 [59] to get construct pCaLRR-RLK1:GUS. The GV3101 cells containing pCaLRR-RLK1:GUS were mixed with 35S:HA-CaHDZ27 and were co-infiltrated into the leaves of *N. benthamiana*. 48 or 72 h after infiltration, the GUS activity was measured. The result showed that GUS activity was much higher in the leaves co-expressing pCaLRR-RLK1:GUS and 35S:HA-CaHDZ27 co-expressed pepper leaves (Fig. 7f). Taken together, these data support the idea that CaHDZ27 activates the transcription of CaLRR-RLK1 by direct binding to the CAATTATTG motif.

**Discussion**

CaLRR-RLK1 plays a positive role in pepper immunity

Bacterial wilt incited by *R. solanacearum* causes heavy losses in pepper, a vegetable of great agricultural importance
worldwide. In the present study, our data indicate that CaLRR-RLK1 act as a positive regulator in pepper immunity toward R. solanacearum and it is transcriptionally modulated directly by CaHDZ27.

Based on sequence motifs in the extracellular domain, typical RLKs can be divided into more than 21 subfamilies [11]. So far, three RLKs including CaRLK1 [60–62], CaPIK11 [63–66] and CaLecRK-S.5 [67] in pepper have been functionally characterized. Like CaLRR-RLK1 in the present study, CaRLK1 contains cytoplasmic kinase domain, a transmembrane span, and an extracellular domain which shows low homology to other known plant RLKs. It functions in cell death [62], hypoxia resistance [61] and cell division [60]. CaPIK1 functions as a positive regulator of defense responses and cell death in an SA-dependent manner [65, 66]. L-type lectin receptor kinase CaLecRK-S.5 positively mediated broad-spectrum resistance in pepper [67]. However, no LRR-RLK in pepper has been functionally characterized in plant immunity to date.

CaLRR-RLK1 contains a predicted extracellular region with an N-terminal signal peptide, 21 extracellular leucine-rich repeats, a transmembrane domain and an intracellular kinase domain. CaLRR-RLK1 appears to be RD Ser/Thr kinase since it contains a conserved arginine-aspartic acid motif in its kinase subdomain. Consistent with the trans-membrane domain, CaLRR-RLK1 exclusively localizes to the plasma membrane. The direct evidence that CaLRR-RLK1 act as a positive regulator came from data that CaLRR-RLK1 silencing by VIGS significantly impaired resistance of pepper plant to RSI and down-regulated the tested immunity associated maker genes. By contrast, the transient overexpression of CaLRR-RLK1 significantly enhanced the HR cell death which is frequently found to occur in ETI and occasionally in PTI [68], the accumulation of H2O2 which is manifested by DAB staining and closely related to HR cell death [69], and the induction of the tested immunity associated marker genes. These data from pepper plants indicate a role of CaLRR-RLK1 as a positive regulator in pepper immunity against RSI, and it

Fig. 7 CaHDZ27 can bind the motif CAATTATTG in the promoter of CalRR-RLK1 and activate CalRR-RLK1 transcription. a Scheme of the CalRR-RLK1 promoter region, primers used in ChIP-qPCR assay were marked by arrows. b ChIP-qPCR assays were performed using the specific primers corresponding to the region containing the motif (CAATTATTG). c The transcript levels of CalRR-RLK1 in HA-CaHDZ27-SRDX or HA-CaHDZ27 transiently overexpressed pepper leaves. d The transcript levels of CalRR-RLK1 in CaHDZ27-silenced leaves challenged by R. solanacearum. e Activities driven by the promoter of CalRR-RLK1 were assayed when transiently overexpressing HA-CaHDZ27-SRDX or HA-CaHDZ27 in pepper leaves. f Activities of GUS driven by the pCalRR-RLK1 or pCalRR-RLK1mut in which CAATTATTG was replaced by CAGGGTTG, when co-transiently overexpressed with HA-CaHDZ27 in pepper leaves. In b, c and e, the values were the mean ± standard error (n = 3), different letters indicate significant differences, as determined by LSD test (P < 0.05). In d and f, asterisks indicate significant difference according to Student’s t test at P < 0.05.
was further supported by the data from ectopic expression of CaLRR-RLK1 in tobacco plants. The overexpression of CaLRR-RLK1 significantly enhanced the resistance of tobacco plants to RSI, accompanied with significant up-regulations of the tested immunity associated marker genes including NtPR2 [55], NtPR3 [56], NtHSR201 and NtHSR15 [57]. RLKs were categorized into RD kinases and non-RD kinases according to the RD motif in their kinase domains [70]. By far, well-studied LRR-RLK PRRs, such as FLS2 or Xa21 [71, 72], that recognize the exogenous PAMPs in disease resistance were non-RD kinases [70]. Characterized plant DAMP receptors all contained RD kinase domains [4]. For example, PEPR1/2 and RLK7 have been implicated in activation of PTI against pathogens through perception of peptides AtPEP1 [73, 74] and PIP1 [75] in Arabidopsis, respectively. As a RD kinase domain containing RLK, we speculate that CaLRR-RLK1 might act as a receptor of unidentified DAMPs in pepper defense response to R. solanacearum.

