The Cassava NBS-LRR Genes Confer Resistance to Cassava Bacterial Blight

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Cassava bacterial blight (CBB) caused by Xanthomonas axonopodis pv. manihotis (Xam) seriously affects cassava yield. Genes encoding nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains are among the most important disease resistance genes in plants that are specifically involved in the response to diverse pathogens. However, the \textit{in vivo} roles of NBS-LRR remain unclear in cassava (\textit{Manihot esculenta}). In this study, we isolated four \textit{MeLRR} genes and assessed their expression under salicylic acid (SA) treatment and Xam inoculation. Four \textit{MeLRR} genes positively regulate cassava disease general resistance against Xam via virus-induced gene silencing (VIGS) and transient overexpression. During cassava-Xam interaction, \textit{MeLRRs} positively regulated endogenous SA and reactive oxygen species (ROS) accumulation and pathogenesis-related gene 1 (\textit{PR1}) transcripts. Additionally, we revealed that \textit{MeLRRs} positively regulated disease resistance in \textit{Arabidopsis}. These pathogenic microorganisms include \textit{Pseudomonas syringae} pv. \textit{tomato}, \textit{Alternaria brassicicola}, and \textit{Botrytis cinerea}. Our findings shed light on the molecular mechanism underlying the regulation of cassava resistance against Xam inoculation.

\textbf{Keywords:} cassava, cassava bacterial blight, resistance genes, salicylic acid, ROS, NBS-LRR

\section*{INTRODUCTION}

Disease resistance genes (\textit{R} genes) usually act as receptors of pathogen-encoded effector proteins, which are often secreted by pathogens directly into host cells (Urbach and Ausubel, 2017). \textit{R} genes are specifically involved in the response to diverse pathogens, including fungi, bacteria, viruses, nematodes, insects, and oomycetes (Dalio et al., 2017). In the past 30 years, more than 300 \textit{R} genes have been cloned from many plant species (Kourelis and van der Hoorn, 2018). Among them, genes encoding nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains are important \textit{R} genes in plants (van der Hoorn and Kamoun, 2008; Pandolfi et al., 2017). The amino terminal (N-terminal) of NBS-LRR proteins usually contain the Toll/interleukin-1 receptor-like (TIR) domain, coiled-coil (CC) domain, or resistance to powdery mildew 8 (RPW8) domain, and the carboxyl terminus (C-terminus) contain a zinc-finger transcription factor-related domain containing the WRKY sequence (WRKY domain) (Shao et al., 2006). Based on the N-terminal domains, NBS-LRR was usually divided into three subclasses, namely TIR-NBS-LRR (TNL), CC-NBS-LRR (CNL), and RPW8-NBS-LRR (RNL) proteins (Shao et al., 2006).

In plant genome, about 0.2–1.6\% of genes are predicted as NBS-LRR-coding genes (Jia et al., 2015). For instance, there are 150–175 NBS-LRR genes in \textit{Arabidopsis thaliana} genome...
NBS-LRR (Masumba et al., 2017). A cluster of similar results were obtained under plants infected by viruses (Chaves et al., 2021), cassava brown streak disease (Utsumi et al., 2016). Within the database, four mapping and genomic selection (Kayondo et al., 2018). However, to cassava brown streak disease via genome-wide associated chromosome 11 of cassava genome was associated with resistance by Utsumi et al. (2016) indicated that the transcript level identify the NBS-LRR proteins in cassava. Results presented cassava is especially vulnerable to pathogens, especially cassava and subtropical regions of the world (Lozano et al., 2015; Utsumi et al., 2016). Among these, four MeLRRs (MeLRR1, MeLRR2, MeLRR3, and MeLRR4) were both induced under CBSV and C. gloeosporioides infection (Lozano et al., 2015; Utsumi et al., 2016). Among these, four MeLRRs (MeLRR1, MeLRR2, MeLRR3, and MeLRR4) were both induced under CBSV and C. gloeosporioides infection and selected for further analysis. The four MeLRR proteins have typical leucine-rich repeats, which are named MeLRR1 (Manes. 11G053000.1), MeLRR2 (Manes. 03G071700.1), MeLRR3 (Manes. 13G036800.1), and MeLRR4 (Manes. 07G107800.1), located on chromosomes 11, 3, 13, and 7, respectively. MeLRR1, MeLRR3, and MeLRR4 belong to CC-NBS-LRR protein, while MeLRR2 is one of the TIR-NBS-LRR protein. Bioinformatics predicted that the MeLRR proteins were unstable and hydrophilic (Supplementary Table 1). The phylogenetic analysis showed that MeLRR1 clustered with XP_012073222.1 of Jatropha curcas, MeLRR2 clustered with XP_021684995.1 of Hevea brasiliensis, MeLRR3 clustered with XP_020535356.1 of J. curcas, and MeLRR4 clustered with KAF2295929.1 of H. brasiliensis based on whole protein sequences (Supplementary Figure 1).

