Planta (2017) 246:351–364
DOI 10.1007/s00425-017-2709-5

REVIEW

Functional roles of the pepper leucine-rich repeat protein and its interactions with pathogenesis-related and hypersensitive-induced proteins in plant cell death and immunity

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Received: 10 April 2017 / Accepted: 6 May 2017 / Published online: 15 May 2017

Abstract

Main conclusion **Pepper leucine-rich repeat protein (CaLRR1) interacts with defense response proteins to regulate plant cell death and immunity. This review highlights the current understanding of the molecular functions of CaLRR1 and its interactor proteins.**

Plant cell death and immune responses to microbial pathogens are controlled by complex and tightly regulated molecular signaling networks. *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*)-inducible pepper (*Capsicum annuum*) leucine-rich repeat protein 1 (*CaLRR1*) serves as a molecular marker for plant cell death and immunity signaling. In this review, we discuss recent advances in elucidating the functional roles of CaLRR1 and its interacting plant proteins, and understanding how they are involved in the cell death and defense responses. CaLRR1 physically interacts with pepper pathogenesis-related proteins (CaPR10 and CaPR4b) and hypersensitive-induced reaction protein (CaHIR1) to regulate plant cell death and defense responses. CaLRR1 is produced in the cytoplasm and trafficked to the extracellular matrix. CaLRR1 binds to CaPR10 in the cytoplasm and CaPR4b and CaHIR1 at the plasma membrane. CaLRR1 synergistically accelerates CaPR10-triggered hypersensitive cell death, but negatively regulates CaPR4b- and CaHIR1-triggered cell death. CaHIR1 interacts with *Xcv* filamentous hemagglutinin (Fha1) to trigger disease-associated cell death. The subcellular localization and cellular function of these CaLRR1 interactors during plant cell death and defense responses were elucidated by *Agrobacterium*-mediated transient expression, virus-induced gene silencing, and transgenic overexpression studies. *CaPR10*, *CaPR4b*, and *CaHIR1* positively regulate defense signaling mediated by salicylic acid and reactive oxygen species, thereby activating hypersensitive cell death and disease resistance. A comprehensive understanding of the molecular functions of CaLRR1 and its interacting protein partners in cell death and defense responses will provide valuable information for the molecular genetics of plant disease resistance, which could be exploited as a sustainable disease management strategy.

Keywords Cell death · Defense response · Leucine-rich repeat (LRR) protein · Pathogenesis-related (PR) protein · Pepper (*Capsicum annuum*) · Protein–protein interaction

Abbreviations

ET	Ethylene
HIR	Hypersensitive-induced reaction
JA	Jasmonic acid
LRR	Leucine-rich repeat
PR	Pathogenesis related
SA	Salicylic acid
SP	Signal peptide

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Introduction

Plants have evolved complex signal perception and resistance mechanisms to defend against microbial pathogen attack. During avirulent pathogen infection, plant resistance (R) proteins recognize pathogen avirulence (Avr) factors and initiate the hypersensitive response (HR) and localized programmed cell death (PCD) at the infection site (Coll et al. 2011). HR is the strongest plant strategy to prevent the growth and spread of invasive pathogens into healthy tissues. The hypersensitive cell death response includes a transient oxidative burst; rapid ion fluxes; mitogen-activated protein kinase (MAPK) signaling; accumulation of signaling molecules such as nitric oxide, salicylic acid (SA), and jasmonic acid (JA); induction of pathogenesis-related (PR) proteins; phytoalexin accumulation; and antimicrobial compound synthesis (Beers and McDowell 2001; Lam et al. 2001; Mur et al. 2008; Spoel and Dong 2008; Melech-Bonfil and Sessa 2010; Coll et al. 2011). By contrast, compatible plant-pathogen interactions lead to susceptible cell death relatively late during the course of infection, which enables the growth and spread of invasive pathogens into healthy tissues. There is convincing experimental evidence to indicate that host-controlled PCD is also closely associated with the onset of susceptible cell death and disease development in plants (Yao et al. 2002; Greenberg and Yao 2004; Choi et al. 2011).

Most resistance (*R*) genes encode members of an extremely polymorphic superfamily of nucleotide-binding leucine-rich repeat (NLR) receptors (Maekawa et al. 2011; Dangl et al. 2013). The leucine-rich repeat (LRR) domain is a conserved feature of many R proteins, including NLR-type proteins (Dangl and Jones 2001). LRRs are present in the sequences of more than 2000 proteins with diverse function and origin in viruses, bacteria, archaea, and eukaryotes (Enkhbayar et al. 2004). The LRR domain provides a structural framework for diverse and specific molecular interactions. LRR proteins function in an array of developmental and immune signaling pathways and are involved in receptor/coreceptor complex formation (Kobe and Deisenhofer 1994; Jaillaisa et al. 2011). Some NLR proteins directly bind to pathogen-associated proteins, primarily pathogen effector molecules, to induce basal defense responses and effector-triggered immunity (ETI) in resistant host genotypes (DeYoung and Innes 2006; Jones and Dangl 2006; Han and Hwang 2017).

The pepper (*Capsicum annuum*)–*Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) pathosystem is useful for exploring the molecular and cellular bases of plant cell death and defense responses to microbial pathogens (Han and Hwang 2017). Infection with the avirulent (incompatible) *Xcv* strain Bv5-4a carrying the effector AvrBsT activates rapid and distinct expression of a repertoire of cell death- and defense-related genes in pepper plants (Hong et al. 2008; Choi and Hwang 2011, 2015; Kim et al. 2014; Kim and Hwang 2015a, b; Han and Hwang 2017). The pepper LRR1 (*CaLRR1*) gene is induced by pathogens and encodes a small extracellular protein containing a single LRR domain with five tandem repeats of a 24 amino acid LRR motif (Jung et al. 2004). CaLRR1 lacks a kinase domain, although it exhibits sequence homology to RLKs, and is involved in plant cell death and defense responses (Jung et al. 2004; Jung and Hwang 2007). *CaLRR1* serves as a molecular marker for microbial perception in pepper and can be detected in pepper leaves infected with the avirulent *Xcv* strain Bv5-4a using an array-based differential hybridization technique (Jung and Hwang 2000).

The pepper PR proteins CaPR10 (Choi et al. 2012) and CaPR4b (Hwang et al. 2014) and the pepper HR-induced protein CaHIR1 (Jung and Hwang 2007; Choi et al. 2011) are host proteins that physically interact with CaLRR1 during cell death and defense responses in plants. CaPR10, CaPR4b, and CaHIR1 can be isolated from pepper leaves and identified using yeast two-hybrid screening (Fields and Song 1989), bimolecular fluorescence complementation (BiFC) (Walter et al. 2004), and co-immunoprecipitation (Co-IP) assays. Cytoplasmic CaPR10 functions in HR-like cell death and defense signaling, and this activity is enhanced by interaction with CaLRR1 (Choi et al. 2012). CaPR4b interacts with CaLRR1 in the plasma membrane to suppress CaPR4b-triggered cell death and defense response (Hwang et al. 2014). CaLRR1 interacts with CaHIR1 during pathogenesis and suppresses CaHIR1-induced cell death (Jung and Hwang 2007). CaLRR1 and CaHIR1 proteins are proposed to act as cell death regulators associated with plant immunity and disease, respectively (Choi et al. 2011).

In this review, we discuss the identification and functional characterization of CaLRR1 and its interacting partner proteins during cell death response and immunity in plants (Table 1). Molecular and cellular responses of transgenic *Arabidopsis* plants overexpressing these CaLRR1-interacting pepper proteins are analyzed to provide insights into the crucial roles of cell death-associated pepper proteins in a heterologous cellular system.

