RESEARCH PAPER

Overexpression of the peanut CLAVATA1-like leucine-rich repeat receptor-like kinase AhRLK1 confers increased resistance to bacterial wilt in tobacco

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Abstract

Bacterial wilt caused by Ralstonia solanacearum is a devastating disease affecting hundreds of plant species, yet the host factors remain poorly characterized. The leucine-rich repeat receptor-like kinase gene AhRLK1, characterized as CLAVATA1, was found to be up-regulated in peanut upon inoculation with R. solanacearum. The AhRLK1 protein was localized in the plasma membrane and cell wall. qPCR results showed AhRLK1 was induced in a susceptible variety but little changed in a resistant cultivar after inoculated with R. solanacearum. Hormones such as salicylic acid, abscisic acid, methyl jasmonate, and ethephon induced AhRLK1 expression. In contrast, AhRLK1 expression was down-regulated under cold and drought treatments. Transient overexpression of AhRLK1 led to a hypersensitive response (HR) in Nicotiana benthamiana. Furthermore, AhRLK1 overexpression in tobacco significantly increased the resistance to R. solanacearum. Besides, the transcripts of most representative defense responsive genes in HR and hormone signal pathways were significantly increased in the transgenic lines. EDS1 and PAD4 in the R gene signaling pathway were also up-regulated, but NDR1 was down-regulated. Accordingly, AhRLK1 may increase the defense response to R. solanacearum via HR and hormone defense signaling, in particular through the EDS1 pathway of R gene signaling. These results provide a new understanding of the CLAVATA1 function and will contribute to genetic enhancement of peanut.

Keywords: AhRLK1; Arachis hypogaea; defense signaling; EDS1; Ralstonia solanacearum; transgenic tobacco.
Introduction

Bacterial wilt (BW) caused by Ralstonia solanacearum is a severe soil-borne disease that affects plants worldwide. The hosts of R. solanacearum include over 450 species belonging to 54 botanical families (Zhang et al., 2017). BW reduces peanut output by 10–30% in infected areas and can lead to total crop failure in heavily infected regions, thus causing significant economic loss (Wicker et al., 2007; Yu et al., 2011). To date, efficient methods to control BW remain unavailable, and farmers rely on rotation, intercropping with other non-host crops, and biological control to reduce the incidence of BW. Plants have evolved a multi-layered innate immune system to defend against pathogens. Pattern recognition receptors (PRRs) on the plant cell surface act as initial detectors that recognize pathogen-associated or damage-associated molecular patterns to elicit the first-layer immune response called pathogen-associated molecular pattern–triggered immunity (PTI) (Jones and Dangl, 2006; Zipfel, 2014). PTI prevents infections by non-adapted pathogens. Some pathogens deploy effectors that contribute to their virulence. Effectors subsequently interfere with PTI and cause effector-triggered susceptibility. In resistant plants, these effectors are recognized by R proteins to induce effector-triggered immunity (ETI) (Jones and Dangl, 2006) and the co-evolution of PTI and ETI has shaped the plant immune system (Böhm et al., 2014).

Most PRRs are characterized as leucine-rich repeat receptor-like protein kinases (LRR-RLKs) that comprise a class of RLKs in plants (Zhang, 1998). LRR-RLK proteins regulate plant growth and development (Morris and Walker, 2003), hormone signal transduction (Hong et al., 1997), and response to biotic or abiotic stresses (Nishiguchi et al., 2002; Torii, 2004). LRR-RLKs are also involved in plant defense-related disease resistance (Song et al., 1995; Godiard et al., 2003). A typical LRR-RLK has extracellular domains (LRR), a single transmembrane domain flanked by juxta membrane regions, and cytoplasmic protein kinase domains (Dardick et al., 2012; Zhang and Thomma, 2013; Böhm et al., 2014). LRR domains function as binding sites that recognize pathogen-derived elicitors to activate downstream signal transduction by the cytoplasmic protein kinase domains, thereby activating the plant’s defensive immune response (Jones and Jones, 1997; Dardick et al., 2012; Böhm et al., 2014).

FLAGELLIN SENSITIVE2 (FLS2), an LRR-RLK protein, is a plasma membrane receptor involved in the recognition of pathogen flagellin (Gómez-Gómez and Boller, 2000). FLS2 can bind to flagellin through its extracellular domain, and the kinase domain is required to trigger an immune response (Gómez-Gómez and Boller, 2000; Gómez-Gómez et al., 2001). Xa21 is a LRR-RLK gene involved in resistance to leaf blight in rice (Wang et al., 1996). Xa21 has 21 LRR motifs that recognize pathogen ligands, eliciting plant defense responses, such as oxidative bursts, hypersensitive cell death, and activation of defense genes through the activity of its intracellular kinases (Song et al., 1995; Wang et al., 1996). ERECTA is an Arabidopsis LRR-RLK involved in the resistance response to R. solanacearum (Godiard et al., 2003), and it activates the expression of downstream resistance-related genes against R. solanacearum by the phosphorylation of its extracellular kinase (Godiard et al., 2003). In addition, ERECTA triggers a resistance response to the necrotrophic fungus Plectosphaerella cucumerina in Arabidopsis (Llorente et al., 2005). An increasing number of LRR-RLKs have been identified, but their resistance mechanisms in plant–pathogen interactions remain elusive.

In the present study, an LRR-RLK gene named AhRLK1 was identified in peanut by microarray analysis. AhRLK1, characterized as CLAVATA1, was up-regulated in a peanut cultivar susceptible to BW but its expression was nearly unchanged in a resistant cultivar. Treatment with different hormones and cold or drought stress altered the expression of AhRLK1. Transient overexpression of AhRLK1 caused a hypersensitive response (HR) in N. benthamiana following agro-infiltration. Furthermore, overexpression of AhRLK1 in N. tabacum produced increased resistance to R. solanacearum. The expression levels of various stress-responsive genes, including those of R gene signaling, were also significantly induced in the AhRLK1-overexpressing transgenic lines. Therefore, these results suggest that AhRLK1 is involved in the defense response of peanut to R. solanacearum and in the resistance conferred by multiple, complex signaling regulatory networks.