Subcellular Localization of the MeLRR Proteins
To investigate the subcellular localization of the MeLRR proteins, the coding sequences (CDs) of MeLRRs were cloned and inserted into the poly-cloning sites of the fusion expression vector pEGAD and fused upstream to a green florescence protein (GFP) fusion partner by the constitutive CaMV35S promoter. The Agrobacterium tumefaciens strain GV3101 cell culture harboring the pEGAD empty vector containing 35S:GFP was used as a control, and tobacco (Nicotiana benthamiana) leaves were infected with 35S:GFP or 35S:GFP-MeLRR1, −2, −3, −4 plasmid as described by Sparks et al. (2006). The fluorescence of transiently expressing MeLRR proteins in tobacco leaf epidermal cells was detected in the nucleus, cytoplasm and cytomembrane, similar to that of 35S:GFP (Figure 1).

Expression Level of MeLRR Genes in Response to SA Treatment and Xam Inoculation
The expression profile of MeLRRs in response to SA treatment and Xam inoculation were analyzed by qRT-PCR (real-time quantitative reverse transcription PCR). Under SA treatment, the expressions of MeLRR1, MeLRR3, and MeLRR4 were induced and
peaked at 1 h post treatment (hpt), while MeLRR2 showed the highest level at 3 hpt (Figure 2). Following infection by Xam, the expression level of MeLRRs at 1–24 hpt hpi was significantly higher than that at 0 hpi (Figure 2). Moreover, the expression of MeLRR1, MeLRR2, and MeLRR3 were induced and peaked at 3 hpt, while the expression of MeLRR4 reached the peak at 12 hpt (Figure 2).

Virus-Induced Gene Silencing of MeLRR Genes
To analyze the function of MeLRRs, we constructed MeLRR-silenced cassava plants by virus-induced gene silencing (VIGS). The partial sequences of MeLRR1 (453 bp), MeLRR2 (441 bp), MeLRR3 (433 bp) and MeLRR4 (423 bp) were individually inserted into pTRV2 plasmid to construct VIGS vector. At 14 days post-infection (dpi) in cassava infected with Agrobacterium GV3101 carrying the pTRV-MeLRR plasmids, qRT-PCR was performed to detect the target gene transcript level. The transcript level of the target MeLRR-1, -2, -3, -4 genes were significantly decreased in the MeLRR-silenced cassava leaves compared to the pTRV empty vector. The silencing efficiency of MeLRR-1, -2, -3, -4 was 46.33 (± 2.31)%, 15.28 (± 0.49)%, 30.22 (± 2.28)%, and 17.45 (± 0.87)% (Mean ± SD, n = 3), respectively (Figure 3A). It was noteworthy that the silenced of MeLRR1 did not affected the transcription of MeLRR2, -3, -4. Similar results were verified in MeLRR2-, MeLRR3-, and MeLRR4-silenced plants (Supplementary Figure 2). When co-silenced four target genes (MeLRR-1, -2, -3, -4) in one VIGS line, the transcript levels of all four target genes were significantly decreased (Supplementary Figure 3). On the contrary, the bacteria number was significantly higher than that in the pTRV empty vector-infected cassava leaves.
**MeLRR-Mediated Cassava Immune Responses via SA Accumulation**