Structures and molecular functions of leucine-rich repeat proteins

LRR domains are present in the primary structures of many biologically active proteins involved in perception and signal transduction networks, including immune receptors, ubiquitin ligases, hormone receptors, enzyme inhibitors, cell

Table 1 Pepper pathogenesis-related and hypersensitive-induced reaction proteins that interact with the pepper leucine-rich repeat protein 1

Protein	Accession no.	Putative function	Localization	References
CaLRR1	AY237117	Positive and negative regulation of HR cell death	Extracellular matrix	Jung and Hwang (2000), Jung et al. (2004)
CaPR4b	HM581975	Induction of HR cell death	Synthesized in ER	Hwang et al. (2014)
		Antifungal activity	Secreted to apoplastic space	
CaPR10	JF345171	Induction of HR cell death	Complex formation with CaLRR1 in cytoplasm	Choi et al. (2012)
		RNase activity	Secreted to apoplastic space	
CaHIR1	AY529867	Induction of HR cell death	Plasma membrane	Jung and Hwang (2007), Jung et al. (2008), Choi et al. (2011)

ER endoplasmic reticulum, *HR* hypersensitive response

adhesion molecules, and ribosome-binding proteins (Kobe and Kajava 2001; Napier 2004; Padmanabhan et al. 2009; Eitas and Dangl 2010). The ubiquity of the LRR domain may be due to its ability to interact with a wide range of substrates, including proteins, nucleic acids, lipids, and small hormone molecules (Helft et al. 2011). The LRR structural motif contains 20–30 amino acids with a characteristic repetitive sequence pattern that is enriched with the hydrophobic amino acid leucine (Bella et al. 2008). A defining feature of the LRR motif is the 24-residue consensus sequence (LxxLxxLxxLxLxxNxLxGxIPxx; x is any amino acid) (Jones and Jones 1997). In plants and animals, LRRs occur in tandem arrays of 1 to >40 LRR motifs. LRRs contain leucine (L) or other hydrophobic residues at regular intervals and regularly spaced proline (P) and asparagine (N) (Kobe and Deisenhofer 1994; Jung et al. 2004).

LRRs are an important feature of the receptors that mediate the two major plant immune systems, pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), and effector-triggered immunity (ETI) (Jones and Dangl 2006). Many plant cell surface receptors that recognize microbe- or pathogen-associated molecular pattern (MAMP or PAMP) molecules contain LRRs as the bulk of their extracellular recognition domain and intracellular protein kinase domain (Boller and Felix 2009; Nicaise et al. 2009). Different classes of extracellular LRR proteins have been identified in plants (Zhou et al. 2009). Receptor-like kinases (RLKs) (Shiu and Bleecker 2003) contain an extracellular LRR domain, a transmembrane α-helical structure, and an intracellular kinase domain. Receptor-like proteins (RLPs) (Wang et al. 2008) contain an extracellular LRR domain and a C-terminal membrane anchor, but lack the intracellular kinase domain. RLKs and RLPs are involved in diverse biological processes in plants, such as innate immunity, phytohormone responses, cell proliferation, and self-incompatibility (Tor et al. 2009). Some RLKs (e.g., FLS2 and Xa21) recognize pathogen-derived molecules and lead to *R* gene-triggered resistance in plants (Boller and Felix 2009; Monaghan and Zipfel 2012). RLPs (e.g., Cf-9, RPP27, and EIX1) mediate disease resistance by recognizing pathogen-derived molecules (van der Hoorn et al. 2005). Polygalacturonase-inhibiting proteins (PGIPs) share high homologies with a class of animal extracellular LRR proteins named small leucine-rich proteoglycans (SLRPs) (Lorenzo et al. 2001). PGIPs are soluble proteins of the extracellular matrix and contain LRRs in the central domain flanked by cysteine-rich clusters. PGIPs recognize and bind fungal polygalacturonases to prevent plant cell wall degradation and they also induce plant defense responses (Alexandersson et al. 2011). The LRR modules are responsible for PGIP recognition of pathogenic factors; solvent-exposed residues in the LRR β-strand/β-turn motifs are the determinants of recognition specificity (Lorenzo et al. 2001).

LRRs are central for recognition specificity in the large, diverse superfamily of plant intracellular nucleotide-binding (NB)-LRR (NLR) receptors known as resistance (R) proteins. R proteins recognize specific pathogen effectors and then modify their host plant targets (or decoys) to initiate strong defense responses such as ETI (Dangl et al. 2013). NLR-type proteins have intracellular LRR domains that determine their cytoplasmic localization (Gururani et al. 2012). Small LRR proteins without any additional functional structures are involved in the regulation of plant resistance to pathogen infection. A number of small LRR proteins are induced in plant cells by biotic and/or abiotic stimuli. The following small LRR proteins have been identified in solanaceous plants: tobacco NtLRR2 (accession no. EF535611; Xu et al. 2009), tobacco NtLRP1 (accession no. DQ118081; Jacques et al. 2006), tomato LeLRP (accession no. X95269; Tornero et al. 1996), potato StRSI7 (accession no. JQ315227; Jung et al. 2004), and pepper CaLRR1 (accession no. AY237117; Jung et al. 2004), as well as rice OsLRR1 (accession no. AAO85403; Zhou et al. 2009).

These small LRR proteins share structural features such as signal peptides (SPs) and leucine-zipper (LZ) and LRR motifs in the conserved regions, although the domain sizes slightly differ (Fig. 1a). The solanaceous small LRR proteins contain five tandem LRR motifs within the consensus sequence (LxxLxxLxxLxLxxNxLxGxIPxx) (Fig. 1a). The first LRR is imperfect and begins with valine (V) in the first position. The N-terminus contains a putative SP of 23–34 amino acid residues, and 4 leucine (L) residues in the LZ domain are conserved in the SP and the first imperfect LRR motif. In CaLRR1, the interstitial variable residues (i.e., the x residues in the LxxLxLxxN β-strand/β-turn motif) are not conserved and there are no transmembrane or kinase domains. Tobacco NtLRR2 and NtLRP1, tomato LeLRP, and potato StRSI7 have one imperfect and four perfect LRR motifs, similarly to the pepper CaLRR1. NtLRP1 is a relatively large small LRR protein with a proline-rich region at the C-terminus. There is high homology in the primary amino acid sequences of CaLRR1 and NtLRR2 (Fig. 1a, b). Small LRR proteins identified in monocots include sorghum SbLRR1 and SbLRR2, rice OsLRR1 and OsLRR2, and maize ZmLRR2 (Zhu et al. 2013). SbLRR2 has an SP at the N-terminus and six LRR motifs at the C-terminus, but it lacks the LZ structure. Rice OsLRR1 contains an SP, an LZ motif, and five LRR motifs, and is structurally similar to pepper CaLRR1 (Fig. 1a, b).

