Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2017) 18(8), 1089–1100 DOI: 10.1111/mpp.12462

A novel leucine-rich repeat protein, CaLRR51, acts as a positive regulator in the response of pepper to *Ralstonia solanacearum* infection

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SUMMARY

The leucine-rich repeat (LRR) proteins play important roles in the recognition of corresponding ligands and signal transduction networks in plant defence responses. Herein, a novel LRR protein from *Capsicum annuum*, CaLRR51, was identified and characterized. It was localized to the plasma membrane and transcriptionally up-regulated by *Ralstonia solanacearum* infection (RSI), as well as the exogenous application of salicylic acid (SA), jasmonic acid (JA) and ethephon (ETH). Virus-induced gene silencing of *CaLRR51* significantly increased the susceptibility of pepper to RSI. By contrast, transient overexpression of *CaLRR51* in pepper plants activated hypersensitive response (HR)-like cell death, and up-regulated the defence-related marker genes, including *PO2*, *HIR1*, *PR1*, *DEF1* and *ACO1*. Moreover, ectopic overexpression of *CaLRR51* in transgenic tobacco plants significantly enhanced the resistance to RSI. Transcriptional expression of the corresponding defence-related marker genes in transgenic tobacco plants was also found to be enhanced by the overexpression of *CaLRR51*, which was potentiated by RSI. These loss- and gain-of-function assays suggest that CaLRR51 acts as a positive regulator in the response of pepper to RSI. In addition, the putative signal peptide and transmembrane region were found to be required for plasma membrane targeting of CaLRR51, which is indispensable for the role of CaLRR51 in plant immunity.

Keywords: *Capsicum annuum*, leucine-rich repeat (LRR) protein, plant defence response, *Ralstonia solanacearum*.

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INTRODUCTION

In their natural habitats, plants inevitably encounter a broad range of microbial pathogens, and have evolved sophisticated defence mechanisms to defend themselves against these potential threats. In addition to physical or chemical barriers, such as a waxy cuticle and preformed antimicrobial compounds, plants also employ innate immunity to actively respond to pathogen attack (Schneider, 2002). This active form of defence consists of two interconnected branches, known as pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI), on perception of PAMPs and effectors by cell surface-localized pattern recognition receptors (PRRs) and disease resistance (R) proteins in host cells, respectively (Jones and Dangl, 2006). The never-ending arms race between plants and pathogens may drive the continuous emergence of interconnected PRRs or R proteins in plants and PAMPs or effectors in pathogens, resulting in a continuum between typical PTI and ETI (Block *et al.*, 2008; Boller and Felix, 2009; Boller and He, 2009; Jones and Dangl, 2006; Lee *et al.*, 2015). However, knowledge of this continuum is still very limited.

Leucine-rich repeats (LRRs) exist widely in plant proteins and are involved in protein–protein interactions and ligand binding, and therefore in pathogen perception and signal transduction (McHale *et al.*, 2006). Based on their subcellular localization, LRRs can be divided into extracellular (eLRR) and intracellular (iLRR) LRRs (Zhou *et al.*, 2009). iLRRs are mainly found in NB-LRR R proteins, named after their central nucleotide-binding (NB) domain, with an LRR domain in the C-terminal region and a Toll/Interleukin-1 Receptor (TIR) homology or predicted coiled-coil (CC) domain in the N-terminal region (Caplan *et al.*, 2008), in which LRRs play important roles in the determination of the recognition specificity of R proteins to pathogen effectors (Dodds *et al.*, 2001, 2006; Hwang and Williamson, 2003; Rairdan and Moffett, 2006), or in the auto-inhibition of the NB-LRR R protein through intramolecular interactions with the region carrying the NB-ARC (nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4) and CC domains (Ade *et al.*, 2007; Moffett *et al.*, 2002; Rairdan and Moffett, 2006; Qi *et al.*, 2012). Interactions

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between R proteins and other signalling components, for example SGT1, can be mediated by the LRR domain in R proteins (Bieri *et al.*, 2004). eLRRs have been found frequently in receptor like kinases (RLKs), which contain an eLRR domain, a single membrane-spanning helix and a cytosolic kinase domain for signal transduction (van der Hoorn and Jones, 2004), and in receptor-like proteins (RLPs), which contain an eLRR domain, a single transmembrane helix and a small cytosolic tail (Zhou *et al.*, 2009). Emerging data have shown that LRR proteins, such as SLRR in sorghum (Hipskind *et al.*, 1996), LeLRP in tomato (Tornero *et al.*, 1996), NtLRP1 in tobacco (Jacques *et al.*, 2006), CaLRR1 in pepper (Jung *et al.*, 2004; Jung and Hwang, 2007) and OsLRR1 in rice (Zhou *et al.*, 2009), exhibit structural similarities to RLKs with different subcellular localizations, and may play a role in plant immunity (Zhou *et al.*, 2009). In rice, the OsLRR1 protein can enter the endosomal pathway and act as a positive regulator in the defence response by interacting with OsHIR1 in the plasma membrane (Zhou *et al.*, 2009, 2010). In pepper, the CaLRR1 protein has been found to act as a positive regulator in plant immunity by interacting with pathogenesis-related protein 10 (PR10) in the cytoplasm and the apoplastic space (Choi *et al.*, 2012); however, it suppresses hypersensitive response (HR)-like cell death and the defence response by interacting with hypersensitive-induced reaction 1 (HIR1) in the plasma membrane (Choi *et al.*, 2011; Jung and Hwang, 2007), or PR4b in the plasma membrane and the apoplast (Hwang *et al.*, 2014). Despite the difference between the sequences of CaLRR1 and OsLRR1, and their modes of action in plant immunity, both CaLRR1 and OsLRR1 can interact with HIR1, and this interaction has been found to be highly conserved among different plant species (Zhou *et al.*, 2009). All these results indicate that eLRR-containing proteins play important roles in plant immunity. However, the data available on the roles of eLRR-containing proteins in plants is very limited, even though a large number of genes encoding eLRR proteins can be found in the genomes of different plant species.

In the present study, a novel LRR protein from pepper, CaLRR51, structurally distinct from the previously characterized CaLRR1, was identified and characterized. It was localized to the plasma membrane and transcriptionally up-regulated by *Ralstonia solanacearum* infection (RSI), as well as by exogenously applied salicylic acid (SA), jasmonic acid (JA) and ethephon (ETH). The data of loss- and gain-of-function assays indicated that CaLRR51 acts as a positive regulator in the response of pepper to RSI. In addition, the signal peptide (SP) and transmembrane region (TR) were found to be required for plasma membrane targeting of CaLRR51, which is required for the role of CaLRR51 in pepper immunity.

RESULTS

Cloning and sequence analysis of a novel LRR protein CaLRR51

A normalized cDNA library of pepper inbred line GZ03 was constructed previously to isolate full-length *Capsicum annuum* cDNAs (our group's unpublished data). One of the positive cDNA clones was isolated by random sequencing and identified as a member of the LRR domain proteins, which is identical to the sequence of *C. annuum* cultivar CM334 PGAv.1.5.contig190568 (accession no: AYRZ01190568.1). The length of this cDNA was 1586 bp, including 80 bp of 5′-untranslated region, 1287 bp of open reading frame (ORF) and 219 bp of 3′-untranslated region. The ORF of this gene was predicted to encode a protein of 428 amino acids with three successive domains from the N-terminus to the C-terminus: a putative SP, an LRR domain and a TR (Fig. 1A). The putative SP was predicted with a cleavage site after the 28th amino acid. There were six tandem repeats of the LRR motif in the central region of the deduced amino acid sequence, containing a domain exhibiting similarity to LRR RLP kinases. The TR was predicted at the C-terminus, starting at residue 402 and ending at residue 424.