To further analyze the mechanism of MeLRRs in response to Xam inoculation, the SA content was measured. As shown in Figure 5, the SA level in MeLRR1,−2,−3,−4-silencing was significantly decreased compared with that in pTRV control cassava leaves (Figure 5A). By contrast, the SA level in MeLRRs overexpression was significantly increased compared with the control cassava leaves (Figure 5B). These results suggested that MeLRR1,−2,−3,−4 positively participated in cassava immune responses via SA accumulation.

**Overexpression of MeLRR Genes in Arabidopsis Enhances Resistance to Plant Pathogens**

To further confirm the MeLRR function, MeLRRs were overexpressed in Arabidopsis. Quantification of endogenous SA levels indicated that MeLRR-overexpressing lines accumulated significantly higher levels than WT leaves (Supplementary Figure 7). The MeLRRs overexpression plants displayed slight symptoms of wilting in response to *P. syringae* pv. *tomento*, *A. brassicicola*, and *B. cinerea* infection support the hypothesis that MeLRRs functions in a pathogen response pathway. A difference was already observed in the WT, suggesting that restricted bacterial entry into the leaves may underlie part of the apparent resistance (Figure 6A). Unlike *P. syringae* pv. *tomento*, *A. brassicicola*, and *B. cinerea* can enter hosts by penetrating the cuticle. Consistently, there was less fungal growth in leaves overexpressing these factors than WT plants by analyzing the transcript levels of the *A. brassicicola* *AbAct* (JQ671669.1) gene and *B. cinerea* *BeActA* (XM_024697950.1) gene (Liao et al., 2016).
FIGURE 3 | The VIGS of MeLRRs reduced disease resistance against cassava bacterial blight. (A) At 14 dpi, the new leaves were used for relative transcript levels of MeLRRs in MeLRR-silenced leaves and the pTRV control leaves. Then, the new leaves were syringe infiltrated with $4 \times 10^8$ cfu/mL of pathogenic bacteria Xam used for disease resistance assay. (B) The number of Xam populations in MeLRR-silenced cassava and the pTRV control leaves at 0 and 1 dpi, respectively. (C) The pathogenesis-related gene (MePR1) transcript level was quantitatively analyzed by qRT-PCR at 1 dpi. The relative transcript level of MePR1 in the pTRV control leaves was normalized to 1.0. (D) Cassava leaves were observed using a Coomassie brilliant blue imaging system Fusion FX7-826 apparatus (Vilber Lourmat, France). (E) Dynamic of ROS accumulation in response to flg22 elicitation in MeLRR-silenced cassava and the pTRV control leaves. The flg22-triggered ROS burst were measured using luminol-based assay by a GloMax 96 Microplate Luminometer. (F) Total photon of MeLRR-silenced cassava and the pTRV control leaves. Multiple comparisons of total photon were calculated by Student’s t-test. Asterisks (*) indicate significant differences at $p < 0.05$. dpi is days post-infection.
FIGURE 4 | Transient overexpression of MeLRRs improved disease resistance against cassava bacterial blight. Cassava leaves inject with recombinant pEGAD plasmids and empty vector of Agrobacterium GV3101, respectively. (A) At 3 days later, the relative transcript levels of MeLRRs in MeLRR-overexpression cassava and the pEGAD control leaves. The relative transcript levels of MeLRRs in the pEGAD control leaves was normalized to 1.0. Then, the cassava leaves were syringe infiltrated with $4 \times 10^8$ cfu/mL of pathogenic bacteria Xam used for disease resistance assay. (B) The number of Xam populations in MeLRR-overexpression cassava and the pEGAD control leaves at 0 and 1 dpi, respectively. (C) The pathogenesis-related gene (MePR1) transcript level was quantitatively analyzed by qRT-PCR at 1 dpi. The relative transcript level of MePR1 in the pEGAD control leaves was normalized to 1.0. (D) Cassava leaves were observed using a Coomassie brilliant blue imaging system Fusion FX7-826 apparatus (Vilber Lourmat, France). (E) Dynamics of ROS accumulation in response to flg22 elicitation in MeLRR-overexpression cassava and the pEGAD control leaves. The flg22-triggered ROS burst were measured using luminol-based assay using a GloMax 96 Microplate Luminometer. (F) Total photon of MeLRR-overexpression cassava and the pEGAD control leaves. Multiple comparisons of total photon were calculated using Student’s t-test. Asterisks (*) indicate significant differences at $p < 0.05$. dpi is days post-infection.
with the Arabidopsis AtAct2 gene as an internal control at 2 and 4 dpi, respectively (Figures 6B,C).