Materials and methods

Plant material and growth conditions

Peanut (Arachis hypogaea) cultivars that were middle resistant (Minhua 6), hyper-resistant (Yuexiu 92), and hypersusceptible (Xinquxiao) to R. solanacearum were obtained from the Oil Crop Institute of Fujian Agriculture and Forestry University. Seeds were sown in sterile sand and in 5×6 cm plastic pots. The Tobacco Research Group of Fujian Agriculture and Forestry University provided the seedlings of transgenic lines, wild-type tobacco lines (Nicotiana tabacum cv. CB-1, cv. Honghuadajinynuan, and cv. Yanyan97) with medium susceptibility, hypersusceptibility, and hyper-resistance to R. solanacearum, respectively) and those of N. benthamiana. All seedlings were grown in a greenhouse. T1 and T2 seeds of transgenic tobacco lines were surface-sterilized with 75% (w/v) alcohol for 20 s and 1% (v/v) H2O2 for 10 min, washed five times with sterile water, and then plated on Murashige–Skoog (MS) medium supplemented with 75 mg l−1 kanamycin for 2–3 weeks. The surviving plants were transferred into a soil mix (peat moss/perlite, 2:1, v/v) in a plastic tray and grown in a greenhouse for another 2–3 weeks. Transgenic and wild-type tobacco plants of the same size were transferred into the same soil mixed in plastic pots and grown for another 3–4 weeks. The peanut and tobacco plants were grown in a greenhouse at 26±2 °C, with 70% relative humidity and a 16 h light/8 h dark cycle.

Pathogens and inoculation procedures

Virulent R. solanacearum strains Rs-P362200-060707-2-2 for peanut and FJ1003 for tobacco were provided by Prof. Bo Liu of the Fujian Academy of Agricultural Sciences. Two strains were streaked on TTC agar medium (Kelman et al., 1954) and incubated at 28 °C for 48 h. The two virulent strains were cultured and inoculated in peanut and tobacco following published protocols (Zhang et al., 2017). The pathogen cell solution used for R. solanacearum infection of peanut and tobacco plants for functional characterization of AhRLK1 was diluted to 109 colony forming units (cfu) ml−1 (OD600=0.5) with ddH2O. The leaves were harvested at the indicated time points for the preparation of RNA.
For the transient overexpression of AhRLK1 in N. benthamiana, 10^5 cfu ml^-1 Agrobacterium was infiltrated into the second leaf of 2-month-old tobacco from the top using a syringe without a needle until the bacterial suspensions were spread over the entire leaf. The third leaf was harvested at the indicated time points, immediately frozen in liquid nitrogen, and stored at ~80°C.

Application of plant hormones or abiotic and biotic stresses
One-month-old peanut (Minhua 6) seedlings were sprayed with 3 mM salicylic acid (SA), 37.8 µM abscisic acid (ABA), 10 mM ethephon (ET), or 100 µM mthyl jasmonate (MeJA) dissolved in distilled water. Control seedlings were sprayed only with distilled water. The leaves of the treated seedlings were harvested, frozen in liquid nitrogen, and stored at ~80°C for 3°C or a normal temperature of 25°C, and leaves were collected at various time points. For drought stress, the seven to eight leaf peanut plants were planted in 5×6 cm plastic pots in the greenhouse at 28°C, the soil moisture was saturated before treatment. The control plants were given water every day as the normal watering. Drought-treated plants were left without water for 4 d after the initial watering, and the samples were collected after 24, 48 and 96 h. All experiments had three biological replicates. All samples were frozen in liquid nitrogen and stored at ~80°C.

Cloning of full-length AhRLK1 cDNA
AhRLK1 was identified as a candidate differentially expressed gene using a high-density peanut microarray with 100,000 unigenes, which was devised by our laboratory and created by the Roche Company (Roche, Branford, CT, USA). AhRLK1 was isolated by chip hybridization using RNA extracted from peanut plants with or without inoculation of R. solanacearum. For cloning of full-length AhRLK1, the AhRLK1-F and AhRLK1-R primers were designed from the available gene fragments. The 5′- and 3′-end sequences of the cDNA were cloned through rapid amplification of cDNA ends (RACE) using a SMART™ RACE cloning kit (Clontech, Palo Alto, CA, USA) following the manufacturer’s instructions with minor modifications. Total RNA was extracted from leaves of the peanut cultivar hyper-resistant to R. solanacearum using the cetyltrimethylammonium bromide (CTAB) method (Chen et al., 2016). The adaptor primers of RACE-F and RACE-R primer were ligated to both ends of the cDNA. The 5′ RACE was generated by PCR with RACE-F and AhRLK1-R primers. Similarly, the 3′ RACE was generated by the set of AhRLK1-F and the 3′ PCR primer. The RACE products were ligated to the pmD18-T vector (Takara, Japan) following the manufacturer’s instructions and sequenced. After assembly, the full-length cDNA sequence of AhRLK1 was cloned from the reverse transcription products using AhRLK1-FL-F and AhRLK1-FL-R. All primers are listed in Supplementary Table S1 at JXB online.

Sequence analysis and phylogenetic tree construction
AhRLK1 sequence similarity analysis was performed using BLASTN and BLASTX (http://www.ncbi.nlm.nih.gov/BLAST). Conserved domains of the AhRLK1-encoded protein were analysed using SMART (Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de/). Multiple sequence alignments were obtained from known functional LRR–RLKs of different species using Clustal2W. A phylogenetic tree of the different subfamilies of LRR–RLKs in Arabidopsis was generated using the MEGA6 program (Tamura et al., 2011).