The deduced amino acid sequence of this isolated cDNA exhibits high similarity (56%–81% identity) with its orthologues in other plant species, including *Arabidopsis thaliana* (AtLRR51, accession no: NP_193611.1), *Gossypium arboreum* (accession no: ACD56661.1), *Glycine max* (accession no: XP_003541695.1), *Nicotiana sylvestris* (accession no: XP_009770243.1), *Nicotiana tomentosiformis* (accession no: XP_009594315.1) and *Solanum lycopersicum* (accession no: XP_004234080.1). As a result of its high sequence similarity to *AtLRR51* from *A. thaliana*, we designated this cDNA clone as *CaLRR51*. Notably, CaLRR51 in pepper and its orthologues in other plant species do not seem to be functionally characterized in the existing literature.

We also compared CaLRR51 with other previously characterized eLRR proteins, such as CaLRR1 (accession number: AY237117.1), OsLRR1 (accession number: AAO85403.1) (Fig. S1, see Supporting Information), AtSERK1 (*A. thaliana* somatic embryogenesis receptor-like kinase; accession number: NP_177328), AtSERK3 (accession number: NP_567920), OsSERK1 (accession number: BAD86793), rice LRR protein (*Oryza sativa* LRR protein; accession no: AP000815), SLRR (*Sorghum bicolor* LRR protein; accession no: U62279) and RLK (*A. thaliana* receptor-like kinase protein; accession no: NM119198) (data not shown). This revealed that these LRR proteins share no appreciable homology with CaLRR51, except for the LRR motifs.

Up-regulation of *CaLRR51* transcript levels by RSI and exogenous phytohormone application

As LRR proteins have been implicated in plant immunity, to test whether this holds for CaLRR51, its transcriptional expression was measured by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) on inoculation of *R. solanacearum*, the causal agent of pepper bacterial wilt (Fig. 2). On leaf inoculation with the highly virulent *R. solanacearum* strain FJC100301, the *CaLRR51* transcripts were up-regulated nearly

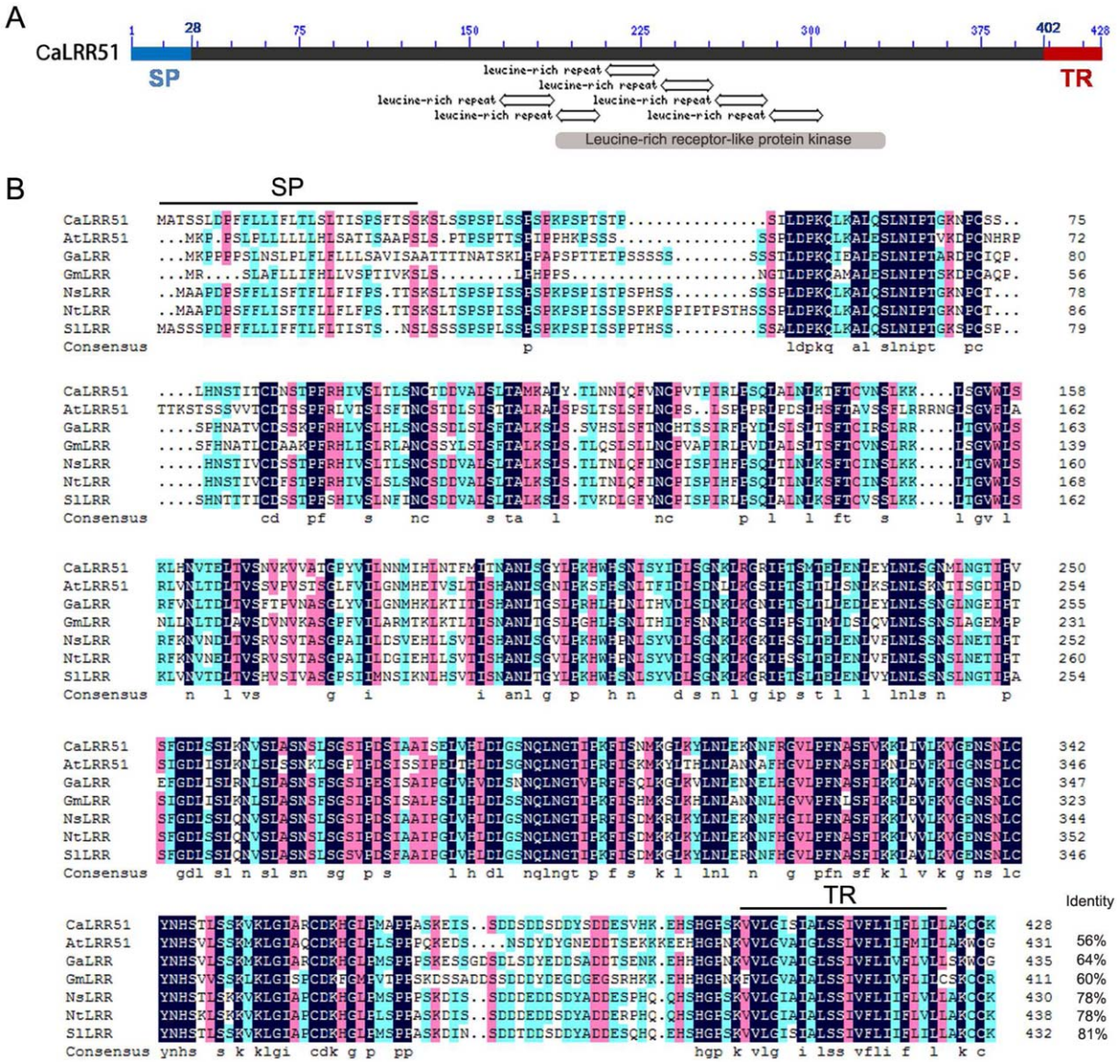

Fig. 1 Structural domains and comparison of amino acid sequences of CaLRR51 with its homologues. (A) The amino acid sequence of CaLRR51 was predicted with three successive domains from N-terminus to C-terminus: a putative signal peptide (SP), a leucine-rich repeat (LRR) domain with six tandem repeats of the LRR motif and a transmembrane region (TR). Domains were detected by BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and SMART (http://smart.embl-heidelberg.de/). (B) Sequence alignment of CaLRR51 with its homologues in some other plant species, including *Arabidopsis thaliana* (AtLRR51, accession no: NP_193611.1), *Gossypium arboreum* (GaLRR, accession no: ACD56661.1), *Glycine max* (GmLRR, accession no: XP_003541695.1), *Nicotiana sylvestris* (NsLRR, accession no: XP_009770243.1), *Nicotiana tomentosiformis* (NTLRR, accession no: XP_009594315.1) and *Solanum lycopersicum* (SlLRR, accession no: XP_004234080.1).

three-fold at 24 and 48 h post-inoculation (hpi) in pepper plants, suggesting that CaLRR51 participates in the defence response of pepper to *R. solanacearum*.