To determine whether the enhanced resistance to plant pathogens was related to changing the defense response genes expression level, we used qRT-PCR to analyze the expression levels of AtICS1, AtPDF1.2, AtPR1, AtPR2, AtPR5, and AtTGA3 in WT and MelRR overexpression lines upon A. brassicicola, B. cinerea, and P. syringae pv. tomato DC3000 infection (Supplementary Figure 8). Particularly, the relative expression levels of genes involved in the SA synthesis pathway and pathogen resistance showed higher level in overexpression MelRR1 and MelRR2 in Arabidopsis plants than in control plants without A. brassicicola, B. cinerea, and P. syringae pv. tomato DC3000 infection. Similar results were observed in plant pathogen-infected overexpression of MelRR3 in Arabidopsis plants compared with control plants. However, AtPDF1.2 and AtPR1 were significantly down-regulated in overexpression of MelRR3 in Arabidopsis plants than in control plants without P. syringae pv. tomato DC3000 infection. On the other hand, the expression levels of AtPR2 and AtTGA3 were significantly up-regulated in overexpression of MelRR4 in Arabidopsis plants than in control plants. AtICS1, AtPDF1.2, AtPR1, and AtPR5 genes were up-regulated or down-regulated under different plant pathogen infections. These results indicate that overexpression of MelRRs resulted in enhanced resistance simultaneously against pathogenic bacteria and pathogenic fungi, demonstrating the requirement of MelRRs for resistance to plant pathogens.

DISCUSSION

NBS-LRR proteins play important roles in pathogen recognition and defense response signal transduction (Urbach and Ausubel, 2017). An increasing number of NBS-LRR proteins that conferred resistance to pathogens have been cloned from higher plants (Liu et al., 2017), such as TaRCK1 (Zhu et al., 2017), ZmNBS25 (Xu et al., 2018), GbNA1 (Li et al., 2018a,b), GhDC1 (Li et al., 2019), and OsRLR1 (Du et al., 2021). In this study, we found that MelRR1,-2,-3,-4 expression could be induced by Xam inoculation. Similar expression patterns have been observed in other plant NBS-LRR genes, such as AhRRS5 (Zhang et al., 2017) and SacMi (Zhou et al., 2018). NBS-LRRs mainly participate in plant resistance against pathogen infection, and we speculated that the up-regulation of MelRRs could help cassava successfully evade Xam inoculation.