Subcellular localization, co-localization, and plasmolysis experiments
For the green fluorescent protein (GFP) fusion vector, the full-length open-reading frame of AhRLK1 without the termination codon was amplified by the gene-specific primers AhRLK1-BamHI-F and AhRLK1-AscI-R harboring BamHI and AscI sites, respectively. The PCR products and the pBl-GFP vector (provided by W-JZ) were both digested with BamHI and AscI. The corresponding bands were recovered and ligated to the 35S::AhRLK1–GFP expression vector. The 35S::GFP vector (provided by W-JZ) and 35S::CaSRC2-1-RFP (specifically targeting the plasma membrane; Liu et al., 2013) were used as a control. For yellow fluorescent protein (YFP) fusion vector, a Gateway cloning technique (Invitrogen, Carlsbad, CA, USA) and a Gateway-compatible destination vector were employed. The full-length open reading frame (ORF) of AhRLK1 was initially amplified by PCR with corresponding specific primer pair (Supplementary Table S1) flanked with attB for Gateway cloning and GXG DNA polymerase (Takara, Japan), and confirmed by sequencing. The full-length cDNA was cloned into the entry vector pDONR207 by BP reaction, and then into destination vectors pEarleyGate101 (Invitrogen) by LR reaction. All vectors were transformed into Agrobacterium strain GV3101. The Agrobacterium strains harboring the above mentioned constructs were grown for 24 h in YEP medium (10 g l^-1 yeast extract, 10 g l^-1 peptone, and 5 g l^-1 NaCl) containing appropriate antibiotics and cultured to OD_600=0.1 in induction medium (10 mM methanesulfonic acid, pH 5.7, 10 mM MgCl_2, and 200 mM acetoxysemine) and diluted to OD_600=0.8. The diluted culture was infiltrated into N. benthamiana leaves using a syringe without a needle. For co-localization experiment, 35S::AhRLK1–GFP and 35S::CaSRC2-1–RFP constructs were mixed at 1:1 ratio, and co-infiltrated into N. benthamiana leaves. For plasmolysis experiments, the tobacco leaves were immersed in 10% sorbitol for 20 min before detection. After 48 h of infection, different fluorescences were visualized using a laser scanning confocal microscope (TCS SP8, Leica, Solms, Germany). Digital images were overlaid using ImageJ.

AhRLK1 overexpression vector construction, transient expression, and tobacco transformation
The complete ORF of AhRLK1 was amplified by high-fidelity PCR polymerase with pMD-T-AhRLK1 as the template using AhRLK1-OE-F and AhRLK1-OE-R primers. The PCR products and the pBl121-GUSA vector (provided by W-JZ) were digested with BamHI and AscI, the corresponding bands were recovered and ligated into pBl121-GUSA, creating the overexpression vector 35S::AhRLK1, which drives expression of AhRLK1 under the 2xCaMV 35S promoter. The 35S::AhRLK1 plasmid was transferred into Agrobacterium tumefaciens strain GV3101. For transient overexpression, Agrobacterium GV3101 with the 35S::AhRLK1-GFP plasmid was injected into N. benthamiana leaves by Agrobacterium infiltration and then transformed into tobacco using the leaf-disc method (Müller et al., 1987). To confirm transgene integration, the initial transgenic T_0 lines were selected by kanamycin and further confirmed by RT-PCR. The T_0 homozygous lines that were generated through this process were used for experiments in this study.

In silico analysis and quantitative real-time PCR
In silico analysis of the AhRLK1 gene expression pattern in peanut was performed using non-amplified double stranded cDNA for hybridization as described previously (Chen et al., 2016). The gene expression intensity of all hybridization experiments was analysed, and expression levels were estimated for different tissues and under diverse stress conditions. Three replicates were performed for all experiments. The data from the tobacco microarray were generated previously (Zhang et al., 2017). Leaves were harvested from hyper-resistant tobacco variety Yuan97 and the hypersusceptible tobacco variety Honghuadaxian that were inoculated with R. solanacearum. Microarray design, hybridization, washing, scanning, and data analysis were conducted as previously described (Zhang et al., 2017). For qRT-PCR analysis, total RNA isolation and real-time RT-PCR were carried out following procedures used in our previous studies (Chen et al., 2016; Zhang et al., 2017). At least three replicates of each experiment were performed. Data were analysed by the method of Schmittgen and Livak (2008) and shown as a normalized relative expression level (2^{-ΔΔCT}) of the respective genes. The relative transcript levels of AhRLK1 were detected under different treatments in peanut, with Ahatin used as
the internal reference as reported previously (Chen et al., 2016; Zhang et al., 2017). The relative transcript levels of related defense genes after *R. solanacearum* treatment were detected between the wild-type and transgenic tobacco plants, with tobacco *NiEF1:GFP* as the internal reference. All primers used in the qPCR analyses are listed in Supplementary Table S1.

**Histochimical staining analysis and ion conductivity determination**

At 48 h after the transient overexpression of AhRLK1 in *N. benthamiana* leaves, the infected plants were stained with 3,3′-diaminobenzidine (DAB; Sigma-Aldrich, St Louis, MO, USA) and lactophenol–ethanol trypan blue. To measure the levels of H2O2, the infected *N. benthamiana* leaves were incubated in 1 mg ml−1 DAB solution overnight at room temperature, boiled for 5 min in a 3:1:1 ethanol–lactic acid–glycerol solution, and then placed in absolute ethanol before observation. To detect cell death, the inoculated leaves were boiled for 2 min in trypan blue solution, which consisted of 10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, 30 ml of absolute ethanol, and 10 mg of trypan blue, dissolved in 10 ml of ddH2O. They were left at room temperature overnight, transferred into a chloral hydrate solution (2.5 g of chloral hydrate dissolved in 1 ml of distilled water), and then boiled for 20 min for de-staining. The leaves were observed under a light microscope. Ion conductivity leakage was measured with a Mettler Toledo 326 apparatus.

**Results**

**Sequence characteristics of AhRLK1 isolated from peanut**

After microarray data analysis, one LRR receptor like kinase gene was selected that was up-regulated after *R. solanacearum* inoculation in peanut, and named AhRLK1 (Supplementary Dataset S1). The unknown 5′ and 3′ cDNA sequences of AhRLK1 were cloned by RACE. The full-length cDNA sequence was isolated from total RNA of peanut leaves using RT-PCR (Supplementary Fig. S1). The full-length cDNA contained a 3292 bp ORF encoding a polypeptide of 992 amino acids with 122 and 251 bp for 5′ and 3′ untranslated terminal regions, respectively (Fig. 1; Supplementary Dataset S2). Sequence analysis showed the deduced AhRLK1 protein contained the typical serine/threonine protein kinase catalytic domain and 10 LRR conserved domains (LxxLxxLxxLxxC/A-xx) (Kobe and Kajava, 2001; Zhou, et al., 2009) (Fig. 1; Supplementary Fig. S2). In addition, the protein had a signal peptide in the N-terminal region (Fig. 1).