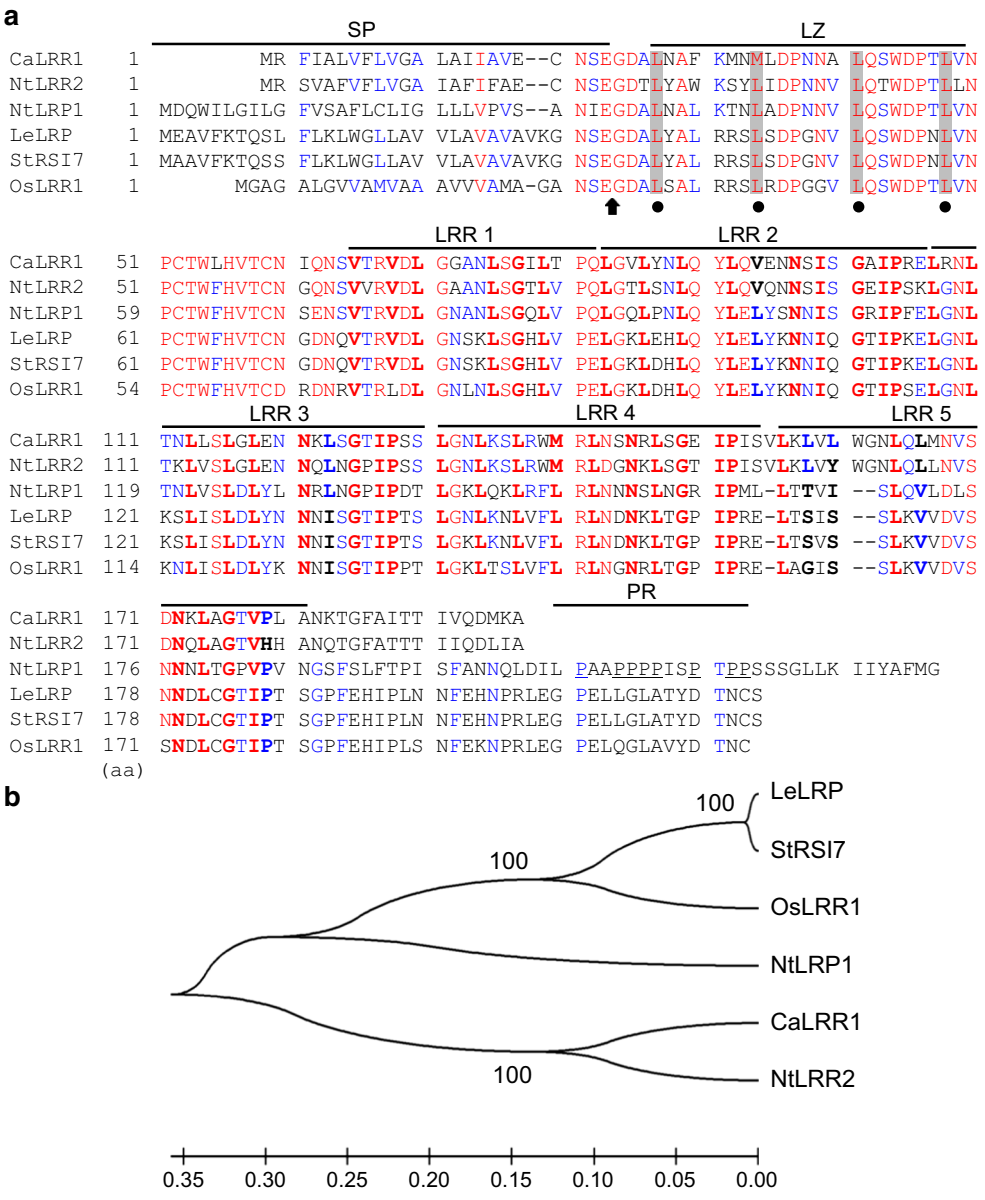

Fig. 1 Structural domains and deduced amino acid sequences of leucine-rich repeat (LRR) proteins from solanaceous plants. *SP* signal peptide, *LZ* leucine-zipper motif, *PR* proline-rich region, *aa* amino acid. **a** Structural domains and amino acid sequence alignment of pepper CaLRR1 (accession no. AY237117, Jung et al. 2004), tobacco NtLRR2 (accession no. EF535611, Xu et al. 2009), tobacco NtLRP1 (accession no. DQ118081, Jacques et al. 2006), tomato LeLRP (accession no. X95269, Tornero et al. 1996), potato StRSI7 (accession no. JQ315227), and OsLRR1 (accession no. AAO85403, Zhou et al. 2009). *Red* and *blue letters* indicate highly and moderately conserved amino acids, respectively. The *arrow* indicates putative cleavage sites for the mature proteins. A periodic repetition of leucine residues at every seventh position to form leucine-zipper motifs is indicated by *black dots*. **b** Phylogenetic tree of some solanaceous LRR proteins and a rice LRR protein. The tree is constructed based on primary amino acid sequences using the neighbor-joining method of MEGA 4.0.2 software

Identification and molecular functions of pepper leucine-rich repeat protein 1

Differential hybridization is used to isolate specific cDNAs that are differentially or strongly expressed in pepper leaves infected with avirulent *Xcv* strain Bv5-4a (Jung and Hwang 2000). This is an effective strategy for isolation of a large number of differentially expressed defense-related genes in plants. Automated partial cDNA sequencing is conducted on randomly selected cDNAs to generate expressed sequence tags (ESTs) for the functional identification, mapping, and comparison of all plant genes. A number of pepper cDNAs have been isolated that appear to correspond to mRNAs that increased in abundance during avirulent *Xcv* strain Bv5-4a infection (Jung and Hwang 2000). The DNA sequence of *CaLRR1* [accession numbers AF082727 (EST clone) and AY237117 (full-length cDNA)], which is strongly induced by pathogen infection in pepper leaves, displays high homology with the small LRR proteins SbLRR and LeLRP (Hipskind et al. 1996; Tornero et al. 1996). These LRRs comprise approximately 65% of the entire mature protein. The first LRR is imperfect and begins with valine at residue 65. CaLRR1 also contains five potential *N*-linked glycosylation sites on asparagine residues at positions 74, 96, 109, 168, and 182. The pepper genome contains at least two or more copies of genes homologous to *CaLRR1* (Jung et al. 2004).

RNA gel-blot analyses of *CaLRR1* expression in pepper leaves infected with various pathogens indicate that *CaLRR1* is induced by pathogens and suggest that *CaLRR1* is involved in limiting the rate of pathogen infection (Jung et al. 2004). More importantly, *CaLRR1* induction is higher in incompatible than compatible interactions after infection with *X. campestris* pv. *vesicatoria*, *Phytophthora capsici*, *Colletotrichum coccodes*, and *Colletotrichum gloeosporioides*. Inoculation with non-plant pathogenic bacteria (*Pseudomonas fluorescens* and *Escherichia coli*) efficiently triggers *CaLRR1* expression in pepper leaf tissues (Jung et al. 2004). These results suggest that diverse types of MAMP or PAMP molecules trigger *CaLRR1* expression and lead to multiple defense responses. *SbLRR2*, *OsLRR1*, *NtLRR2*, and *LeLRP* are differentially induced by *Colletotrichum sublineolum*, *X. oryzae* pv. *oryzae*, *tobacco mosaic virus*, and *citrus exocortis viroid*, respectively (Tornero et al. 1996; Xu et al. 2009; Zhou et al. 2009; Zhu et al. 2015). However, it is not fully understood whether MAMP/PAMP molecules are required for the induction of small *LRR* genes in host plant cells.