Phytohormones, such as SA, JA and ethylene (ET), serve as important signalling molecules and are involved ubiquitously in the responses of plants to pathogen attack. To assess the possible association of CaLRR51 with signalling mediated by these hormones, the transcript abundance of *CaLRR51* was determined by qRT-PCR in leaves of pepper plants (at the four-leaf stage) exogenously treated with SA, methyl jasmonate (MeJA) and ETH (Fig. 2). The results showed that the transcript levels of *CaLRR51* were rapidly induced by 2.5-fold at 6 h post-treatment (hpt) of SA, and gradually decreased to the basal level at 48 hpt. On MeJA treatment, the transcript levels of *CaLRR51* were significantly increased up to 5.3-fold at 6 hpt and 2.8-fold at 12 hpt. In response to ETH treatment, the transcript levels of *CaLRR51* were elevated nearly

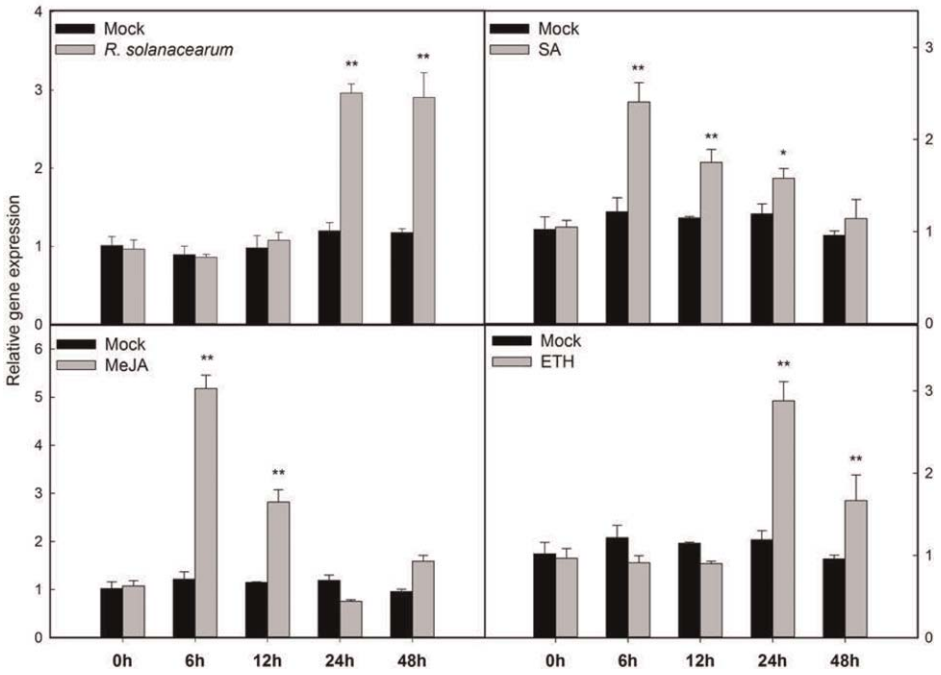

Fig. 2 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *CaLRR51* in pepper plants during *Ralstonia solanacearum* infection and exogenous phytohormone application. For *R. solanacearum* infection assay, the third leaves from the top of pepper plants at the eight-leaf stage were infiltrated with 10 μL of the highly virulent *R. solanacearum* strain FJC100301 suspension [optical density at 600 nm (OD_{600}) = 0.8] using a syringe without a needle, and the mock was inoculated with 10 mM $MgCl_2$. For exogenous phytohormone application, pepper plants at the four-leaf stage were sprayed with 1 mM salicylic acid (SA), 100 μM methyl jasmonate (MeJA) or 100 μM ethephon (ETH), and the mock treatment was sprayed with a corresponding solvent or sterile double-distilled H_2O. The leaves were collected at the indicated time points for RNA extraction and qRT-PCR analysis. The relative transcript levels of *CaLRR51* were compared with those in mock-treated control plants. Data are the means ± standard deviation from three independent experiments. Asterisks indicate statistically significant differences compared with mock treatment by the least-significant difference (LSD) test (*$P < 0.05$; **$P < 0.01$).

three-fold at 24 hpt and 1.6-fold at 48 hpt. These results suggest that CaLRR51 plays a role in the response of pepper to RSI and is involved in the signalling pathways mediated by these hormones.

Silencing of *CaLRR51* enhances the susceptibility of pepper to *R. solanacearum*

To further study the role of CaLRR51 in the response of pepper to RSI, we performed loss-of-function experiments in pepper seedlings by virus-induced gene silencing (VIGS) of *CaLRR51*. qRT-PCR analysis showed that *CaLRR51* was effectively silenced in *Tobacco rattle virus* (TRV):*CaLRR51* pepper plants; the transcript levels in TRV:*CaLRR51* plants were reduced to approximately 28% of those in TRV:*00* plants (Fig. 3A). On leaf inoculation with *R. solanacearum* strain FJC100301, the transcript levels in TRV:*CaLRR51* plants were only 3%–18% of those in TRV:*00* plants at 24 and 48 hpi, and the growth of *R. solanacearum* was significantly enhanced in *CaLRR51*-silenced pepper plants compared with that in TRV:*00* plants (Fig. 3B). Consistently, the *CaLRR51*-silenced pepper plants exhibited more severe disease symptoms compared with control plants after inoculation with *R. solanacearum* in pepper roots. These results suggest that CaLRR51 plays a role in host defence against *R. solanacearum*.

Transient overexpression of *CaLRR51* induces cell death and defence-related marker gene expression in pepper plants

To further confirm that CaLRR51 acts as a positive regulator in the defence response of pepper to *R. solanacearum*, *CaLRR51* was transiently expressed in pepper leaves by infiltration with *Agrobacterium tumefaciens* strain GV3101 carrying 35S:*CaLRR51* or 35S:*00* (empty vector). As shown in Fig. 4A, transient overexpression of *CaLRR51* triggered an obvious HR-like cell death response at 4 days post-inoculation (dpi), but not in empty vector control plants. The HR-like cell death response was then assessed by trypan blue staining to identify necrotic cells. There was no or a very weak HR-mediated necrotic response in leaves infiltrated with *Ag. tumefaciens* carrying the empty vector, whereas transient overexpression of 35S:*CaLRR51* strongly induced the necrotic response in pepper leaves. Hydrogen peroxide production in pepper leaves was also detected by diaminobenzidine (DAB) staining. There was visible DAB staining in leaves with transient expression of