The expression of CaLRR-RLK1 was activated by CaHDZ27 direct binding the CAATTATTG motif in its promoter

Our data indicate an inducible expression of CaLRR-RLK1 upon challenge of R. solanacearum, this enhanced expression might be crucial for profound amplification of the defense signaling to avoid the negative effect of the constitutive immune reaction. Some PRRs such as FLS2 [76] and R genes such as Xa1 [77] were reported to exhibit inducible expression upon pathogen attack. To dissect the mechanism underlying up-regulation of CaLRR-RLK1 upon RSI, its upstream transcription factor is identified. Our data showed that CaHDZ27, a HD-Zip protein that acts as positive regulator in pepper resistance against R. solanacearum [42], directly targets the promoter of CaLRR-RLK1, and activate the expression of CaLRR-RLK1 in a motif CAATAATTG dependent manner. Similar to CaHDZ27 [42], the transcription of CaLRR-RLK1 was also up-regulated by exogenously applied SA, MeJA and ETH, indicating that CaHDZ27 and CaLRR-RLK1 act as a positive regulators in plant immunity during pepper response to RSI. CaLRR-RLK1 contains 21 LRRs in the extracellular domain. Although the ligand of LRRs is not known, CaLRR-RLK1 may play a role in the pepper defense signaling pathway through these LRR domains binding with ligand, and the enhanced expression caused by the transcript factor CaHDZ27 is beneficial to pepper resistance against R. solanacearum.

Conclusions

Our data in the present study indicate that expression of CaLRR-RLK1 was induced by R. solanacearum inoculation or exogenous application of SA, MeJA or ETH. Inhibition of its expression in pepper disrupted the defense ability of plants against R. solanacearum infection. Transit expression of this gene in pepper leaves can lead to cell death, and overexpression of this gene rendered the transgenic tobacco plants with increased disease tolerance. Furthermore, the expression of CaLRR-RLK1 is activated by CaHDZ27 in a pseudopalindromic DNA (CAATTATTG) dependent manner. The experimental evidence collectively demonstrates that CaLRR-RLK1 act as a positive regulator in pepper response to R. solanacearum, and it is transcriptionally activated by CaHDZ27.

Methods

Plant materials and conditions of growth

Pepper (Capsicum annuum cultivar L. cv. Fj8), with medium-resistance to R. solanacearum, was obtained from the pepper breeding group at Fujian Agriculture and Forestry University. Tobacco (Nicotiana tabacum L. cv. Honghuadajinyuan) was kindly supplied by the tobacco breeding group in Fujian Agriculture and Forestry University. Plants were grown under the conditions of 26°C temperature, 60% relative humidity, 60–70 μmol photons m⁻² s⁻¹ and 16 h light/8 h dark.