Salicylic acid (SA) acts as a key endogenous signal molecule for the induction of defense-related genes and systemic acquired resistance (SAR) in a variety of plant species (Durrant and Dong 2004; Vlot et al. 2009; Dempsey et al. 2011). Exogenous application of SA or SA analogs induces *PR* gene expression and enhances disease resistance in plants (Cao et al. 1994). JA and ethylene (ET) act either independently or cooperatively to induce resistance and defense-related gene expression. In addition to these synergistic interactions, JA and/or ET can interact antagonistically with SA (Grant and Jones 2009). Treatment with SA, methyl jasmonate (MeJA) and ET does not activate *CaLRR1* expression in pepper plants (Jung et al. 2004), indicating that *CaLRR1* expression is not regulated by defense signaling pathways activated by these molecules. *SbLRR2* encodes a small extracellular LRR protein that is strongly induced by treatment with exogenous MeJA, but not by SA or the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC), in sorghum seedlings (Zhu et al. 2015). *CaLRR1* is induced by treatment with high salinity, abscisic acid (ABA), and wounding. *NtLRR2* is induced in tobacco by high salinity (Xu et al. 2009), and wounding of rice leaves triggers *OsLRR1* expression (Zhou et al. 2009). ABA is proposed to be a crucial signaling molecule to activate disease resistance in many plant species (Fan et al. 2009). An in situ hybridization study reported that *CaLRR1* transcripts were localized in leaf phloem tissues, stems, and green fruits during pathogen infection and after treatment with ABA in pepper plants (Jung et al. 2004). The localization of *CaLRR1* transcripts in phloem cells suggests that CaLRR1 functions in phloem cells.

Identification and molecular functions of the pepper pathogenesis-related and hypersensitive-induced reaction proteins that interact with CaLRR1

Physical interactions between proteins have a critical role in the signaling cascades that activate plant defense responses to microbial pathogens (Cantu et al. 2013). LRR proteins may be involved in the protein–protein communication networks for plant innate immunity (Padmanabhan et al. 2009; Rebsamen et al. 2013). To identify proteins that interact with pepper small LRR proteins, a GAL 4-based yeast two-hybrid system was used with *CaLRR1* as bait to screen a prey cDNA library prepared from pepper leaves infected with avirulent *Xcv* strain Bv5-4a (Jung and Hwang 2007; Choi et al. 2012; Hwang et al. 2014). Bimolecular fluorescence complementation (BiFC; Walter et al. 2004) and co-immunoprecipitation (Co-IP) assays can be used to verify specific interactions between CaLRR1 and other proteins *in planta*. These yeast and *in planta* studies determined that CaLRR1 (Jung et al. 2004) physically interacted with the pepper PR proteins CaPR10 (Choi et al.

2012) and CaPR4a (Hwang et al. 2014) and the pepper HR-induced protein CaHIR1 (Jung and Hwang 2007; Choi et al. 2011). Therefore, CaPR10, CaPR4b, and CaHIR1 are identified as CaLRR1-interacting partners in pepper.

Pepper pathogenesis-related protein 10 (CaPR10)

CaPR10 is crucial for plant defense and cell death responses against microbial pathogens such as *Colletotrichum acutatum*, *X. campestris* pv. *vesicatoria* (*Xcv*), and tobacco mosaic virus (Park et al. 2004; Choi et al. 2012; Soh et al. 2012). Avirulent *Xcv* infection induces *CaPR10* expression associated with HR-induced cell death (Choi et al. 2012). Transient expression of *CaPR10* in pepper leaves induces partial necrotic cell death. Virus-induced gene silencing (VIGS) of *CaPR10* in pepper disrupts the resistance responses to avirulent *Xcv* infection (Choi et al. 2012). Heterologous *CaPR10* overexpression in *Arabidopsis* enhances resistance to *Pseudomonas syringae* pv. *tomato* and *Hyaloperonospora arabidopsidis* infection (Choi et al. 2012). *CaPR10* is also distinctly induced by SA, JA, ET, and oxidative and osmotic stresses (Park et al. 2004). The PR10 protein level is significantly higher in the resistant maize inbred line CO441 infected with *Fusarium graminearum* than in the susceptible inbred B73 line (Mohammadi et al. 2011). *ZmPR10* overexpression in transgenic *Arabidopsis* increases resistance to *P. syringae* pv. *tomato* infection (Xie et al. 2010). Transgenic peanut overexpressing *ARAhPR10* exhibits reduced *Aspergillus flavus* colonization and aflatoxin content in seeds (Xie et al. 2013). Overexpression of pea *PR10.1* in transgenic potato confers resistance to early dying disease caused by *Verticillium albo-atrum* or *V. dahlia* (Chang et al. 1993; Wang et al. 1999).

The PR protein PR10 is a member of the Bet v 1 allergen family and has ribonuclease and antimicrobial activity (Zhou et al. 2002; Park et al. 2004). Recombinant CaPR10 distinctly degrades torula yeast (*Candida utilis*) RNA (Choi et al. 2012), suggesting that CaPR10 possesses RNase activity. RNase activity is thought to be essential for the resistance response to microbial pathogens (Galiana et al. 1997; Shivakumar et al. 2000; Park et al. 2004). Recombinant CaPR10 exhibits antimicrobial activity against TMV and *P. capsici* in vitro (Park et al. 2004). Conserved PR10 motifs include phosphorylation sites that are characteristic of protein kinases (Bantignies et al. 2000; Ziadi et al. 2001). CaPR10 has a glycine-rich motif (GxGGxG) that forms an ATP- or a GTP-phosphate-binding loop (called the P-loop) at amino acid residues 46–51, and a consensus amino acid sequence (KAxExYL) in the C-terminal helix (Park et al. 2004). CaPR10 contains several putative phosphorylation sites at serine, threonine, and tyrosine residues. CaPR10 is phosphorylated by crude protein extracts from pepper leaves infected with avirulent *Xcv* Bv5-4a (Choi et al. 2012). TMV infection also leads to CaPR10 phosphorylation, a modification that may affect its RNase activity (Park et al. 2004). Together, we conclude that CaPR10 phosphorylation, RNase activity as well as antimicrobial activity are required for triggering cell death and defense responses in plants.

Pepper pathogenesis-related proteins 4b (CaPR4b)

The pepper PR protein CaPR4b is synthesized in the endoplasmic reticulum (ER), interacts with CaLRR1 at the plasma membrane, and is secreted into the apoplastic space (Hwang et al. 2014). CaPR4b contains a C-terminal BARWIN domain, which is a structural characteristic of BARWIN family proteins. CaPR4b also contains an ER signal containing 24 amino acid residues and an N-terminal chitin-binding domain (CBD). The CAPR4b BARWIN domain shares high sequence similarity with the BARWIN domain in BARWIN family proteins, which is referred to as PR4 (Friedrich et al. 1991). Notably, the BARWIN domain of PR4b is suggested to be involved in plant defense response to microbial pathogens such as *Rhizoctonia solani* and *Magnaporthe oryzae* (Zhu et al. 2006; Mukherjee et al. 2010). The CBD of CaPR4b is essential for CaPR4b binding to CaLRR1 in yeast and *in planta* (Hwang et al. 2014). BiFC and Co-IP assays show that $CaPR4b^{CBD}$ specifically binds to CaLRR1 at the plasma membrane. The BARWIN domain of CaPR4b is not required for in vitro and in vivo binding to CaLRR1.

CaPR4b has antifungal activity against pathogenic fungi, such as *Alternaria brassicicola*, *Botrytis cinerea*, *Colletotrichum orbiculare*, and *Fusarium oxysporum* f.sp. *matthioli* (Hwang et al. 2014). The in vitro antifungal activity against plant pathogenic fungi has been demonstrated for some plant PR4 proteins (Zhu et al. 2006; Mukherjee et al. 2010). Recombinant Wheatwin1, a wheat PR protein of class 4, exhibits antifungal activity against *Fusarium culmorum*, *F. graminearum*, and *B. cinerea* (Caruso et al. 2001; Bertini et al. 2009). Purified OsPR4b and *Lycoris radiata* LrPR4 have antifungal activity against *R. solani* (Zhu et al. 2006) and *M. oryzae* (Li et al. 2009), respectively.