SA is a secondary messenger for systemic acquired resistance (SAR), and its production in plants represents the successful recognition of pathogen infection and pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Divi et al., 2010; Peng et al., 2021). In cassava, SA also plays an important role in the regulation of cassava resistance to CBB (Liu et al., 2019; Chang et al., 2020; Wei et al., 2021a,b) and to whitefly (Irigoyen et al., 2020). Wei et al. (2018) found that MeHsf3 regulates cassava resistance to cassava bacterial blight through modulation of SA accumulation. Cassava co-chaperones MeHSP90.9 interacts with MeSR51 and MeWRKY20 to activate SA biosynthesis, accumulation of SA, and thus improve resistance to CBB (Wei et al., 2021b). Therefore, endogenous SA accumulation levels are an indicator of resistance to CBB. We found that the expression levels of MelRR were significantly increased by SA treatment, which showed the similar expression pattern of NPR1 in Arabidopsis, ZmNBS25 in maize, and GhDC1 in cotton. In response to pathogen infection, plant endogenous SA is quickly and strongly induced.

Moreover, multiple transcription activator-like (TAL) effectors and type III effectors (T3Es) of Xam regulate plant immune (Castiblanco et al., 2013; Medina et al., 2018). Such as, TALE1_Xam (Castiblanco et al., 2013), Xop (Arrieta-Ortiz et al., 2013), avrBS2, xopQ, XopR, XopAO1, and similar factors (Bart et al., 2012; Cohn et al., 2016; Medina et al., 2018; Mondal et al., 2020). Flagellin peptide (flg22) treatment regulates the expression of MeZIP3, -5 (Li et al., 2017), MeBKI (Li et al., 2018c), MeDELLAs (Li et al., 2018d), MeWHYs (Liu et al., 2018), and MeASMT2 (Wei et al., 2017). Moreover, these genes mediated cassava resistance to CBB. Flg22 is a bacterial PAMP. In Arabidopsis and tomato, flg22 was used to instead of P. syringae and Xanthomonas to measure the ROS burst, respectively (de Torres Zabala et al., 2015; Bhattachary et al., 2016). Interestingly, MelRRs regulated ROS burst was induced by flg22 (Zipfel et al., 2004). As a homolog protein of MelRR3, AtLRRAC1 is induced by flg22 treatment and leads to production ROS and induction of
pathogen-responsive genes (Bigeard et al., 2015; Bianchet et al., 2019). Therefore, we hypothesized that MeLRRs and effectors of Xam conform to the gene for gene theory.

AtPDF1.2, AtPR1, AtPR2, and AtPR5 are widely known as marker genes for innate immune response (Wang et al., 2017; Xu et al., 2018). AtICS1 is a key enzyme for SA biosynthesis (Macaulay et al., 2017). AtTGA3 showed strong affinity for the NPR1 protein (Zhou et al., 2000; Yuan et al., 2009). In pathogenic microorganism infection, the SAR defense response is triggered by elevated SA through an SA-NPR1-TGA-PR1 signaling pathway (Zhang, 2003). Further analysis of gene expression in overexpression of MeLRR1, −2, −3, −4 at Arabidopsis leaves suggested that these genes might exert their function through SA biosynthesis and immune responses. This is similar to the function of MeHsf3 (Wei et al., 2018), and MebZIP3, -5 (Li et al., 2017), which were confirmed to regulate cassava resistance against cassava bacterial blight. Hence, we conclude that MeLRR1, −2, −3, −4 may regulate the plant immune response through SA and ROS accumulation, and the transcription of disease resistance genes. Taken together, the MeLRR genes encode a class of NBS-LRR proteins, which controls immunity to Xanthomonas axonopodis pv. manihotis in cassava. Further investigation of the role of the MeLRRs will build an important foundation for future development of resistant cultivars, which may be the most effective means of controlling this devastating disease.