Pathogen inoculation

Virulent strains FJC100301 and FJ1003 strain of R. solanacearum were from pepper and tobacco, respectively. The pathogen strains were amplified according to the method described previously [78]. The R. solanacearum strain was grown in PSA medium (200 g/L of potato, 20 g/L of sucrose, 3 g/L of beef extract, 5 g/L of tryptone). Bacteria were harvested with sterile ddH₂O and re-suspended to 1.0 × 10⁸ CFU/mL. Seedlings were root inoculated by pouring either 30 mL bacterial inoculum or sterile ddH₂O as the mock into each pot. Before inoculation, the roots were damaged by making holes in the soil of each pot. For leaf inoculation, the third leaf from the top of each pepper plant at the eight-leaf stage were infiltrated with 10 μL of the R. solanacearum suspension using a syringe without a needle, and the mock treatment was performed with sterile ddH₂O.

Exogenous hormones treatments

Six leaf-stage pepper plants were used to investigate gene expression. For salicylic acid (SA), methyl jasmonate (MeJA) and ethephon (ETH) treatments, 1 mM SA, 100 μM MeJA or 100 μM ETH were sprayed onto pepper leaves. In parallel experiments, corresponding solvent or sterile water was sprayed as a control. Pepper leaves were sampled at various time points, frozen and stored at −80°C for RNA isolation.

Subcellular localization

The open reading frame (ORF) of CaLRR-RLK1 without the stop codon was PCR amplified from plant cDNA
and cloned into pMDC83 to yield 35S:CaLRR-RLK1-GFP. Agrobacterium tumefaciens strain GV3101 containing the constructs 35S:CaLRR-RLK1-GFP or 35S:GFP (used as a control) was re-suspended (OD600 = 0.8) in the induction medium (10 mM MES, 10 mM MgCl2, 200 μM acetosyrингone, pH 5.6) and infiltrated into leaves of Nicotiana benthamiana. After 2 days post infiltration, GFP fluorescence was imaged using a Laser Scanning Confocal Microscope (TCS SP8, Leica, Solms, Germany) with an excitation wavelength of 488 nm and a 505–530 nm band-pass emission filter.

**VIGS in pepper plants**

TRV (tobacco rattle virus)-based VIGS analysis was performed as described previously [42]. A 182-bp gene specific 3’ untranslated region of CaLRR-RLK1 was inserted into the TRV2 vector to yield pTRV2:CaLRR-RLK1 by gateway cloning technique. TRV1 and TRV:00 (control) or pTRV2:CaLRR-RLK1 in A. tumefaciens strain GV3101 were co-infiltrated into the fully expanded cotyledons of approximately 12–14 days old pepper plants. Then, the seedlings were incubated at 16°C for 56 h, and grown at 26°C.

**Agrobacterium-mediated transient expression in pepper leaves**

The full length cDNA of CaLRR-RLK1 or CaHDZ27 was cloned into pEarleyGate201 to yield 35S:HA-CaLRR-RLK1 construct or 35S:HA-CaHDZ27 by gateway cloning technique. The CaHDZ27 dominant repression construct (35S:HA-CaHDZ27-SRDX) was created by fusing the CaHDZ27 cDNA in frame with the dominant EAR repression sequence [79], which was ligated downstream of the 35S promoter into the vector pEarleyGate201.

*A. tumefaciens* strain GV3101 harboring the above vector was infiltrated into the pepper leaves at the eight-leaf stage using a syringe without a needle. After 48 or 72 h post infiltration, injected leaves were harvested for further use.

**Histochemical staining and ion conductivity measurement**

Diaminobenzidine (DAB) and trypan blue staining, as well as ion conductivity measurement assays were performed according to methods described previously [51, 78]. Bacteria-infiltrated leaves were harvested and incubated in 1 mg mL⁻¹ DAB in the dark overnight and destained with lactic acid/glycerol/ethanol (1:1:3). To visualize the cell death, the infiltrated leaves were stained with trypan blue and destained with chloral hydrate solution. Cell death was quantified by ion conductivity measurement, eight discs (11 mm in diameter) were excised and washed in sterile ddH2O. After incubation in 20 mL of double distilled water with gentle shaking for 3 h, ion conductivity was measured using a Mettler Toledo 326 (Mettler, Zurich, Switzerland).