The *Arabidopsis PR4* gene depends on JA/ET and is involved in the defense response against infection by the necrotrophic fungus *F. oxysporum* (Trusov et al. 2009). Infection with another necrotrophic fungal pathogen, *A. brassicicola*, also strongly induces *PR4* expression in *Arabidopsis* (Mukherjee et al. 2010). *CaPR4b* expression is rapidly and strongly induced in pepper leaves during *Xcv* infection (Hwang et al. 2014). Elicitor treatment with ET, SA, ABA, MeJA, NaCl, methyl viologen, wounding, and drought significantly induces *CaPR4b* gene expression.

Among these elicitors of *CaPR4b* expression, the effect of ET is most pronounced in pepper leaves. Transient *CaPR4b* expression significantly induces programmed cell death (PCD) in *Nicotiana benthamiana* leaves. The *PR4* gene is strongly induced in incompatible plant–pathogen interactions (Jacquard et al. 2009). Knock-down suppression of *CaPR4b* via VIGS leads to enhanced susceptibility to *Xcv* infection, which is accompanied by reductions in electrolyte leakage and oxidative burst. *CaPR4b* overexpression in *Arabidopsis* also enhances resistance to infection by virulent *P. syringae* pv. *tomato* DC3000 and *H. arabidopsidis*. The combined results indicate that CaPR4b acts as a positive regulator of plant basal defense and cell death responses to microbial pathogens (Hwang et al. 2014).

Pepper hypersensitive-induced reaction protein (CaHIR1)

The hypersensitive-induced reaction (HIR) proteins, along with the prohibitins and stomatins, belong to a PID (proliferation, ion, and death) superfamily involved in cell proliferation, ion channel activity, and cell death (Nadimpalli et al. 2000). These PID proteins share common features in their SPFH (stomatins, prohibitins, flotillins, and HflK/C) domains. The pepper CaHIR1 has an SPFH domain that contains putative transmembrane helices between amino acid residues 30 and 49 (Jung and Hwang 2007; Choi et al. 2011). Most HIR proteins including pepper CaHIR1 contain an *N*-myristoylation site at the N-terminus and putative transmembrane regions embedded in the band-7 domain. The plant HIR proteins are predicted to have an α-helical coil near their C-termini, which may act as a plug to regulate potassium ion channels (Nadimpalli et al. 2000).

HIR genes are differentially expressed in plant leaves during the development of spontaneous micro-HR lesions (Nadimpalli et al. 2000; Rostoks et al. 2003; Jung and Hwang 2007). *HIR* genes have been identified in plant cell death and defense responses to biotic and abiotic stresses in several plant species, including tobacco, maize, barley, rice, wheat, pepper, soybean, and *Arabidopsis* (Karrer et al. 1998; Nadimpalli et al. 2000; Rostoks et al. 2003; Jung and Hwang 2007; Yu et al. 2008; Zhou et al. 2009, 2010; Qi et al. 2011; Xiang et al. 2015). The three maize *HIR* genes *Zm-hir1*, *Zm-hir2*, and *Zm-hir3* share strong sequence homology with NG1 (Nadimpalli et al. 2000). *Zm-hir3* is strongly induced in the maize disease lesion mimic mutant *Les9*, suggesting a possible role in HR-induced cell death. Constitutively elevated barley *Hv-hir3* expression in lesion mimicking barley mutants is suggested to mediate spontaneous cell death (Rostoks et al. 2003). Pepper CaHIR1 elicits spontaneous cell death in plants (Jung and Hwang 2007). *CaHIR1* expression triggers cell death responses associated with disease and immunity during *X. campestris* pv. *vesicatoria* (*Xcv*) infection (Choi et al. 2011). Wheat *TaHIR2* and *TaHIR3* are strongly induced in wheat leaves by *Puccinia striiformis* infection (Zhang et al. 2009, 2011). Rice *OsHIR1* is induced in rice leaves by *Xanthomonas oryzae* pv. *oryzae* infection (Zhou et al. 2010). Defense responses activated by the OsHIR1–OsLRR1 protein complex are well documented in rice plants during *X. oryzae* pv. *oryzae* infection (Zhou et al. 2009). Soybean *GmHIR1*, *GmHIR3*, and *GmHIR4* are more rapidly expressed in response to *Phytophthora sojae* infection in the resistant line than in the susceptible line (Xiang et al. 2015). The *Arabidopsis HIR* genes are significantly induced by MAMPs such as the bacterial flagellin fragment flg22 (Qi et al. 2011). AtHIR proteins are physically associated with the immune receptor RPS2 and quantitatively contribute to RPS2-mediated ETI (Qi et al. 2011).

Pepper CaHIR1 acts as a positive regulator of PCD associated with plant immunity and disease (Jung and Hwang 2007; Choi et al. 2011). *CaHIR1*-overexpressing transgenic *Arabidopsis* plants exhibit elevated defense responses to challenge with the virulent bacterial pathogen *P. syringae* pv. *tomato* and the oomycete *H. arabidopsidis* (Jung and Hwang 2007; Jung et al. 2008). However, *CaHIR1*-overexpressing transgenic plants are highly susceptible to infection by the necrotrophic fungus *B. cinerea* (Jung et al. 2008). Augmented SA-dependent defenses of *CaHIR1*-overexpressing plants antagonistically suppress JA/ET-dependent signaling, which is usually responsible for defense against necrotrophic pathogen infection. Overexpression of *AtHIR1* and *AtHIR2*, and rice *OsHIR1* in *Arabidopsis* enhances resistance to virulent *P. syringae* pv. *tomato* infection, but an enhanced susceptibility to necrotrophic pathogens could not be excluded (Zhou et al. 2010; Qi et al. 2011). By contrast, *CaHIR1* silencing distinctly disrupts hypersensitive and susceptible cell death in pepper plants (Choi et al. 2011). In addition, CaHIR1 interacts with the *Xcv* virulence factor filamentous hemagglutinin-like protein (Fha1), which induces disease susceptibility and cell death and suppresses *PR* gene expression in pepper plants (Choi et al. 2013). Fha proteins of plant pathogens such as *Erwinia chrysanthemi*, *Xanthomonas axonopodis* pv. *citri*, and *Xylella fastidiosa* function as virulence factors by modulating surface attachment, biofilm formation, and cell-to-cell aggregation during host plant infection (Rojas et al. 2002; Guilhabert and Kirkpatrick 2005; Gottig et al. 2009). Pepper cell death is triggered in leaf tissues by *Xcv*-releasing Fha1 (Choi et al. 2013). *Xcv* Fha1 enhances susceptible host cell death, suppresses basal immunity, and increases bacterial spot disease in pepper plants.