MATERIALS AND METHODS
Plant Materials, Growth Conditions, and Treatments
Cassava (M. esculenta), variety South China 124 (SC124), and N. benthamiana were cultivated in mixed soil (vermiculite/nutritional soil = 2:1, v.v.) in a greenhouse with 16/8 h light/dark at 28/22 °C, 60–70% relative humidity with irradiance of 130–150 uE.m$^{-2}$.s$^{-1}$. A. thaliana ecotype Col-0 (Columbia-0) seedlings were cultivated in the mixed soil under fluorescent light (130–150 uE.m$^{-2}$.s$^{-1}$) and were grown under 16/8 h light/dark at 22 °C. For axenic growth,

FIGURE 6 | Overexpression of MeLRRs in Arabidopsis enhances resistance to plant pathogens. (A) The number of P. syringae pv. tomato populations in overexpression Arabidopsis leaves and the wild type. The relative transcript levels of AbAct/AtAct2 (B) and BcActA/AtAct2 (C) in overexpression Arabidopsis leaves and the wild type after infection with A. brassicicola and B. cinerea, respectively. Asterisks (*) indicate significant differences at $p < 0.05$. Col-0 is A. thaliana ecotype Columbia-0. #1 and #2, #3 and #4, #5 and #6, and #7 and #8 are overexpression of MeLRR3 in A. thaliana Col-0 lines, respectively. dpi is days post-infection.
N. benthamiana, and A. thaliana seeds were sterilized (10% NaClO for 1 min, washed five times with sterile water) and sown on half-strength MS (Murashige and Skoog) medium (PhytoTechnology Laboratories, Kansas, United States) with 0.4% agar powder and 2% (w/v) sucrose. The seeds were grown in chambers under 16/8 h light/dark at 22°C and 130–150 μE.m⁻².s⁻¹. For expression analysis, 4-week-old cutting seedlings of cassava leaves were sprayed with 5 mmol/L salicylic acid or Xam suspension for 0, 1, 3, 6, 12, or 24 h, and the bacterial solution was diluted to 4 × 10⁸ colony-forming units/mL (cfu/mL) using 10 mmol/L MgCl₂ with 0.05% Silwet L-77.

Comprehensive Characterization and Bioinformatics Analysis of MeLRR Genes

The sequences of MeLRR genes were searched and obtained from the cassava genome database, M. esculenta v6.1 (Phytozome v13) (Muñoz-Bodnar et al., 2014; Lozano et al., 2015; Bredeson et al., 2016). The ProtParam tool² was used to predict the number of amino acids, relative molecular mass of protein, isoelectric point, total average hydrophilicity stability index, fat coefficient, and instability index (Gasteiger et al., 2003). Alignments between MeLRRs and other NBS-LRR proteins were validated through comparisons of the protein basic local alignment search tool (BLASTP) with the National Center for Biotechnology Information (NCBI). The 24 NBS-LRR protein amino acid sequences in 13 species were derived from A. thaliana (PhytoTechnology Laboratories, Kansas, United States) with C and 130ñ150 μE.m⁻².s⁻¹. For expression analysis, 4-week-old cutting seedlings of cassava leaves were sprayed with 5 mmol/L salicylic acid or Xam suspension for 0, 1, 3, 6, 12, or 24 h, and the bacterial solution was diluted to 4 × 10⁸ colony-forming units/mL (cfu/mL) using 10 mmol/L MgCl₂ with 0.05% Silwet L-77.

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RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

Total RNA was extracted from three independent pools, and DNA contamination was removed using the Tiangen RNA prep pure plant plus kit (Tiangen Biotech, Beijing, China, Cat# DP441). cDNA synthesis was performed using the Tiaragen FastQuant RT kit (Tiangen Biotech, Beijing, China, Cat# KR116) with 20-μl reaction mixture. qRT-PCR analysis was performed using UltraSYBR Mixture (low ROX) (CoWin Biosciences, Beijing, China, Cat# CW0956) in an ABI QuantStudio™ 6 flex Real-Time PCR System (ABI, CA, United States). The PCR cycling conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The Arabidopsis and cassava gene transcripts were normalized to the AtAct2 gene (AT3G18780) and elongation factor 1α (EF1a, Me.15G054800) using the comparative 2⁻ΔΔCt method, respectively (Livak and Schmittgen, 2001). The qRT-PCR primers of MeEF1a, MePR1 were obtained from Wei et al. (2018), AtPR1, AtPR2, AtPR5, AtPDF1.2, AtICS1, AtAct2, and BcAct2 were obtained from Mhamdi and Noctor (2016), and AtTGA3 was obtained from Ndamukong et al. (2017), respectively. The qRT-PCR primers of MeLRRs, and AbAct (IQ671669.1) of A. brassicicola were designed by Primer3Plus³ to find optimal primers (Untergasser et al., 2007), and then the specificity of the melt curve analyzed performed to determine. In addition, the qRT-PCR fragments and VIGS fragments are different CDS regions of MeLRRs. The primers used are listed in Supplementary Table 2.