**Quantitative real-time RT-PCR**

For quantitative real-time PCR analysis, total RNA from plant leaves was extracted using a TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized using One Step PrimeScript TM cDNA Synthesis Kit (TaKaRa, Shigo, Japan). Real-time PCR experiments were performed according to the manufacturer’s instructions for the BIO-RAD Real-time PCR system (Foster City, CA, USA) and the SYBR Premix Ex Taq II system (TaKaRa). To normalize the transcript levels, Pepper CaActin (GQ339766) or 18S ribosomal RNA (EF564281) and Tobacco NtEF1a (D63396) or NtActin (U60489) expression was monitored as reference genes in each reaction. The gene-specific primers used for the quantitative real-time RT–PCR analysis are listed in Additional file 4: Table S1 and Table S2. Each gene expression was calculated from three experimental replicates to ensure reproducibility of the results.

**ChIP-qPCR analysis**

ChIP-qPCR assays were performed essentially as described previously [51]. Briefly, the chromatin was isolated from the pepper leaves infiltrated with 35S:HA-CaHDZ27. The isolated chromatin was cross-linked with 1% formaldehyde, sheared, and precipitated using anti-HA. Enrichment of DNA samples was analyzed by quantitative real-time PCR. Each ChIP value was normalized to its respective input DNA value.

**β-Glucuronidase (GUS) enzymatic assay**

The CaLRR-RLK1 promoter or its mutation was cloned into pMDC163 to yield pCalLRR-RLK1-GUS or pCalLRR-RLK1mut-GUS construct. The frozen plant material collected from leaves of transiently transformed pepper, was ground with a pestle in the extraction buffer (50 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, and 10 mM β-mercaptoethanol) and centrifuged at 11000 g for 10 min at 4 °C. Soluble crude protein in the supernatant was quantified using the Bradford method [80]. Quantitative analysis of GUS activity was measured with 4-methylumbelliferyl-D-glucuronide (4-MUG) as the substrate [54].

**Plant transformation**

The full length cDNA of CaLRR-RLK1 was cloned into plant binary vector pK7WG2 to yield 35S:CaLRR-RLK1 construct. Agrobacterium-mediated transformation of tobacco (Nicotiana tabacum L. cv. Honghudajinyuan) was performed. Transformants were selected on Mura-shige and Skoog (MS) agar plates containing 50 μg mL⁻¹ kanamycin. Successful transformation was confirmed by RT-PCR using the CaLRR-RLK1 gene-specific primers. Among the selected transgenic plants, T2 lines #2 and #8 were used in this study.
Additional files

**Additional file 1: Figure S1.** The phylogenetic relationship of CaLRR-RLK1 protein with other plant LRR-RLKs. An unrooted neighbor-joining tree was built by MEGA 4.0. (TIF 30 kb)

**Additional file 2: Figure S2.** Transient overexpression of HA-CaLRR-RLK1 in pepper leaves detected by immunoblot. (TIF 36 kb)

**Additional file 3: Figure S3.** The expression of CaLRR-RLK1 in representative T1 transgenic tobacco plants was checked by RT-PCR: Wild-type (WT) tobacco plants as the blank control, and Nef1 α served as an endogenous control. (TIF 21 kb)

**Additional file 4: Table S1.** Pepper primers used for qPCR in this study. Tobacco primers used for qPCR in this study. (DOCX 21 kb)

Abbreviations

- ChIP: Chromatin immunoprecipitation
- ETL: Effector-triggered immunity
- ETS: Effectors triggered susceptibility
- GFP: Green fluorescent protein
- hpa: H post application
- LRR-RLK: Leucine-rich repeat receptor-like kinase
- non-RD: Non-arginine-aspartate
- ORF: Open reading frame
- PAMP: Pathogen-associated molecular pattern
- PTI: PAMP-triggered immunity
- RSI: Ralstonia solanacearum
- RD: Non-arginine-aspartate
- ORF: Open reading frame
- PAMP: Pathogen-associated molecular pattern
- PAMP-TRV: PAMP-triggered immunity
- SRL: Ralstonia solanacearum inoculation
- TAR: Systematic acquired resistance
- TMD: Transmembrane domain
- VIGS: Virus-induced gene silencing

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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 Additional file 4).

Authors’ contributions

SM and SH conceived and designed the research. SM, FG, LS, SY and WH contributed to the acquisition of transgenic tobacco lines. SH and YW made key comments on the design of the trial, the article writing and the revisions. SM and SH wrote the article. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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