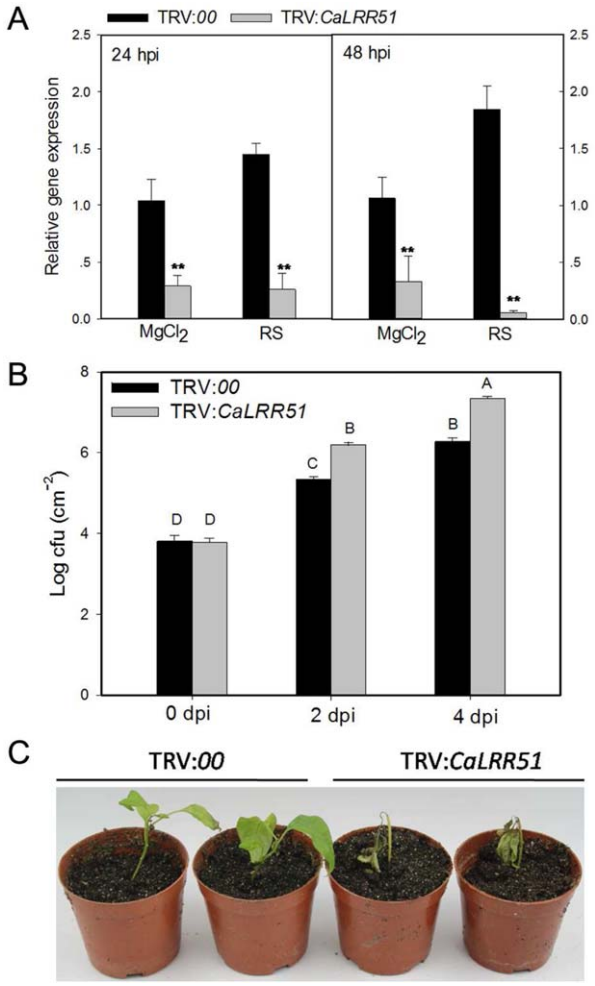

Fig. 3 Virus-induced gene silencing (VIGS) of *CaLRR51* enhances the susceptibility of pepper to *Ralstonia solanacearum*. (A) Relative transcriptional expression of *CaLRR51* in leaves of *CaLRR51*-silenced pepper plants (TRV:*CaLRR51*) versus empty vector control plants (TRV:*00*) by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) at 24 and 48 h post-inoculation (hpi) with *R. solanacearum* or $MgCl_2$ (mock). (B) Bacterial growth (colony-forming units, cfu) in *CaLRR51*-silenced or empty vector control pepper leaves inoculated with *R. solanacearum* at 2 and 4 days post-inoculation (dpi). Data are the means ± standard deviation of three independent experiments. Different letters indicate statistically significant differences compared with the empty vector control plants by the least-significant difference (LSD) test ($P < 0.01$). (C) Disease symptoms of *CaLRR51*-silenced and empty vector control plants at 7 dpi after root inoculation with *R. solanacearum*.

35S:*CaLRR51*, but not in leaves infiltrated with GV3101 strain containing the empty vector. We also performed an ion leakage test to analyse the severity of cell necrosis caused by plasma membrane damage in leaves expressing 35S:*CaLRR51* (Fig. 4B). Greater ion leakage at 48 and 72 h after agroinfiltration was observed in leaves expressing 35S:*CaLRR51* than in the empty vector control.

To test whether transient overexpression of *CaLRR51* could alter defence-related gene expression in pepper plants, we also examined the transcript abundances of defence-related marker genes, including the HR-associated gene *CaHIR1*, reactive oxygen species (ROS) detoxification-associated gene *CaPO2*, SA-responsive gene *CaPR1*, JA-responsive gene *CaDEF1* and ET-associated gene *CaACO1* (Fig. 4C). The results showed that the relative transcription levels of these defence-related genes were increased in plants by transient overexpression of *CaLRR51*. In particular, the transcript level of *CaPR1* was increased over 10-fold compared with that in control plants.

Stable overexpression of *CaLRR51* enhances resistance of tobacco to *R. solanacearum* inoculation

As pepper is very recalcitrant to genetic transformation, we generated stable transgenic tobacco plants to determine the *in vivo* function of CaLRR51. We obtained 15 independent T_0 transgenic tobacco lines that constitutively expressed *CaLRR51* driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter. Two T_2 transgenic lines, CaLRR51-OX-5 and CaLRR51-OX-6, which exhibited the highest levels of *CaLRR51* transcripts, displayed visible normal morphology and set viable seeds, were selected for further analyses. Eight-week-old plants of the two transgenic lines and wild-type (WT) line were inoculated in roots with the highly virulent *R. solanacearum* strain FJC100301. Seven days after inoculation, severe wilting symptoms had developed on wild-type plants; however, the transgenic lines displayed less severe disease symptoms (Fig. 5A). To quantify the extent of disease in *R. solanacearum*-infected plants, bacterial growth in the third leaves of WT and transgenic tobacco plants was assessed at 36 hpi. Significantly decreased bacterial growth was observed in transgenic tobacco plants compared with wild-type plants (Fig. 5B). We also examined local defence responses by DAB (as an indicator of H_2O_2 accumulation) and trypan blue (as an indicator of cell death or necrosis) staining of *R. solanacearum*-inoculated leaves. The infected leaves of CaLRR51-OX transgenic lines exhibited clearly increased intensities of H_2O_2 accumulation (dark brown) and HR-like cell death (dark blue) compared with wild-type plants (Fig. 5C).

To further investigate the possible mode of action of CaLRR51, the expression of defence-related marker genes was analysed in CaLRR51-OX transgenic tobacco plants and wild-type plants by qRT-PCR. We examined the transcript levels of the HR-associated gene *NtHSR201*, ROS detoxification-associated gene *NtCAT1*, SA-responsive genes *NtPR1a* and *NtCHN50*, JA-responsive gene *NtLOX1* and ET-associated gene *NtACO1*. The results showed that the relative transcription levels of these defence-related genes were constitutively increased in CaLRR51-OX transgenic plants, except for *NtPR1a* and *NtACO1* (Fig. 6A). We also detected the transcript levels of these defence-related genes in tobacco plants

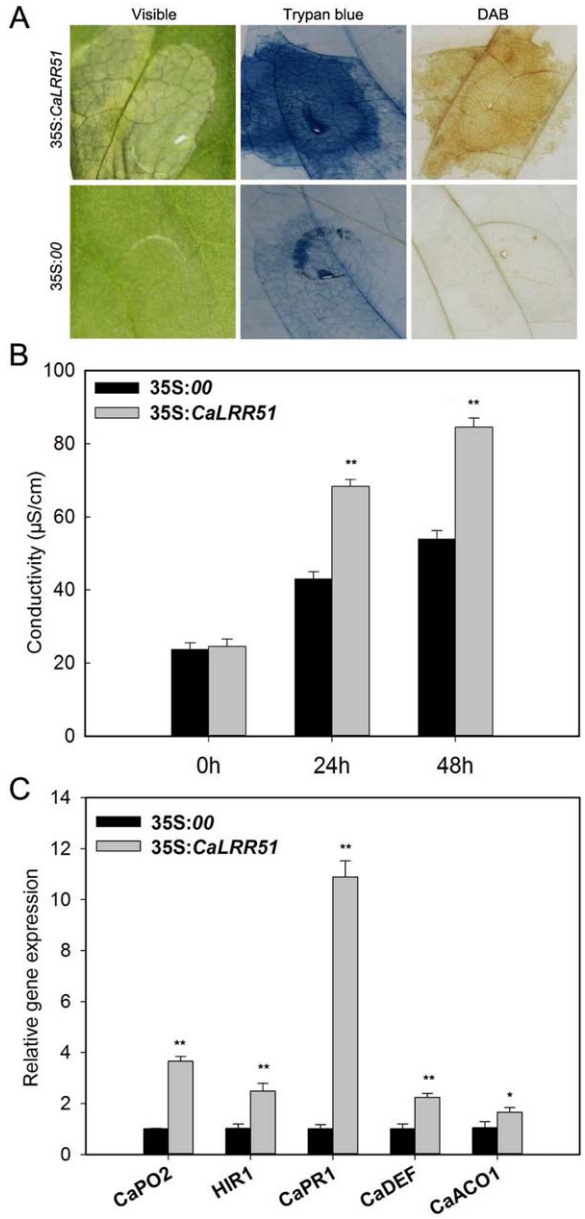

Fig. 4 Transient overexpression of *CaLRR51* induces hypersensitive response (HR)-like cell death and defence-related gene expression in pepper plants. (A) Pepper leaves were infiltrated with *Agrobacterium* GV3101 carrying the 35S:*00* (empty vector) or 35S:*CaLRR51* construct. Phenotypes of infiltrated pepper leaves at 4 days after agroinfiltration (left), by trypan blue staining (middle) and by diaminobenzidine (DAB) staining (right). (B) Electrolyte leakage assay of pepper leaves after agroinfiltration with the 35S:*00* or 35S:*CaLRR51* construct at 24 and 48 h post-inoculation (hpi). (C) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of defence-related gene expression in pepper leaves transiently overexpressing 35S:*CaLRR51* or 35S:*00* at 48 hpi. The relative transcript levels were normalized to the pepper *CaActin* gene. Data are the means ± standard deviation of three independent experiments. Asterisks indicate statistically significant differences compared with the empty vector controls by the least-significant difference (LSD) test (*$P < 0.05$; **$P < 0.01$).