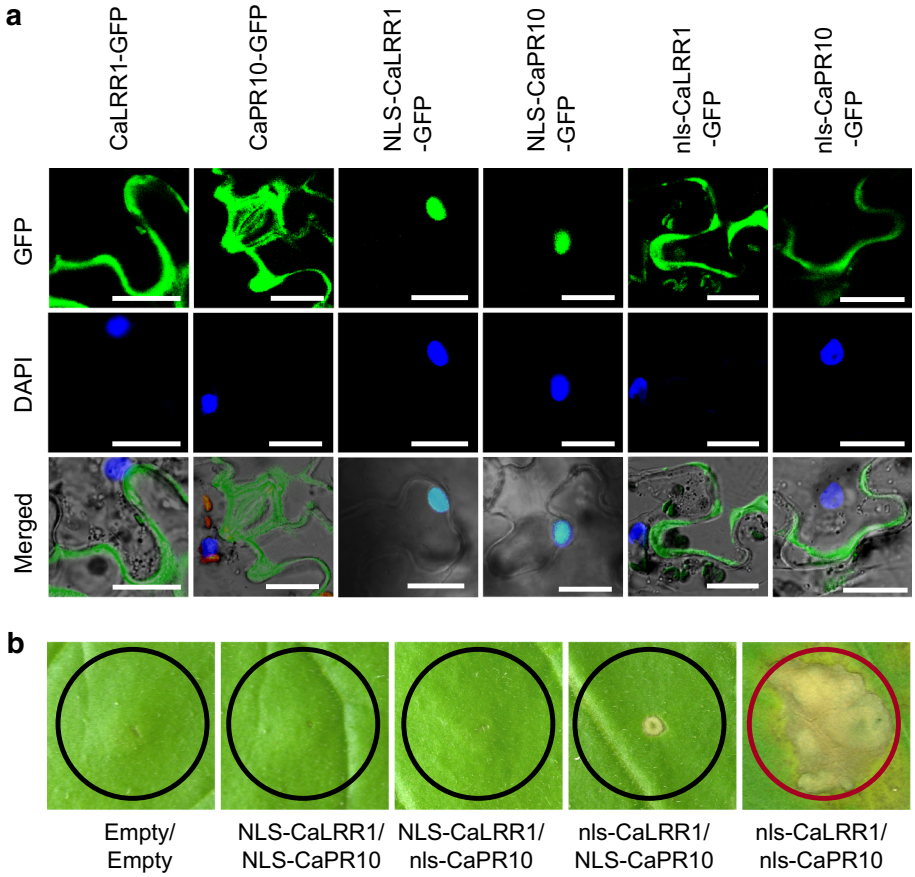

Fig. 2 The cytoplasmic CaLRR1/CaPR10 complex is required for cell death induction in *N. benthamiana* leaves (Choi et al. 2012). **a** Subcellular localization of CaLRR1 and CaPR10 proteins with wild-type (NLS) and modified (nls) nuclear localization signal peptides. For nuclear localization, the nuclear localization signal (NLS) is fused to the N-terminus of *CaLRR1* or *CaPR10*. The nls sequence contains a lysine (Lys) residue substituted with an asparagine (Asn) residue. The nls sequence, which fails to target to nuclei, is included as a negative control. The sequences used are NLS (5′-GGCCCTAAAAAGAAGCGTAAGGTT-3′) and nls (5′-GGCCCTAAAAACAAGCGTAAGGTT-3′) (Hodel et al. 2001). **b** Induction of cell death response by transient expression of the nls-CaLRR1/nls-CaPR10 complex in the cytoplasm via agroinfiltration

Subcellular localization and cellular functions of CaLRR1–CaPR10/CaPR4b/CaHIR1 complexes

Cellular trafficking of pepper CaLRR1 and its interacting partner proteins is crucial for fine-tuned regulation of plant cell death and immunity to *Xcv* infection. CaLRR1 is produced in the cytoplasm, located in the cytoplasm and plasma membrane, and secreted into the apoplastic space (Jung and Hwang 2007; Choi et al. 2011, 2012; Hwang et al. 2014). Different subcellular locations of CaLRR1 play important roles in host cell death and immunity. Small LRR proteins are generally located in the plant plasma membrane, and LRR domains may be exposed to the extracellular space, which contains active components capable of regulating cell–cell interactions during development and in response to biotic or abiotic stresses (Steinmayer et al. 1994; Hipskind et al. 1996; Tornero et al. 1996).

The subcellular localization sites of the CaLRR1–CaPR10 complex are determined to investigate whether the cellular trafficking of the LRR and PR protein complexes is essential for the cell death-mediated defense signaling. CaLRR1 and CaPR10 are located in the cytoplasm and bind with each other (Choi et al. 2012). The CaLRR1–CaPR10 complex translocates to the plasma membrane and is secreted into the extracellular space. Cytoplasmic localization of the CaLRR1–CaPR10 complex is required for induction of the hypersensitive cell death response (Choi et al. 2012). Fusion of a *simian vacuolating virus 40* nuclear localization signal (NLS) sequence (PKKKRKV) with CaLRR1 and CaPR10 enables these proteins to localize in the nucleus (Hodel et al. 2001). Confocal microscopy images show that NLS fusion targets NLS-CaLRR1-GFP or NLS-CaPR10-GFP to the nuclei (Fig. 2a). Nuclear or nucleocytoplasmic co-expression of NLS or nls-fused CaLRR1 and CaPR10 does not trigger the

cell death response in *N. benthamiana* leaves. By contrast, co-expression of nls-CaLRR1 and nls-CaPR10 3 days after agroinfiltration induces hypersensitive cell death response (Fig. 2b). *Agrobacterium*-mediated transient co-expression of *CaLRR1* and *CaPR10* enhances *CaPR10*-triggered hypersensitive cell death, similar to that triggered by *Bax* or *avrPto/Pto* expression in pepper and *N. benthamiana* leaves (Choi et al. 2012). Their co-expression results in high levels of electrolyte leakage and callose deposition in the pepper leaves. Mammalian Bax triggers cell death in plants (Lacomme and Santa Cruz 1999), and physical interaction of *P. syringae* pv. *tomato* AvrPto and tomato Pto kinase triggers HR-induced disease resistance (Tang et al. 1996). CaLRR1 promotes the ribonuclease activity and phosphorylation of CaPR10, which leads to enhanced cell death signaling (Choi et al. 2012).

CaPR4b has a putative signal peptide (SP) (24 amino acids) followed by the CBD at the N-terminus (Hwang et al. 2014). CaPR4b lacking the SP (CaPR4bΔSP) is present throughout the cell except in the endoplasmic reticulum (ER). Thus, CaPR4b SP is responsible for the ER localization and secretion of CaPR4b. CaPR4b is synthesized in the ER, interacts with CaLRR1 in the plasma membrane, and is secreted into the extracellular space through the plasma membrane (Hwang et al. 2014). Secretion of CaPR4b into the apoplastic region is a pivotal cellular event to trigger rapid cell death in pepper leaf tissues. However, formation of the CaLRR1–CaPR4b complex disturbs the secretion of CaPR4b into the apoplastic space and inhibits the CaPR4b-triggered cell death response. Similar to the antagonistic activity of CaLRR1 against CaPR4b in pepper, transient co-expression of *CaLRR1* with *CaPR4b* significantly suppresses the induction of cell death by *CaPR4b* expression in *N. benthamiana* leaves, although transient expression of *CaLRR1* alone does not trigger cell death in the leaves. There is convincing evidence that LRR proteins inhibit elicitin-induced (Jacques et al. 2006) and CaHIR1-triggered (Jung and Hwang 2007; Choi et al. 2011) cell death in plants. *CaLRR1* expression may block the signaling pathways involved in the *CaPR4b*-triggered cell death response in plants.