Plasmid Construction and Transient Expression in Plant Leaves

For overexpression, the full-length coding regions of MeLRR1, 2, 3, 4 were amplified and cloned into the pEGAD vector (Promoter CaMV35S:GFP) via appropriate restriction enzyme digestion and T4 DNA ligase. The recombinant plasmids and empty vector were transformed into Agrobacterium GV3101. Then, the A. tumefaciens suspension was used to infect the leaves of cassava or tobacco as described by Sparkes et al. (2006) and Zeng et al. (2019). Tobacco leaves injected with Agrobacterium GV3101 for 2 days, the GFP fluorescence and DAPI (4',6-diamidino-2-phenylindole, Thermo Fisher Scientific, Shanghai, China)-stained cell nuclei were imaged under a fluorescence microscope (Leica TCS SP8, Solms, Germany), with an excitation wavelength of 488 nm and a 505–530-nm bandpass emission filter. Cassava leaves inject with recombinant pEGAD plasmids or empty vector of Agrobacterium GV3101. Then, 3 days later, the cassava leaves were syringe infiltrated with 4 × 10⁸ cfu/mL of pathogenic bacteria Xam used for disease resistance assay, include number of Xam populations, MePR1 transcript level, and symptoms of cassava bacterial blight at 0 and 1 dpi, respectively.

VIGS constructs are usually prepared using 300–500 bp partial CDS regions of MeLRRs and the online siDirect 2.0 tools (Naito et al., 2009) are available for predicting regions with high siRNA generating capability (Naito et al., 2009; Ui-Tei and Naito, 2013). Zeng et al. (2019) constructs the method about Agrobacterium-mediated Tobacco Rattle Virus (TRV)-based gene silencing in cassava. For VIGS in cassava, the specific CDS fragments of MeLRR1, 2, 3, 4 were amplified and cloned into the pTRV2 vector through appropriate restriction enzyme digestion and T4 DNA ligase. The recombinant plasmids and empty vectors were transformed into Agrobacterium GV3101. Then, the Agrobacterium suspension, as well as pTRV1, was used to infect the leaves of cassava as previously described (Zeng et al., 2019). At 14 dpi, the new leaves were syringe infiltrated.

¹https://phytozome-next.jgi.doe.gov/ ²http://web.expasy.org/protpasam/ ³https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi ⁴http://siDirect2.RNAi.jp/
with $4 \times 10^8$ cfu/mL of pathogenic bacteria *Xam* used for disease resistance assay. The sequences of primers used for vector construction in this study are listed in Supplementary Table 2.

**Arabidopsis Transformation**

*Arabidopsis thaliana* ecotype Col-0 was used as wild-type. Overexpressing lines were transformed by floral dip transformation method of 35S:GFP-MeLRR recombinant plasmids constructs with Agrobacterium GV3101 (Bechtold and Pelletier, 1998). The overexpressing lines were selected by 100 mg/L kanamycin and 20 mg/L glufosinate (Basta; Sangon Biotech. Shanghai, China) resistance and further confirmed by PCR. Single insertion transgenic lines were chosen for further analysis in transgenic third generations (T3).