The amino acid sequences of AhRLK1 were then compared with homologous genes, and AhRLK1 shared 61% identity and 78% similarity to Arabidopsis CLAVATA1, 34% identity and 50% similarity to ERECTA, 50% identity and 67% similarity to BAM1, and 49% identity and 67% similarity to BAM2 (Supplementary Dataset S3; Supplementary Fig. S2). A phylogenetic tree of AhRLK1 and its homologs was constructed, which confirmed that AhRLK1 is a member of the LRR X1 subfamily, with highest similarity to At1g75820 (Accession Number: NP_177710), which encodes the CLAVATA1 (CLV1) protein (Fig. 2; Supplementary Dataset S4; Supplementary Fig. S3). CLAVATA1 mainly plays an important role in shoot and root meristem and flower development in Arabidopsis (Clark et al., 1997), but participates in the signaling pathway during nodulation in *Medicago truncatula* (Laffont et al., 2018). Interestingly, a mutant of the CLAVATA1 (CLV1) gene conferred enhanced disease resistance to bacterial wilt (Hanemian et al., 2016). However, these two kinases may have significantly diverged in their functions; thus, AhRLK1 may be related to disease resistance and meristem development.

**Subcellular localization of AhRLK1**

Sequence analysis predicted that AhRLK1 is a plasma membrane-bound kinase (Query Protein WoLFPSORT prediction plas: 29.29 by http://www.genscript.com/psort/wolf_psort.html). A vector expressing the AhRLK1–GFP fusion protein under the constitutive CaMV35S promoter was generated and the 35S::GFP vector was used as the negative control. AhRLK1–GFP was mainly localized to the plasma membrane, whereas GFP was localized broadly in different subcellular compartments, including the cytoplasm and nuclei (Fig. 3). Co-expression of AhRLK1–GFP and CaSR.C2-1–red fluorescent protein (RFP), a protein that binds to the plasma membrane, showed complete overlap of the green fluorescence signal of AhRLK1–GFP and the red fluorescence signal in the plasma membrane (Supplementary Fig. S4). Plasmolysis experiments for AhRLK1 subcellular localization showed that AhRLK1 was primarily localized to cell wall and plasma membrane, and occasionally to membrane vesicles (Supplementary Fig. S4). These results suggest that AhRLK1 is a plasma membrane- and cell wall-associated kinase.

**AhRLK1 showed diverse expression patterns among tissues**

*In silico* analysis of AhRLK1 expression with three unigenes was performed using a high-density microarray. These unigenes, with more than 97% sequence identity, appeared to belong to the same AhRLK1 gene family. Non-amplified double strand cDNA was used to evaluate the transcript levels of unigenes in the microarray. The expression profiles of these three members were similar in different tissues, with the highest expression in the roots and stem, followed by leaves, flowers, pegs, and tests. However, expression was weak in the pericarp, and embryos had the lowest expression levels (Fig. 4; Supplementary Dataset S5). Semi-quantitative RT-PCR also showed that AhRLK1 expression was higher in the root and stem, and low in embryos of different developmental stages (Fig. 4).

**AhRLK1 responds to exogenous phytohormones and bio/abiotic stresses**

The expression of AhRLK1 under exogenous phytohormone treatments was determined using the medium resistance variety Minhua 6 at the eight-leaf stage (Fig. 5). When treated with 3 mM SA, the AhRLK1 transcripts increased up to 6.6-fold at 6 h post-treatment (hpt) and then gradually decreased to levels slightly higher (<3-fold) than those of the control plants (Fig.
AhRLK1 confers increased resistance to \( R. \) solanacearum | 5411

The expression of \( \text{AhRLK1} \) also increased when plants were treated with 37.8 \( \mu \text{M} \) ABA, reaching a single peak of 4.5-fold at 6 hpt (Fig. 5B). In response to 10 mM ET, \( \text{AhRLK1} \) expression increased with two peaks (2.6- and 2.8-fold) at 3 and 24 hpt, respectively, after which the expression level returned to baseline (Fig. 5C). Plants treated with 100 \( \mu \text{M} \) MeJA showed progressive increase in \( \text{AhRLK1} \) expression, with the highest expression level (3.8-fold induction) at 6 hpt (Fig. 5D).

The expression of \( \text{AhRLK1} \) under low temperature and drought was also examined in eight-leaf Minhua 6 seedlings (Fig. 5E, F). \( \text{AhRLK1} \) expression changed at 6 hpt under low temperature stress, and at 24–48 hpt upon drought stress. Specifically, the transcript level of \( \text{AhRLK1} \) under low temperature was the most reduced, decreasing 6.13-fold at 6 hpt (Fig. 5E). Under drought treatment, \( \text{AhRLK1} \) expression level decreased by 2-fold at 24 and 48 hpt, but increased by 1.54-fold at 96 hpt (Fig. 5F).

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**Fig. 1.** Sequence and structure analysis of the \( \text{AhRLK1} \) gene. (A) Complete cDNA and deduced amino acid sequences of the \( \text{AhRLK1} \) gene. The full-length cDNA was 3292 bp with an ORF encoding 992 amino acids. The gray-shaded portion shows the signal peptide domain, and the green-shaded portion indicates the LRR units; the transmembrane domain is in the yellow-shaded region. The underlined red sequences show the serine/threonine protein kinase catalytic domain. (B) LRR domain of \( \text{AhRLK1} \), including several degenerate LRR units. The consensus sequence for the \( \text{AhRLK1} \) LRR is given at the bottom. The core leucines and prolines (or equivalent amino acids) are highlighted in gray. X represents an arbitrary amino acid residue. The L-residues in the consensus sequence represent several residues at that position. (This figure is available in color at JXB online.)
Transient overexpression of AhRLK1 in N. benthamiana leaves induced a hypersensitive response

To verify whether AhRLK1 overexpression caused hypersensitive response (HR) cell death, 35S::AhRLK1 was transformed into Agrobacterium GV3101 and transiently expressed in N. benthamiana leaves by infiltration. At 48 h after infiltration, the transient overexpression of AhRLK1 in N. benthamiana leaves induced an intensive HR that mimicked cell death, whereas no visible HR cell death was found in the plants infiltrated with GV3101 harboring the empty vector 35S::00. Electrolyte leakage measurement and dark trypan blue staining showed that AhRLK1 overexpression triggered HR in N. benthamiana leaves (Fig. 6A, B). DAB staining revealed high H$_2$O$_2$ accumulation in N. benthamiana leaves after AhRLK1 overexpression (Fig. 6B). Therefore, the transient overexpression of AhRLK1 in tobacco leaves likely induced HR and H$_2$O$_2$ generation, as it would in response to stress.