CaLRR1 and CaHIR1 localize to the extracellular matrix and plasma membrane, respectively (Choi et al. 2011). CaLRR1 has a putative SP at the N-terminus, and CaHIR1 has a transmembrane domain at the N-terminus. CaLRR1 is localized in the extracellular matrix of pepper leaf tissues. CaHIR1 remains primarily in the microsomal fraction, indicating a membrane-associated subcellular localization. CaLRR1 and CaHIR1 are localized in small patches at the plasma membrane, which is characteristic of many membrane microdomain proteins (Solis et al. 2007). Mature CaLRR1 protein is secreted to the extracellular matrix and specifically binds to the plasma membrane-localized CaHIR1 to form a heterodimer complex *in planta*. CaLRR1 physically interacts with CaHIR1 *in planta* and suppresses CaHIR1-triggered cell death response (Choi et al. 2011). Co-expression of *CaHIR1* with *CaLRR1* greatly suppresses cell death in the pepper tissue. Electrolyte leakage and callose deposition are higher in *CaHIR1*-expressing pepper leaf tissues than in *CaLRR1*/*CaHIR1*-co-expressing tissues (Choi et al. 2011). *CaLRR1* and *CaHIR1* positively and negatively regulate SA induction in pepper, respectively (Choi et al. 2011). SA in plants regulates disease resistance mechanisms, including host cell death and defense gene expression (Vlot et al. 2009). *CaHIR1* overexpression in *Arabidopsis* induces the spontaneous cell death phenotype, together with increased K^+ efflux and SA pathway-dependent *PR* gene induction (Jung and Hwang 2007). Co-overexpression of both *CaLRR1* and *CaHIR1* in transgenic *Arabidopsis* leaves compromises *CaHIR1*-mediated *PR* gene expression and cell death phenotype (Choi et al. 2011). Rice OsLRR1 interacts with OsHIR1, which is localized at the plasma membrane (Zhou et al. 2009). OsHIR1 triggers hypersensitive cell death, and its localization to the plasma membrane is enhanced by OsLRR1 (Zhou et al. 2010), which suggests that OsLRR1 acts as a positive regulator for OsHIR1 function.

Regulation of plant cell death and defense responses by the CaLRR1–CaPR10/CaPR4b/CaHIR1 complex

Among the pepper defense response genes, *CaLRR1*, *CaPR10*, *CaPR4b*, and *CaHIR1* are strongly up-regulated in pepper leaf tissues with similar temporal kinetics. CaLRR1 physically interacts with CaPR10 (Choi et al. 2012), CaPR4b (Hwang et al. 2014), and CaHIR1 (Jung and Hwang 2007) to tightly modulate cell death and immunity in pepper plants (Fig. 3). *Xcv* effector AvrBsT triggers HR cell death in pepper and *N. benthamiana* leaves (Orth et al. 2000; Escolar et al. 2001; Kim et al. 2010; Han and Hwang 2017). CaLRR1 alone does not function as a regulator of cell death and disease resistance, but coordinates defense responses with its interacting partner proteins CaPR10, CaPR4b, and CaHIR1 in the different subcellular locations (Fig. 3). CaLRR1 is produced in the cytoplasm and trafficked to the extracellular matrix. It binds to CaPR10 in the cytoplasm and CaPR4b and CaHIR1 at the plasma membrane (Fig. 3). CaLRR1 may possess a dual function in the regulation of cell death responses. CaLRR1 positively regulates CaPR10-triggered hypersensitive cell death in pepper and *N. benthamiana* leaves (Choi et al. 2012). However, CaLRR1 negatively regulates not only CaPR4b-triggered hypersensitive cell death (Hwang et al.

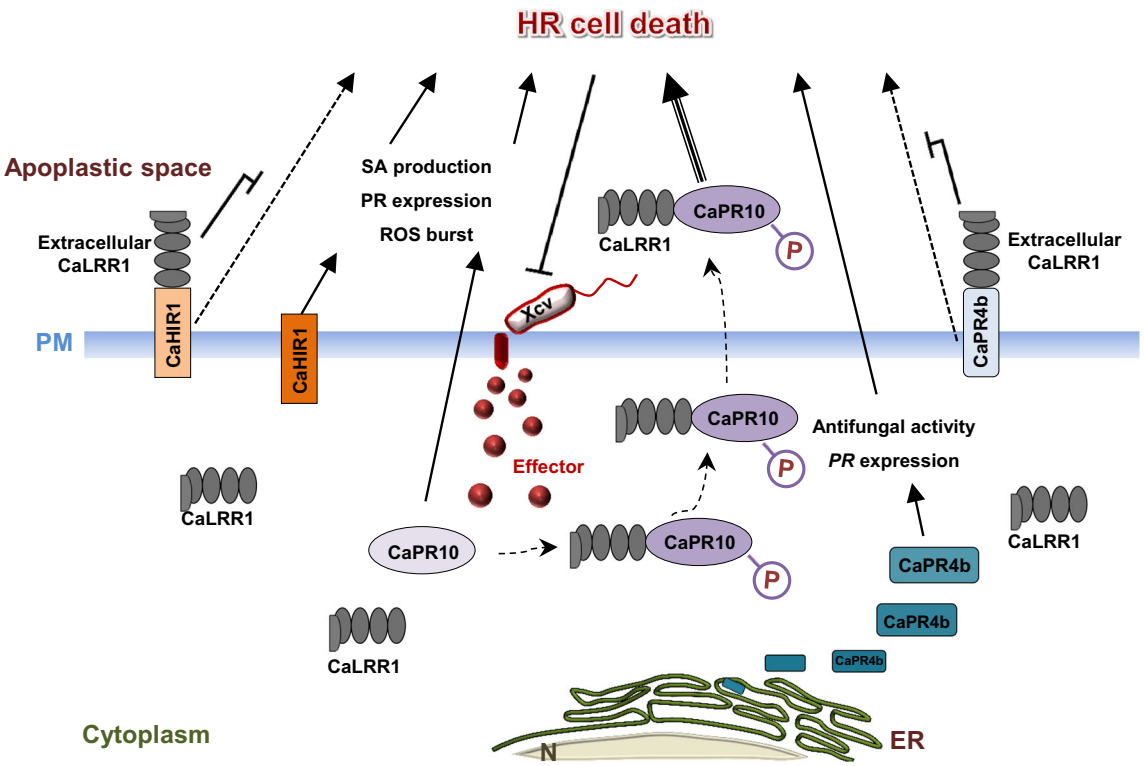

Fig. 3 Roles of CaLRR1, CaPR10, CaPR4b, and CaHIR1 in molecular and cellular signaling of HR cell death and defense responses in pepper cells in response to *X. campestris* pv. *vesicatoria* (Xcv) invasion. *ER* endoplasmic reticulum, *HR* hypersensitive response, *N* nucleus, *P* phosphate, *PM* plasma membrane, *PR* pathogenesis related, *ROS* reactive oxygen species, *SA* salicylic acid. CaLRR1 is produced in the cytoplasm and trafficked to the extracellular matrix and binds to CaPR10 in the cytoplasm and CaPR4b and CaHIR1 at the plasma membrane. CaLRR1 enhances CaPR10 phosphorylation and RNase activity. CaLRR1 synergistically accelerates CaPR10-triggered HR cell death, but suppresses CaPR4b- and CaHIR1-triggered cell death. CaPR10, CaPR4b, and CaHIR1 positively regulate defense signaling mediated by SA production, PR expression, and ROS burst, thereby activating HR cell death and disease resistance. The CaLRR1–CaPR10 complex is secreted into the apoplastic space to synergistically accelerate CaPR10-triggered cell death. CaPR4b is produced in the ER and is translocated into the apoplast via the plasma membrane. CaPR4b has strong antifungal activity against fungal pathogens

2014), but also disease-associated cell death response in pepper leaves via interaction with CaHIR1 (Jung and Hwang 2007; Choi et al. 2011).