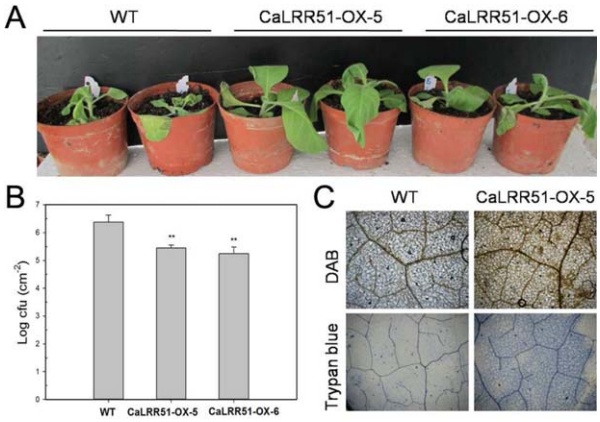

Fig. 5 Transgenic tobacco plants overexpressing *CaLRR51* show increased resistance to *Ralstonia solanacearum* infection. (A) Disease symptoms of 8-week-old transgenic tobacco lines (CaLRR51-OX-5 and CaLRR51-OX-6) and the wild-type (WT) line at 7 days after root inoculation with the highly virulent *R. solanacearum* strain FJC100301. (B) Bacterial growth (colony-forming units, cfu) in the third leaves of WT and transgenic tobacco plants at 36 h after leaf inoculation with *R. solanacearum*. Data are the means ± standard deviation of three independent experiments. Asterisks indicate statistically significant differences compared with the WT controls by the least-significant difference (LSD) test (**$P < 0.01$). (C) Increased H_2O_2 accumulation and hypersensitive response (HR)-like cell death in CaLRR51-OX-5 transgenic tobacco leaves compared with WT leaves inoculated with *R. solanacearum* at 24 h post-inoculation. DAB, diaminobenzidine.

after leaf inoculation with *R. solanacearum* at 24 and 48 hpi. All the defence marker genes were strongly induced in the transgenic lines in at least one of the two tested time points during *R. solanacearum* infection (Fig. 6B). Taken together, the overexpression of *CaLRR51* in tobacco plants appears to enhance disease resistance against *R. solanacearum* by elevated HR-like cell death and H_2O_2 levels, accompanied by increased transcript levels of HR-associated genes and other defence genes, which is consistent with the positive role of *CaLRR51* observed in pepper plants.

The SP/TR deletion mutants of CaLRR51 attenuate HR-like cell death in pepper plants

To determine the subcellular localization of CaLRR51, we generated a CaLRR51-GFP fusion construct (35S:*CaLRR51-GFP*) driven by the constitutive CaMV 35S promoter. As computational analysis of the CaLRR51 protein highlighted putative SP and TR at the N-terminus and C-terminus, respectively (Fig. 1), we also constructed vectors of SP and/or TR deletion mutants of CaLRR51 fused with the green fluorescent protein (GFP). *Nicotiana benthamiana* leaves were used to express these constructs by co-agroinfiltration with a *35S:CBL1n-RFP* fusion construct (as an additional control targeting to the plasma membrane), and were visualized at 48 hpi using a confocal microscope (Batistic *et al.*, 2008) (Fig. 7A). The green fluorescent signals of 35S:*GFP* control

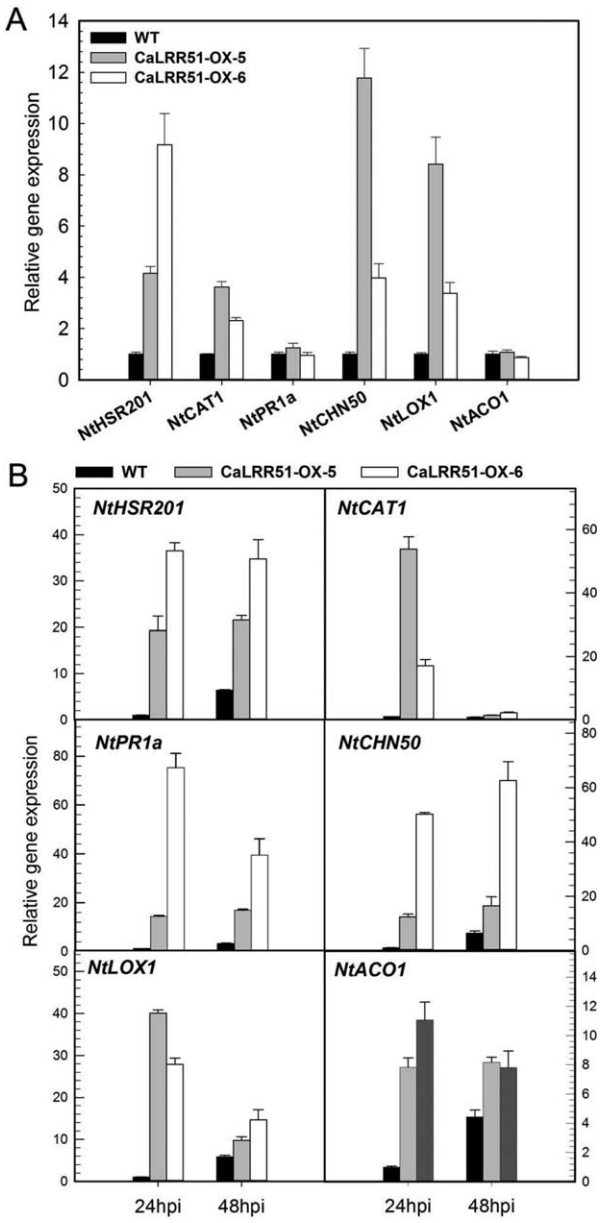

Fig. 6 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of defence-related gene expression in transgenic tobacco plants overexpressing *CaLRR51*, with or without *Ralstonia solanacearum* inoculation. (A) The relative transcript levels of defence-related genes in the third leaves of CaLRR51-OX transgenic tobacco plants and wild-type plants without *R. solanacearum* inoculation. (B) The relative transcript levels of defence-related genes in the third leaves of CaLRR51-OX transgenic tobacco plants and wild-type plants at 24 and 48 h after leaf inoculation with *R. solanacearum*. *NtHSR201*, hypersensitive response (HR)-associated gene; *NtCAT1*, reactive oxygen species (ROS) detoxification-associated gene; *NtPR1a* and *NtCHN50*, salicylic acid (SA)-responsive genes; *NtLOX1*, jasmonic acid (JA)-responsive gene; *NtACO1*, ethylene (ET)-associated gene. The relative transcript levels were normalized to the pepper *CaActin* gene. Data were the means ± standard deviation of three independent experiments.

construct in *N. benthamiana* leaves were observed in the cytoplasm and nuclei. However, CaLRR51-GFP was localized primarily at the plasma membrane, similar to our observation with CBL1n-RFP. The subcellular localization of ΔSP-CaLRR51-GFP was shown to be similar to that of the 35S:*GFP* control construct, except that the intensity of green fluorescence was discontinuous in the plasma membrane. In *N. benthamiana* leaves expressing the CaLRR51-ΔTR-GFP fusion protein, the GFP signals were observed in multiple subcellular compartments, including the cytoplasm and nuclei. Significantly, the fluorescent signals of CaLRR51-ΔTR-GFP exhibited a punctate pattern in the cytoplasm. For the ΔSP-CaLRR51-ΔTR-GFP deletion mutant construct, the subcellular localization was similar to that of ΔSP-CaLRR51-GFP and also exhibited some punctate signals in the cytoplasm.