Overexpression of AhRLK1 in tobacco increased resistance to R. solanacearum

To evaluate whether AhRLK1 is involved in resistance to R. solanacearum, the conventional tobacco cultivar CB-1 that has medium susceptibility to bacterial wilt was transformed with a vector containing AhRLK1 under the CaMV35S promoter using the Agrobacterium-mediated method. The vector frame is shown in Fig. 7A. Transgenic T$_0$ and T$_1$ tobacco plants were generated and examined for tobacco–R. solanacearum interaction. Compared with the wild-type cv. CB-1, the T$_1$ transgenic generation AhRLK1-OE plants showed no morphological difference. The transgenic plants showed significantly increased resistance to bacterial wilt at 40 d post-inoculation (dpi) with R. solanacearum. Most control plants died, with only 4 of the 65 wild-type plants surviving after R. solanacearum inoculation. However, most transgenic plants showed high

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**Fig. 2.** Phylogenetic tree constructed with AhRLK1 and different subfamilies of LRR-RLK proteins in Arabidopsis. The phylogenetic tree confirms that AhRLK1 is a member of the LRR XI family; AhRLK1 is indicated by a red circle. Alignments were conducted in ClustalW, and the phylogenetic tree was constructed by the neighbor-joining algorithm in MEGA 6 software. Bootstrap values (1000 replicates) are shown as percentages at the branch nodes. (This figure is available in color at JXB online.)
AhRLK1 confers increased resistance to *R. solanacearum*. Resistance to bacterial wilt, and the death rate was greatly reduced (Supplementary Fig. S5). Three T2 transgenic homozygous lines were obtained and inoculated with the pathogen (*AhRLK1-OE*, Fig. 7B). All transgenic lines exhibited increased resistance against *R. solanacearum*. Obvious wilting symptoms were observed on the leaves of wild-type plants at 7 dpi, but only slight wilting symptoms were observed on the *AhRLK1-OE* leaves (Fig. 7C, D). Severe wilting symptoms were observed in the wild-type plants at 15 dpi but not in the *AhRLK1-OE* transgenic lines. *AhRLK1* resistance was further evaluated in the hypersusceptible cultivar Honghuadajinjuan and six transgenic T2 homozygous lines, which were inoculated and compared with the wild-type. Semi-quantitative PCR analysis results showed that *AhRLK1* was constitutively expressed in six T2 tobacco lines (Supplementary Fig. S6), and these lines showed increased resistance to *R. solanacearum* (Fig. 7E; Supplementary Fig. S7). Line 1 displayed the highest resistance with a low infection index (21.84%) and death rate (6.80%) at 21 dpi. In comparison, the wild-type showed serious wilting, with a 95.64% index and death rate of 86.05% at 21 dpi (Table 1; Supplementary Table S2). Taken together, *AhRLK1* overexpression greatly increased disease resistance against *R. solanacearum* in tobacco.

Specific marker genes were up-regulated in *AhRLK1*-transgenic tobacco in response to *R. solanacearum*. To confirm the role of *AhRLK1* in plant disease resistance and to elucidate its possible molecular mechanism, we examined the transcriptional responses of defense-related genes, HR-responsive genes, and marker genes for SA, MeJA, and ET responses in *AhRLK1-OE* transgenic tobacco and wild-type CB-1 plants infected with *R. solanacearum* (Fig. 8). We found transcript levels of the HR-associated genes *NhHIN1*, *NhHSR201*, and *NhHSR515* to be increased by 11.6-, 14.9-, and 5.1-fold, respectively, in the *AhRLK1-OE*-1 line compared with wild-type.
plants. The HR-responsive genes \( \text{NtH1N1, NtHSR201, NtHSR203, and NtHSR515} \) were significantly up-regulated in transgenic plants \((P<0.01 \text{ or } P<0.05)\) at 48 h after inoculation with \( \text{R. solanacearum} \). In contrast, the transcripts level of these marker genes showed no significant change or a decrease in response to pathogen infection in wild-type plants (Fig. 8A). The expression levels of the SA-responsive genes \( \text{NtPR2, NtPR3, and NtCHN50} \) significantly increased in the \( \text{AhRLK1-OE-1} \) plants by 13.0-, 594.2- and 10.5-fold, respectively, and the expression of these genes was much higher in the \( \text{AhRLK1-OE-1} \) plants compared with \( \text{CB-1} \) when infected with the pathogen. However, \( \text{NtRP4} \) expression was similar in the transgenic lines compared and wild-type after inoculation with the pathogen (Fig. 8B). MeJA-responsive genes \( \text{NtLOX1, NtPR1b, and NtDEF1} \) were induced 4.3-, 19.0-, and 1.4-fold, respectively, in \( \text{CB-1} \), but levels of the transcript of these genes were significantly increased in transgenic plants in response to the pathogen by 46.7-, 37.1-, and 7.3-fold, respectively (Fig. 8C). The transcript levels of the ET-responsive genes \( \text{NtEFE26} \) and \( \text{NtACS6} \) also increased significantly at 48 h after \( \text{R. solanacearum} \) infection in transgenic plants. In wild-type plants, the increment of \( \text{NtEFE26} \) was less, and \( \text{NtACS6} \) was
AhRLK1 confers increased resistance to *R. solanacearum* | 5415