The CaLRR1–CaPR10 complex is formed in the cytoplasm. *CaLRR1* expression enhances CaPR10 phosphorylation and RNase activity, which may be required for HR cell death (Choi et al. 2012). The CaLRR1–CaPR10 complex is secreted into the apoplastic space of pepper cells to synergistically accelerate CaPR10-triggered cell death (Choi et al. 2012). The CaLRR1–CaPR10 complex may mediate the synergistic activation of cell death and defense responses. During avirulent *Xcv* infection, CaPR10 and the positive regulator CaLRR1 trigger early cell death and defense responses, including callose accumulation, SA and ROS burst, and PR- or defense-related gene expression (Fig. 3). These eventually lead to the promotion of CaPR10-triggered HR cell death response. CaPR4b is produced in the endoplasmic reticulum (ER) and is translocated into the apoplast via the plasma membrane (Hwang et al. 2014). CaPR4b has strong antifungal activity against fungal pathogens. Plant PR4 proteins such as wheatwin1 (Bertini et al. 2009) and OsPR4b (Zhu et al. 2006) have in vitro antifungal activity against plant pathogenic fungi. CaLRR1 also binds both CaPR4b and CaHIR1 at the plasma membrane and suppresses CaPR4b- or CaHIR1-triggered pepper cell death from the apoplastic space (Fig. 3; Jung and Hwang 2007; Choi et al. 2011; Hwang et al. 2014). When free CaLRR1 translocates into the extracellular matrix without CaPR10, it may interact with CaPR4b or CaHIR1 at the plasma membrane. The CaLRR1–CaPR4b interaction suppresses CaPR4b-triggered HR-like cell death in plants. Silencing of *CaLRR1* together with *CaPR4b* reduces cell death in pepper leaves. Transient overexpression of *CaHIR1* induces cell death in pepper leaves in the absence of pathogen attack; however, co-expression of *CaLRR1* with *CaHIR1* mitigates CaHIR1-triggered cell death. Interestingly, CaLRR1 overexpression suppresses pepper cell death caused by a mock inoculation.

CaLRR1 may have other host partners and increase cell death in response to mechanical wounding or physiological imbalance.

Concluding remarks

Protein–protein complexes mediate many of the host molecular cell death and immune (defense) responses that occur at the transcriptional and translational level (Rebsamen et al. 2013; Choi and Hwang 2015). Recent technological advances can be applied for better understanding of the ability of plant cells to mount the appropriate cell death and immune responses. An array of differential molecular and biochemical events is triggered in pepper plants during *X. campestris* pv. *vesicatoria* (*Xcv*) infection (Choi and Hwang 2015). The HR cell death response in pepper leaf tissues challenged with the avirulent *Xcv* strain Bv5-4a carrying AvtBsT is characteristic of the incompatible *Xcv*-pepper interaction (Kim et al. 2010). A number of cell death- and pathogenesis-related (*PR*) genes are positively regulated in pepper during incompatible *Xcv* interactions (Choi and Hwang 2015). The *Xcv*-inducible pepper gene *CaLRR1* encoding the small LRR-containing extracellular protein is involved in plant cell death and immunity signaling (Jung et al. 2004; Jung and Hwang 2007). *CaLRR1* expression is strongly up-regulated in pepper during *Xcv* infection. However, *CaLRR1* expression is not inducible by the defense hormones SA, ET, and JA, or by abiotic stresses such as drought and cold stress. The pepper pathogenesis-related proteins CaPR10 (Choi et al. 2012) and CaPR4a (Hwang et al. 2014) and the pepper hypersensitive-induced reaction protein CaHIR1 (Jung and Hwang 2007; Choi et al. 2011) (Table 1) physically interact with CaLRR1 to regulate plant cell death and immune (defense) responses; these proteins have been identified using a yeast two-hybrid screen (Fields and Song 1989), BiFC (Walter et al. 2004), and Co-IP assays (Choi et al. 2012). These CaLRR1–pepper interactor proteins have been functionally characterized in plant cells using *Agrobacterium*-mediated transient expression, VIGS (Liu et al. 2002), and transgenic overexpression techniques.

Pathogen-induced plant cell death is intimately linked to disease and plant immunity (Greenberg and Yao 2004; Choi et al. 2011). Cell death and defense response genes, such as *CaPR10* (Choi et al. 2012), *CaPR4b* (Hwang et al. 2014), and *CaHIR1* (Jung and Hwang 2007), contribute positively to the regulation of SA- and ROS-mediated defense signaling, ultimately leading to enhanced HR cell death and disease resistance. Avirulent *Xcv* infection induces *CaPR10* expression associated with the HR cell death response (Choi et al. 2012). Transient *CaPR10* expression triggers HR cell death in pepper and *N. benthamiana* leaves, which is promoted by *CaLRR1* co-expression as a positive regulator. The cytoplasmic CaLRR1–CaPR10 complex is involved in cell death and defense signaling in plants (Choi et al. 2012). By contrast, CaLRR1 interacts with CaPR4b to suppress cell death and defense responses (Hwang et al. 2014). CaPR4b positively regulates plant cell death and defense responses. Purified CaPR4b protein inhibits spore germination and mycelial growth of plant fungal pathogens, including *A. brassicicola*, *B. cinerea*, *C. orbiculare*, and *F. oxysporum* f.sp. *matthioli*. CaLRR1 also functions as a negative regulator of CaHIR1-triggered cell death response in plants (Jung and Hwang 2007). CaLRR1 and CaHIR1 regulate plant cell death associated with immunity and disease (Choi et al. 2011). Notably, CaHIR1 interacts with *Xcv* Fha1 to induce disease-associated cell death and suppress *PR* gene expression in plants (Choi et al. 2013).

Ectopic expression of *CaLRR1* and its interactor protein genes in transgenic plants paves the way to mine valuable genetic resources for disease-resistant crop breeding. *CaLRR1* overexpression does not confer any resistance to plant pathogens (Jung and Hwang 2007). However, overexpression of *CaPR10*, *CaPR4b*, and *CaHIR1* in transgenic *Arabidopsis* plants confers enhanced resistance against hemibiotrophic bacterial and biotrophic oomycete infection. These CaLRR1 interactor protein genes are available for molecular breeding of disease resistance of economically important crops. Resistance of transgenic *Arabidopsis* plants to *H. arabidopsidis* is synergistically enhanced by constitutive co-expression of *CaPR10* with *CaLRR1* (Choi et al. 2012). Enhanced resistance of transgenic *Arabidopsis* expressing *CaHIR1* to *P. syringae* pv. *tomato* and *H. arabidopsidis* infection is accompanied by the strong expression of *PR* genes, the accumulation of both SA and H_2O_2, and K^+ efflux (Jung and Hwang 2007). The CaLRR1–CaHIR1 complex suppresses CaHIR1-triggered cell death and *PR* gene expression in *Arabidopsis* and tobacco plants that overexpress both *CaHIR1* and *CaLRR1* (Choi et al. 2011). By contrast, transgenic *Arabidopsis* expressing *CaHIR1* is not only susceptible to the necrotrophic fungal pathogen *B. cinerea*, but is also sensitive to high salinity and drought (Jung et al. 2008). Consequently, plant HIR proteins may be valuable candidates for improving resistance to biotrophic and hemibiotrophic pathogens in crops.

Author contribution statement BKH designed the outline of the article. JKH, ISH, and BKH wrote the manuscript. JKH composed the table and figures. BKH did the revisions of the manuscript. All authors read and approved the manuscript.

Acknowledgements This work was carried out with support from the Cooperative Research Program for Agriculture Science and Technology (Project No. PJ00802701), the Rural Development

Administration, and the Advanced Production Technology Development Program (Project No. 115051-2), Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET), Republic of Korea.

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