To determine whether the location sites of CaLRR51 and its SP/TR deletion mutants are important for function, we investigated HR-like cell death responses by transient overexpression of a set of vectors, including 35S:*CaLRR51*, 35S:Δ*SP-CaLRR51*, 35S:*CaLRR51*-Δ*TR* and 35S:Δ*SP-CaLRR51*-Δ*TR*, in pepper leaves (Fig. 7B). At 4 days after agroinfiltration, the transient overexpression of *CaLRR51* triggered a severe visible cell death response. However, all three SP/TR deletion mutants of CaLRR51 attenuated HR-like cell death, in accordance with the results by trypan blue and DAB staining.

DISCUSSION

Although conserved LRRs have been found to exist widely in plant proteins and have been implicated in pathogen perception and signal transduction, these studies have focused on iLRRs (Zhou *et al.*, 2009), and eLRRs remain largely uncharacterized. The data in this study provide evidence that CaLRR51, a novel eLRR protein, acts as a positive regulator in the response of pepper to RSI.

The evidence that CaLRR51 acts as a positive regulator in the response of pepper to RSI comes from the following aspects. First, the transcriptional expression of *CaLRR51* was found to be significantly enhanced by inoculation of *R. solanacearum* into pepper leaves compared with that in control plant leaves, implying that CaLRR51 may play a role in the response of pepper to RSI. As shown by previous studies, the responses of plants to pathogens are largely regulated at the transcriptional level (Bartsch *et al.*, 2006; Buscaill and Rivas, 2014; Eulgem, 2005; Katagiri, 2004; Lewis *et al.*, 2015; Tsuda and Somssich, 2015), and genes up-regulated by pathogen challenge are frequently found to play important roles in plant immunity (Bartsch *et al.*, 2006; Cai *et al.*, 2015; Dang *et al.*, 2013, 2014; Ramonell *et al.*, 2005). As CaLRR1, which has been found previously to be involved in pepper immunity (Choi *et al.*, 2011, 2012; Hwang *et al.*, 2014; Jung and Hwang, 2007), was also found to be up-regulated by RSI in the present study (Fig. S2, see Supporting Information), this suggests that the response of CaLRR51 to RSI is not specific. Second, the

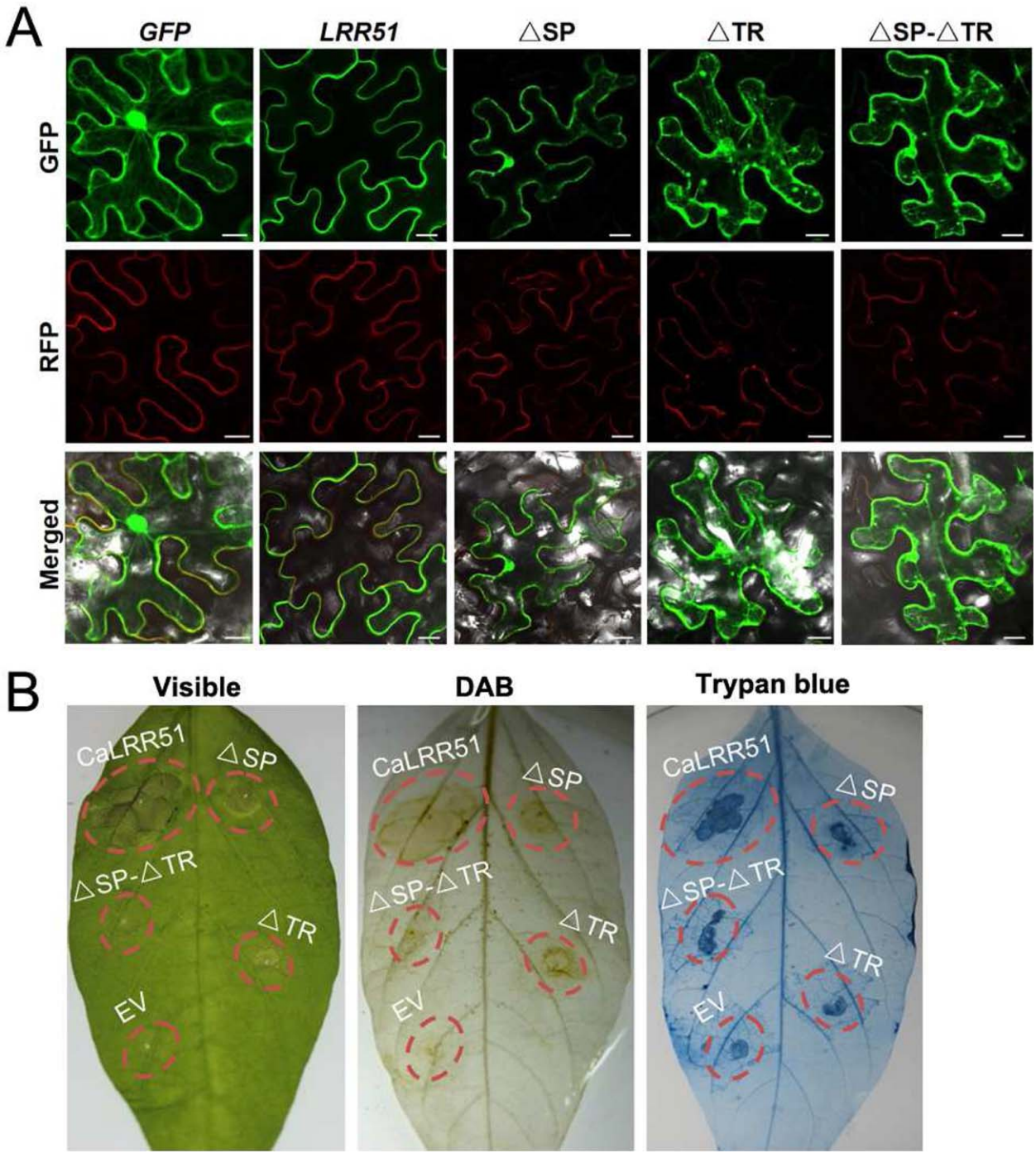

Fig. 7 Subcellular localization and hypersensitive response (HR)-like cell death responses of CaLRR51 and its signal peptide/transmembrane region (SP/TR) deletion mutants. (A) *Nicotiana benthamiana* leaves were co-infiltrated with *Agrobacterium tumefaciens* strains containing *35S:CBL1n-RFP* and *35S:CaLRR51-GFP* or their ΔSP/ΔTR-GFP fusion constructs (*35S:*Δ*SP-CaLRR51-GFP*, *35S:CaLRR51-*Δ*TR-GFP* or *35S:*Δ*SP-CaLRR51-*Δ*TR-GFP*). Fluorescence was imaged using a confocal microscope at 48 h post-inoculation (hpi). GFP, green fluorescent protein; RFP, red fluorescent protein. Bars, 30 μm. (B) Pepper leaves were infiltrated with *Agrobacterium* GV3101 carrying the *35S:CaLRR51*, *35S:*Δ*SP-CaLRR51*, *35S:CaLRR51-*Δ*TR*, *35S:*Δ*SP-CaLRR51-*Δ*TR* or *35S:00* construct. Phenotypes of infiltrated pepper leaves 4 days after agroinfiltration (left), by diaminobenzidine (DAB) staining (middle) and by trypan blue staining (right).