AhRLK1 was identified as a typical LRR-RLK family gene from peanut by microarray hybridization, where it showed an up-regulation response to a *R. solanacearum* challenge (Supplementary Dataset S1) (Torii et al., 1996). The full length coding sequence of this gene was isolated by RACE, and it contained 12 conserved LRRs and a kinase domain. Phylogenetic analysis showed that it was similar to CLAVATA1, which is responsible for shoot and root meristem development and other functions (Clark et al., 1993, 1997; Williams and De Smet, 2013; Hanemian et al., 2016; Laffont et al., 2018). Microarray data showed that the three unigenes in the *AhRLK1* family were expressed most strongly in stem and roots, and only traces were found in the embryo and pericarp (Fig. 4), suggesting that *AhRLK1* may play a role in root and stem development (Alvarez et al., 2013; Williams and De Smet, 2013; Hazak and Hardtke, 2016). Sequence alignment results showed high similarity with several known LRR-RLK genes, such as *FLS2* identified from flg22-sensitive Arabidopsis mutants, which has receptor activity that can induce pathogen response (Gómez-Gómez and Boller, 2000; Gómez-Gómez et al., 2001), and Xa21, a resistance gene in rice, which specifies the gene-for-gene resistance of rice against *Xanthomonas oryzae* (Song et al., 1995; Wang et al., 1996). ERECTA is another Arabidopsis LRR-RLK protein that enhances resistance to *R. solanacearum* (Godiard et al., 2003). Real-time PCR results showed that *AhRLK1* was up-regulated with time in response to *R. solanacearum* inoculation in Xinhuixiaoli, but its expression remained almost unchanged in Yueyou92. Therefore, *AhRLK1* might not only function similarly to CLAVATA1 to regulate shoot meristem determination but may participate in the defense response to down-regulated upon pathogen infection (Fig. 8D). Clearly, these results show that *AhRLK1* overexpression enhances the expression of defense-related genes compared with wild-type tobacco. Specifically, the expression of most pathogen-inducible genes associated with HR and hormone defense signaling significantly increased in *AhRLK1* overexpressing plants inoculated with *R. solanacearum*.

To further characterize the increased resistance to *R. solanacearum* caused by *AhRLK1* overexpression in transgenic tobacco, we examined the expression pattern of several marker genes in R-gene signaling (Fig. 8E). Compared with the wild-type, the transcripts of *NiEDS1* and *NiPAD4* were induced 37.5- and 57.2-fold, respectively, in the transgenic plants. In plants infected with *R. solanacearum*, the increments were more significant in transgenic plants compared with wild-type, showing 122.9- and 73.3-fold higher expression for *NiEDS1* and *NiPAD4*, respectively. However, *NiNDR1* expression was not affected by pathogen infection in transgenic plants, and *NiNPR1* expression declined to levels lower than the wild-type controls. We also investigated the expression of these genes in the resistant cultivar Yanyan97 and the susceptible cultivar Honghuadajinyuan in response to *R. solanacearum* infection with microarray hybridization using non-amplified double stranded cDNA (Fig. 8F). The two NDR1 genes were down-regulation in resistant and susceptible varieties, whereas the transcripts of two *PAD4* genes increased significantly in response to *R. solanacearum* infection. Nevertheless, NPR1-like genes were down-regulated in the susceptible variety and up-regulated in the resistant variety. Therefore, the overexpression of *AhRLK1* in transgenic tobacco contributed to *R. solanacearum* resistance by altering the expression of a series of signaling pathways, in addition to employing the *EDS1* pathway in R-gene signaling, and resistance against *R. solanacearum* in the wild-type plants was mediated by the *EDS1* and *NPR1* pathways.

**Discussion**

*AhRLK1* characterized as CLAVATA1 participates in defense response to *R. solanacearum*

*AhRLK1* was identified as a typical LRR-RLK family gene from peanut by microarray hybridization, where it showed an up-regulation response to a *R. solanacearum* challenge (Supplementary Dataset S1) (Torii et al., 1996). The full length coding sequence of this gene was isolated by RACE, and it contained 12 conserved LRRs and a kinase domain. Phylogenetic analysis showed that it was similar to CLAVATA1, which is responsible for shoot and root meristem development and other functions (Clark et al., 1993, 1997; Williams and De Smet, 2013; Hanemian et al., 2016; Laffont et al., 2018). Microarray data showed that the three unigenes in the *AhRLK1* family were expressed most strongly in stem and roots, and only traces were found in the embryo and pericarp (Fig. 4), suggesting that *AhRLK1* may play a role in root and stem development (Alvarez et al., 2013; Williams and De Smet, 2013; Hazak and Hardtke, 2016). Sequence alignment results showed high similarity with several known LRR-RLK genes, such as *FLS2* identified from flg22-sensitive Arabidopsis mutants, which has receptor activity that can induce pathogen response (Gómez-Gómez and Boller, 2000; Gómez-Gómez et al., 2001), and Xa21, a resistance gene in rice, which specifies the gene-for-gene resistance of rice against *Xanthomonas oryzae* (Song et al., 1995; Wang et al., 1996). ERECTA is another Arabidopsis LRR-RLK protein that enhances resistance to *R. solanacearum* (Godiard et al., 2003). Real-time PCR results showed that *AhRLK1* was up-regulated with time in response to *R. solanacearum* inoculation in Xinhuixiaoli, but its expression remained almost unchanged in Yueyou92. Therefore, *AhRLK1* might not only function similarly to CLAVATA1 to regulate shoot meristem determination but may participate in the defense response to...
Table 1. Comparison of disease index and death ratio of different OE lines and the wild-type after inoculation with R. solanacearum

| OE line | 7 dpi Disease index (%) | Death ratio (%) | 21 dpi Disease index (%) | Death ratio (%) |
|---------|-------------------------|-----------------|--------------------------|-----------------|
| Wild-type | 72.97 | 27.91 | 95.64 | 86.05 |
| OE-1    | 14.32** | 0.00 | 21.84** | 6.80 |
| OE-7    | 28.79** | 6.98 | 42.73** | 12.79 |
| OE-19   | 34.76** | 7.32 | 58.84** | 45.12 |
| OE-32   | 26.09** | 9.78 | 34.51** | 11.96 |
| OE-43   | 27.60** | 7.79 | 41.56** | 23.38 |
| OE-46   | 31.25** | 11.96 | 54.62** | 30.43 |