silencing of *CaLRR51* by VIGS significantly enhanced the susceptibility of pepper plants to RSI relative to the observation in control plants. By contrast, HR-like cell death was found to be significantly triggered by transient overexpression of *CaLRR51* in pepper plants, manifested by the coupled darker trypan blue staining and DAB staining, as HR-like cell death has been found previously to be coupled with the accumulation of H_2O_2, a signalling molecule leading to HR (Torres *et al.*, 2006). We speculate that the HR-like cell death and H_2O_2 accumulation in pepper plants by local transient assays may be closely related because of the high expression levels of CaLRR51. Moreover, transient overexpression of *CaLRR51* in pepper plants triggered the expression of defence-related marker genes, including *CaHIR1* (Jung and Hwang, 2007), *CaPO2* (Choi *et al.*, 2007), *CaPR1* (Kim and Hwang, 2014), *CaDEF1* (Hwang and Hwang, 2010) and *CaACO1* (Aizat *et al.*, 2013; Lee *et al.*, 2014), which have been found previously to be transcriptionally up-regulated by pathogens and to play important roles in plant immunity. Third, ectopic overexpression of *CaLRR51* significantly enhanced the resistance of transgenic tobacco plants to RSI, with more intensive trypan blue and DAB staining compared with that in control plants. Transcriptional expression of defence-related genes, including *NtHSR201* (Takahashi *et al.*, 2004), *NtCAT1* (Takahashi *et al.*, 1997), *NtPR1a* (Sohn *et al.*, 2007), *NtCHN50* (Menke *et al.*, 2005), *NtLOX1* (Fammartino *et al.*, 2007, 2010) and *NtACO1* (Kim *et al.*, 2003), was also found to be enhanced by the overexpression of *CaLRR51*, which was potentiated by RSI. All these data strongly suggest that CaLRR51 acts as a positive component in the response of pepper to RSI.

Although CaLRR51 acts as a positive regulator in the HR-like response by local transient assays, the CaLRR51-OX transgenic tobacco lines described here did not display visible abnormal morphology. This may be because the expression levels of CaLRR51 in transgenic tobacco plants were too low to trigger visible HR-like responses. One explanation may be that, despite the random nature of insertion of the 35S:*CaLRR51* construct into the tobacco genome, high-expression-level CaLRR51 transgenic plants failed to grow because of severe growth retardation or lethal effects. Thus, only those transgenic plants with lower CaLRR51 expression levels would be recoverable. Of note, the transformation efficiency of CaLRR51-OX tobacco lines was very low in this study.

It was predicted that CaLRR51 contains an LRR domain with six tandem repeats of LRR motifs and a TR domain, which are distinct from that in previously characterized eLRR proteins, such as OsLRR1 (Zhou *et al.*, 2009), CaLRR1 (Jung *et al.*, 2004), NtLRP1 (Jacques *et al.*, 2006), LeLRP (Tornero *et al.*, 1996) and SLRR (Hipskind *et al.*, 1996). These eLRR proteins contain LRR domains with only four to five LRR motifs and no TR domain. The function of a given protein is generally related closely to its structurally dependent subcellular localization, and data from previous studies have shown diverse subcellular localizations of eLRR proteins. For example, CaLRR1 localizes to the extracellular matrix (Choi *et al.*, 2011), NtLRP1 localizes to the endoplasmic reticulum (Jacques *et al.*, 2006) and OsLRR1 targets the plasma membrane, clathrin-coated vesicles, early endosome and late endosome (Zhou *et al.*, 2009). Unlike these LRR proteins, our data showed that CaLRR51 was exclusively localized to the plasma membrane. Both TM and SP in CaLRR51 are required for plasma membrane targeting. The SP deletion mutant of CaLRR51 was localized to whole cells including nuclei; however, the intensity of green fluorescent signals was discontinuous in the plasma membrane. The TR deletion mutant of CaLRR51 was localized to the cytoplasm with punctate GFP signals, similar to that of OsLRR1, which lacks the TR domain (Zhou *et al.*, 2009). The membrane targeting of CaLRR51 was found to be crucial for its function in immunity, as the blocking of its membrane targeting by either SP or TR deletion triggered lower levels of HR-like cell death, manifested by lower levels of DAB and trypan blue staining, compared with that of the wild-type CaLRR51. As it has been generally established that PTI is triggered by the perception of PAMPs by cell surface-localized PRRs (Jones and Dangl, 2006), we speculate that CaLRR51 might act as a cell surface-localized PRR. As CaLRR51 is predicted to be an LRR RLP kinase, it might fulfil its function in plant immunity via interaction with certain unidentified ligands, as LRR proteins generally serve as protein interaction platforms; for example, CaLRR1 has been implicated via interaction with HIR1 (Choi *et al.*, 2011; Jung and Hwang, 2007), PR10 (Choi *et al.*, 2012) and PR4b (Hwang *et al.*, 2014).To elucidate the underlying mechanism of immunity mediated by CaLRR51, further studies to identify and characterize its possible interacting proteins from either pathogens or pepper are required.

Plant hormones, such as SA, JA and ET, have been implicated ubiquitously in defence signalling networks that are recruited on perception of an invader (Clarke *et al.*, 2000; Klessig *et al.*, 2000; Pieterse *et al.*, 2012). Although signalling pathways mediated by these hormones act as common signalling cascades in both PTI and ETI (Tsuda and Katagiri, 2010), the relationships among the signalling sectors in PTI and ETI are different. Synergistic relationships are evident in PTI, which may amplify the signal; compensatory relationships among the sectors dominate in ETI (Tsuda *et al.*, 2009). The aforementioned membrane targeting of CaLRR51 and its high sequence similarity to LRR RLP kinases strongly suggest that CaLRR51 acts as a probable receptor of unidentified PAMPs to trigger PTI. Consistent with this, our data showed that exogenously applied SA, MeJA and ETH significantly induced the transcriptional expression of *CaLRR51*. Moreover, the ectopic overexpression of *CaLRR51* in tobacco plants and transient overexpression in pepper plants synergistically activated the transcriptional expression of SA-, JA- and ET-dependent immunity-associated marker genes, suggesting that CaLRR51 might be involved in PTI. Notably, in addition to the above-mentioned

structural differences between CaLRR1 and CaLRR51, there are significant differences in the responses of CaLRR1 and CaLRR51 to the exogenous application of SA, MeJA and ETH (Jung *et al.*, 2004). It can be speculated that CaLRR1 and CaLRR51 have evolved in pepper's co-evolution with pathogens with different lifestyles or invasion strategies. Further investigations on the evolution and roles of different LRR proteins as well as their relationships would provide new insights into plant immunity.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Seeds of pepper (*C. annuum*) cultivar GZ03, tobacco (*Nicotiana tabacum*) cultivar Honghuadajinyuan and *N. benthamiana* were sown in a soil mix [peat moss : perlite, 2 : 1 (v/v)] in plastic pots and placed in a growth room at 25°C, 60–70 mmol photons/m^2/s, relative humidity of 70% and a 16-h light/8-h dark photoperiod.

Pathogens and *R. solanacearum* inoculation

A highly virulent *R. solanacearum* strain FJC100301 was isolated from wilted samples of pepper in Fujian province (China) by our laboratory and amplified according to the method described previously (Dang *et al.*, 2013). The *R. solanacearum* strain was cultured at 28°C, 200 rpm in PSA medium (200 g/L potato, 20 g/L sucrose, 3 g/L beef extract, 5 g/L tryptone) and resuspended in 10 mM $MgCl_2$ solution. The bacterial cell solution used for inoculation was diluted to 10^8 colony-forming units (cfu)/mL [optical density at 600 nm (OD_{600}) = 0.8]. For root inoculation, pepper or tobacco plants at the eight-leaf stage were irrigated with 1 mL of the resulting *R. solanacearum* suspension, and the disease symptoms were scored at 7 dpi. For leaf inoculation, the third leaves from the top of pepper or tobacco plants at the eight-leaf stage were infiltrated with 10 μL of the *R. solanacearum* suspension described above using a syringe without a needle, and the mock was inoculated with 10 mM $MgCl_2$. The leaves were collected at the indicated time points for further analysis.

Treatment of plants with exogenous hormones

Pepper plants at the four-leaf stage were sprayed with 1 mM SA (in 10% distilled ethanol), 100 μM MeJA (in 10% distilled ethanol) or 100 μM ETH (in sterile double-distilled H_2O). Mock treatment was performed by spraying with the corresponding solvent or sterile double-distilled H_2O.

Vector construction

To construct vectors for overexpression, the full-length ORF of *CaLRR51*, or SP and/or TR deletion mutants of *CaLRR51* (Δ*SP-CaLRR51*, *CaLRR51-ΔTR* and Δ*SP-CaLRR51-ΔTR*), were cloned into the entry vector pDONR207 by BP reaction, and then cloned into destination vectors pEarleyGate201 by LR reaction using a Gateway cloning technique (Invitrogen, Carlsbad, CA, USA). To construct the vector for VIGS, a specific 351-bp fragment in the ORF of *CaLRR51* was searched for by BLAST against the genome sequence in the databases of CM334 (http://peppergenome.snu.ac.kr/) and Zunla-1 (http://peppersequence.genomics.cn/page/species/blast.jsp). The specific fragment was cloned into the entry vector pDONR207, and then cloned into the PYL279 vector. For subcellular localization, *CaLRR51* and its SP/TR deletion mutants (Δ*SP-CaLRR51*, *CaLRR51-ΔTR* and Δ*SP-CaLRR51-ΔTR*) were fused with GFP. These GFP fusion constructs were driven by the constitutive CaMV 35S promoter. As the N-terminal 12-amino-acid peptide of CBL1 (CBL1n) had been demonstrated to confer plasma membrane targeting (Batistic *et al.*, 2008), a CBL1n-RFP fusion construct (35S:*CBL1n-RFP*) was also generated for use as an additional control.

VIGS of *CaLRR51* in pepper plants

For VIGS of *CaLRR51* in pepper plants, the TRV-based VIGS system was employed according to previous studies (Dang *et al.*, 2013). *Agrobacterium tumefaciens* strains GV3101 harbouring PYL192 and PYL279-*CaLRR51*, or PYL279 as a negative control, were resuspended in induction medium [10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 10 mM $MgCl_2$, 200 μM acetosyringone, pH 5.6; OD_{600} = 0.8] at a 1 : 1 ratio, and then co-infiltrated into cotyledons of 2-week-old pepper plants. The agro-infiltrated pepper plants were grown in the dark at 16°C for 56 h, and then transferred into a growth room at 25°C, 60–70 mmol photons/m^2/s, relative humidity of 70% and a 16-h light/8-h dark photoperiod for 3–4 weeks for further analysis.

Transient expression of *CaLRR51* in pepper leaves

For transient expression analysis, *Ag. tumefaciens* strains GV3101 harbouring the *35S:CaLRR51*, *35S:*Δ*SP-CaLRR51*, *35S:CaLRR51-ΔTR*, *35S:*Δ*SP-CaLRR51-ΔTR* or *35S:00* vector (empty vector used as a control) were grown overnight, and then resuspended in induction medium. A bacterial suspension (OD_{600} = 0.8) was infiltrated into leaves of pepper plants at the eight-leaf stage using a syringe without a needle, and the injected leaves were collected at the indicated time points for further use.

Generation of transgenic *CaLRR51*-overexpressing tobacco plants

Tobacco cultivar Honghuadajinyuan was used to generate *CaLRR51*-overexpressing tobacco plants by the transformation of leaf discs with *Ag. tumefaciens* strain GV3101 harbouring the *35S:CaLRR51* vector. Fifteen independent T_0 transgenic tobacco lines were selected by hygromycin, and further confirmed by PCR and qRT-PCR. Two T_2 transgenic lines exhibiting the highest levels of *CaLRR51* transcripts were selected for analyses in this study.

Subcellular localization

Agrobacterium tumefaciens strains GV3101 containing *35S:CaLRR51-GFP*, *35S:*Δ*SP-CaLRR51-GFP*, *35S:CaLRR51-ΔTR-GFP*, *35S:*Δ*SP-CaLRR51-ΔTR-GFP*, *35S:GFP* (used as a control) or 35S:*CBL1n-RFP* (used as an additional control targeting to the plasma membrane) were grown overnight, and then resuspended in induction medium (OD_{600} = 0.8). *Agrobacterium tumefaciens* strains harbouring each of the GFP fusion constructs and the 35S:*CBL1n-RFP* construct were mixed in a 1 : 1 ratio and co-infiltrated into *N. benthamiana* leaves using a syringe without a needle. At 48 hpi,

fluorescence was imaged using a laser scanning confocal microscope (TCS SP8, Leica, Solms, Germany).

Histochemical staining

Leaves were stained with trypan blue and DAB according to the method described by Choi *et al.* (2012), following the detailed process reported previously (Cai *et al.*, 2015; Dang *et al.*, 2013; Liu *et al.*, 2015).

qRT-PCR

To determine the relative transcription levels of selected genes, real-time PCR was performed with specific primers (Table S1, see Supporting Information) 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, Dalian, China). Total RNA preparation and real-time RT-PCR were carried out following the procedures described in our previous studies (Cai *et al.*, 2015; Dang *et al.*, 2013). Four independent biological replicates of each treatment were performed. Data were analysed by the Livak method (Livak and Schmittgen, 2001) and expressed as a normalized relative expression level ($2^{-\Delta\Delta CT}$) of the respective genes. The relative transcript level of each sample was normalized to *CaActin*.

ACKNOWLEDGEMENTS

We thank Mark D. Curtis for kindly providing the Gateway destination vectors. This work was supported by grants from the National Natural Science Foundation of China (31372061, 31401890, 31401312, 31501767 and 31572136).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Comparison of the amino acid sequence of CaLRR51 with that of other representative related leucine-rich repeat (LRR) proteins. Sequence alignment of CaLRR51 with *Arabidopsis thaliana* AtLRR51 (accession no: NP_193611.1), *Capsicum annuum* CaLRR1 (accession number: AY237117.1) and *Oryza sativa* OsLRR1 (accession number: AAO85403.1).

Fig. S2 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *CaLRR1* in pepper plants during *Ralstonia solanacearum* infection. The third leaves from the top of pepper plants at the eight-leaf stage were infiltrated with 10 μL of the highly virulent *R. solanacearum* strain FJC100301 suspension [optical density at 600 nm (OD_{600}) = 0.8] using a syringe without a needle, and the mock was inoculated with 10 mM $MgCl_2$. The leaves were collected at 24 and 48 h after leaf inoculation with *R. solanacearum* or $MgCl_2$ for RNA extraction and qRT-PCR analysis.

Table S1 Primers for polymerase chain reaction (PCR) and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) used in this study.