**Highly significant difference. dpi, days post inoculation.
AhRLK1 confers increased resistance to R. solanacearum

**Fig. 8.** Transcript levels of the defense marker genes in transgenic or non-transgenic tobaccos and resistant and susceptible varieties after inoculation of R. solanacearum based on qPCR and microarray analysis. (A–E) The transcript levels of some defense marker genes of the 35S::AhRLK1 transgenic tobacco plants and the wild-type CB-1 by qRT-PCR. (A) NtHIN1, NtHSR201, NtHSR203, and NtHSR515 in HR signaling pathway. (B) NtIPR2, NtIPR3, NtIPR4, and NtCHN50 in SA signaling pathway. (C) NtLOX1, NtIPR1b, and NtNDEF1 in MeJA signaling pathway. (D) NtEFE26 and NtACS6 in ET signaling pathway. (E) NtEDS1, NtPAD4, NtNDR1, and NtNPR1 in R-gene resistance signaling pathway. Transcript levels determined by qRT-PCR and normalized to NtEF1α. The transcript levels of non-inoculated WT plants were used as the controls and assigned the value of 1. AhRLK-R. solanacearum and WT-R. solanacearum are transgenic or wild-type plants with inoculation of pathogen, respectively; AhRLK-Mock and WT-Mock are transgenic or wild-type without inoculation, respectively. (F) In silico analysis of marker gene expression in R gene signal with or without inoculation of pathogen in resistant Yueyou 97 and susceptible Honghuadajinyuan. FG622894 and TC104336 are two NDR1-like genes; FG156504 and TC79797 are NPR1/NIM1-like genes; TC108802 and FG133223 are PAD4 genes. RRS-R. solanacearum indicates hyper-resistant tobacco variety Yanyan 97 under inoculation; RRS-Mock indicates hyper-resistant variety Yanyan 97 without inoculation; SRS-R. solanacearum indicates hypersusceptible variety Honghuadajinyuan with inoculation; SRS-Mock indicates hypersusceptible variety Honghuadajinyuan without inoculation. The experiments were repeated three times with at least three independent repetitions of the biological experiments. Different letters indicate significant differences, as determined by Fisher’s protected LSD test (lowercase indicates, P<0.05; uppercase indicates, P<0.01). Error bars indicate the standard error; data are means ±SE, n=3. (This figure is available in color at JXB online.)
**AhRLK1** expression is associated with defense responses to bio/abiotic stresses

The LRR–RLK gene family participates widely in the regulation of plant growth and development, and also in the resistance to pathogens and environmental stresses (Clark et al., 1993; Godiard et al., 2003; Sun et al., 2004; Wu et al., 2009; Xu et al., 2009; Hanemian et al., 2016). Both AtCLV1 and AtCLV2 in Arabidopsis are involved in regulating meristem identity, and their mutants, clv1 and clv2, are resistant to bacterial pathogens. This increased resistance does not require CLV signaling modules involved in regulating meristem homeostasis, and is not conditioned by defense-related hormones, such as SA, ethylene, and methyl jasmonate (Hanemian et al., 2016).

In peanut, we found that AhRLK1 responded differentially to *R. solanacearum* inoculation in resistant and susceptible varieties (Fig. 5G). AhRLK1 was up-regulated when plants were treated with hormones such as SA, ABA, ET, and MeJA, although the changes in expression pattern were slightly different depending on the hormone. However, the response patterns of transcripts to cold and drought stress were completely different (Fig. 5A–F). Clearly, the expression of peanut AhRLK1 is differentially affected by exposure to various hormones and environmental stresses. Both Arabidopsis and soybean CLV1 function as receptor subunits in the CLAVATA2–CORYNE (CRN) heterodimer complex, although, receptor-like protein kinase 2 is required for perception of CLEs, which are secreted from the nematodes *Heterodera schachtii* and *Heterodera glycines* (Guo et al., 2015). The expression of AhRPK2 is induced at the feeding sites of nematodes on roots, and CLV1 mutants show increased resistance to the nematode in soybean (Guo et al., 2015). However, AhRLK1, the ortholog of Arabidopsis AtCLV1, may be widely involved in defense response against biotic stress and in shoot and root meristem homeostasis.

**AhRLK1 confers resistance to bacterial wilt in transgenic tobacco**

Here, transient expression and co-localization of AhRLK1::GFP fusion protein in *N. benthamiana* showed that AhRLK1 was localized to the plasma membrane and cell wall (Fig. 3; Supplementary Fig. S4). Overexpression of AhRLK1 in a medium-susceptible tobacco cultivar, CB-1, increase the resistance to bacterial wilt significantly. Furthermore, six different transgenic T3 homozygous lines were derived from the hypersusceptible tobacco cultivar Honghuadajinyuan and carrying an overexpression cassette of AhRLK1 increased resistant to *R. solanacearum*, albeit to different levels (Fig. 7E; Supplementary Figs S5, S7; Table 1). These lines demonstrated that overexpression of AhRLK1 could confer resistance to bacterial wilt in a heterologous crop. Transient overexpression of AhRLK1 in *N. benthamiana* suggested it was induced by a hypersensitive response, based on trypan blue staining and H$_2$O$_2$ accumulation, indicating that AhRLK1 expression could result in the cell death caused by a HR. Thus, ROS may be involved in the increased resistance driven by AhRLK1. However, the mechanism of AhRLK1 is apparently different from *Atclv1, Atclv2*, and Atclv mutants, which are null alleles of different genes and show increased resistance through a decrease in miR169 accumulation (Hanemian et al., 2016). Wild-type genotypes, including *AtCLV1, AtCLV2*, and CRN, demonstrate susceptible phenotypes (Hanemian et al., 2016). In contrast, AhRLK1 is a functional gene. AhRLK1 expression was affected by hormone treatment, including ABA, ET, and SA, suggesting that AhRLK may confer resistance to *R. solanacearum* through these mechanisms, unlike *AtCLV1* and *AtCLV2*, which increase resistance via changes in miR169 accumulation (Hanemian et al., 2016). Therefore, our report is the first study showing that a peanut RLK is involved in resistance to *R. solanacearum* and can confer resistance in a heterologous crop.

**AhRLK1 resistance is associated with the R gene and defense signaling in transgenic tobacco**

A complex network of many defense signaling pathways is involved in plant–pathogen interactions, each of which is associated with certain marker genes (Divi et al., 2010; Nahar et al., 2012; Yang et al., 2013; Vos et al., 2015). Comparing AhRLK1-OE and wild-type tobacco variants infected with *R. solanacearum* using real-time PCR, marker genes NtHIN1, HSR201, and HSR515 of HR signaling (Sohn et al., 2007) were significantly up-regulated in transgenic lines that were inoculated with the pathogen (Fig. 8A). This result was consistent with the phenotype of the transient overexpression of AhRLK1 in *N. benthamiana*, which led to the HR and cell death (Fig. 6A, B), indicating that the resistance to the pathogen employed HR signaling. Some pathogen-related genes involved in SA signaling, such as NtPR2, NtPR3, and NtCHN50, were highly up-regulated in overexpression lines of AhRLK1 (Dong, 1998; Glazebrook, 2005), suggesting that SA signaling is also associated with the resistance against pathogens through AhRLK1. The ET signaling marker genes NtACS6 and NtEFE26 and the MeJA signaling marker genes NtPR1b, NtDEF1, and NtLOX1 were all induced in AhRLK1 overexpression lines (Fig. 8). This result was consistent with those observed in peanut, where AhRLK1 was up-regulated by the exogenous applications of SA, ET, MeJA, and ABA. Based on these lines of evidence, the interplay of different hormone signals may be involved in the increased resistance of transgenic tobacco with peanut AhRLK1. In rice, *Xas21* is a receptor-like kinase that confers resistance against most strains of *Xanthomonas oryzae* pv. *Oryzae* (Xoo) (Song et al., 1995). SA is required for *Xa21*-mediated resistance to Xoo, and the resistance to Xoo decreases in *Xa21*/NahG plants, though it is not completely abolished (Lee et al., 2009). However, *Atclv1, Atclv2*, and *clv1* mutants of *AtCLV1, AtCLV2*, and CRN1, respectively, all show increased resistance to bacterial wilt, which does not require hormone signaling through the ABA, ET,
MeJA, and SA pathways (Hanemian et al., 2016). Therefore, the resistance against pathogens in peanuts mediated by **AhRLK1** may be different from that of the orthologs *Atcrn1*, *Atclv1*, and *Atclv2*. As **AhRLK1** is involved in meristem determination, the mechanism by which **AhRLK1** interacts with multiple hormone pathways in fine-tuning immune responses in peanut requires further study.

**NDR1** and **EDS1** are important regulators for R-gene-mediated resistance signaling in plants (Day et al., 2006; Bhattacharjee et al., 2011; Lu et al., 2013). Usually, **NDR1** is involved in pathogen resistance mediated by CC-NBS-LRR-type of R genes, and **EDS1** and **PAD4** are implicated in Tir-NBS-LRR resistance signaling (Aarts et al., 1998; Wang et al., 2014). However, **RRS1-R**, a Tir-NBS-LRR gene in Arabidopsis, and **AhRRS5**, a NBS-LRR gene in peanut, require **NDR1** for the resistance phenotype (Deslandes et al., 2002, 2003; Zhang et al., 2017). In this study, we compared the wild-type and **AhRLK1** overexpression tobacco plants inoculated with *R. solanacearum* and found that **NtEDS1** and **NiPAD4** were significantly up-regulated in the **AhRLK1** overexpression tobacco plants. However, **NDR1** remained similar between wild-type and transgenic plants, and **NPR1** was down-regulated in the transgenic plants infected with the pathogen (Fig. 8E). **NPR1** is a key regulator of systemic acquired resistance and is essential for SA signal transduction to activate pathogen-related gene expression associated with R-gene resistance (Pieterse and Van Loon, 2004; Sandhu et al., 2009; Xia et al., 2013). Thus, the results indicated that **AhRLK1** may be associated with the **EDS1** pathway in the R-gene signal to regulate resistance against pathogens in transgenic tobacco, although **NPR1** is likely not required for this resistance.

For comparison, *in silico* hybridization with double strands of cDNA showed that the expression of two **NDR1** genes declined in hyper-resistant non-transgenic Yanyan97 when infected with the pathogen. In contrast, **NiPAD4** of the **NiEDS1** pathway was up-regulated in response to pathogen infection, and this was consistent with the results from transgenic tobacco overexpressing **AhRLK1**. However, the two **NPR1** genes were down-regulated in the hypersusceptible cultivar, and up-regulated in the hyper-resistant cultivar in response to pathogen infection. This result is consistent with the report that **NPR1** mediated resistance to viral and bacterial pathogens, and that repressing **NPR1** transcripts increases the susceptibility of plants to pathogens (Xiao and Chye, 2011; Li et al., 2012). In contrast, in this study, with high resistance conferred to *R. solanacearum* by **AhRLK1**, **NPR1** was down-regulated. Thus, we further suggest that **AhRLK1** participated in pathogen resistance by employing the R-gene pathway in association with **NiEDS1**, but independent of **NiNPR1**.

### Supplementary data

Supplementary data are available at JXB online.

Dataset S1. Microarray data of **AhRLK1** gene expression after *R. solanacearum* infection in Minhua 6.

Dataset S2. Sequences of **AhRLK1** full-length cDNA, genomic DNA, and protein.

Dataset S3. Amino acid sequences of five homolog LRR-RLKs.

Dataset S4. Thirty-five known functional Arabidopsis LRR-RLK proteins used for phylogenetic analysis.

Dataset S5. *In silico* study of expression characteristics of three members in the **AhRLK1** family in peanut.

Fig. S1. Cloning of **AhRLK1** from peanut. Electrophoresis photos represent 5’ RACE, 3’ RACE, and full-length cDNA PCR product of **AhRLK1**.

Fig. S2. Multiple sequence alignment with known functional LRR receptor kinase proteins.

Fig. S3. Phylogenetic tree constructed using **AhRLK1** and 180 different subfamily LRR RLKs of Arabidopsis.

Fig. S4. Co-localization of **AhRLK1**–GFP and CaSR-C2–1–RFP and plasmolysis experiments of **AhRLK1** protein subcellular localization.

Fig. S5. Phenotype of **AhRLK1-OE** transgenic T1 lines and non-transgenic control plants in tobacco cultivar CB-1 after inoculation with *R. solanacearum* for 40 d.

Fig. S6. Semi-quantitative PCR analysis of **AhRLK1** expression in different overexpression tobacco lines of Honghuadajinyuan background.

Fig. S7. Resistance phenotype of T2 **AhRLK1-OE** transgenic homozygous lines and the control plants.

Table S1. Primary primers used in this study.

Table S2. Detailed data of disease indexes and death ratios of different OE lines and the wild-type after inoculation with *Ralstonia solanacearum*.

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