**Quantification of Endogenous SA Contents**

The endogenous SA content in leaves was determined as previously described (Wei et al., 2018). Briefly, leaves were flash-frozen in liquid nitrogen and ground to a very fine powder. SA was extracted from 0.1 g powder using phosphate-buffered solution (PBS, pH 7.4, 0.15 M) on ice. Then, the supernatant was used for SA quantification using a plant SA ELISA (enzyme-linked immunosorbent assay) kit (Jiangsu Meimian Industrial, Jiangsu, China, Cat#HLE01901) according to the manufacturer's instructions.

**Reactive Oxygen Species Burst Measurements**

The ROS burst in leaves was determined as described previously (de Torres Zabala et al., 2015; Chang et al., 2020; Yan et al., 2021). In tomato, flg22 was used to instead of *Xanthomonas* to measure the ROS burst (Bhattarai et al., 2016). Similar methods were applied to study the cassava resistance to *Xam*, such as MeCAMTA3 (Chang et al., 2020), MeRAV5 (Yan et al., 2021). Herein, to measure the ROS burst, 48 leaf discs (5 mm in diameter) of cassava were placed in 48 single wells of 96-well black plates and placed in the dark for 12 h in 100 µL double-distilled water. After 12 h, the 48 leaf discs were divided into two groups. In one group, the water was replaced with 100 µL incubation solution containing 0.2 µmol/L luminol (AppliChem, Darmstadt, Germany) and 10 µg/mL horseradish peroxidase (AppliChem, Darmstadt, Germany). In the other group, the water was then replaced with 100 µL incubation solution containing 0.2 µmol/L luminol, 10 µg/mL horseradish peroxidase and 1 µmol/L flg22 (Phyto Technology Laboratories, Lenexa, KS, United States). Luminescence was measured immediately for 30 min using a GloMax 96 Microplate Luminometer (Promega, Madison, WI, United States). Luminescence readout is given in relative light emitting units (RLU).

**Trypan Blue Staining**

The cassava or *N. benthamiana* leaves were boiled for 1 min in the trypan blue working solution (100 mL lactic acid, 100 mL glycerol, 100 g phenol, and 0.2 g trypan blue, dissolved in 100 mL distilled water) for 24 h at room temperature (Luo et al., 2017). The leaves were transferred into a chloral hydrate solution (2.5 g/mL) and repeatedly reduced until the background was gone (Luo et al., 2017).

**Pathogen Culture and Disease Assays**

The pathogenic bacterium *P. syringae pv. tomato* (Pst) DC3000 was streaked on LB medium with 50 mg/L of rifampicin at 28°C and shaken to OD600 reached 0.6. Thereafter, a fresh bacterial culture of Pst DC3000 was diluted to $4 \times 10^8$ cfu/mL in 10 mmol/L MgCl2 and 0.05% Silwet L-77 and then sprayed on 24-day-old *Arabidopsis* leaves. The *A. brassicicola* and *B. cinerea* strains were cultured on potato dextrose agar (PDA) medium with 2% (w/v) sucrose at 28°C. Conidia were suspended in distilled water for plant infection. Spore suspensions (about $4 \times 10^6$ spores/mL) of *A. brassicicola* and *B. cinerea* were sprayed on *Arabidopsis* leaves. The infected plants were grown in an incubator at 90% RH and 22°C. At 0, 2, and 4 dpi, the number of Pst DC3000 bacteria was determined, as well as the fungal actin gene transcript in leaves of Col-0 and mutants infected with *B. cinerea* and *A. brassicicola* (Veronese et al., 2006; Mhamdi and Noctor, 2016).

**Analysis of Experimental Data**

Mean and standard deviations are displayed as representative values for data in the figures. Analysis of variance (ANOVA) with Duncan’s test and Student’s t-test were applied to the obtained data with the help of IBM SPSS v20. Statistical significance (*) was set at $p < 0.05$. Each assay contained three independent replicates.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

**AUTHOR CONTRIBUTIONS**

YW and HZ designed the research. HZ did most experimental works and wrote the manuscript. ZY, ZL, and YS did experimental works and database analysis. XL, JW, and GZ did experimental works. YW supervised this project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.790140/full#supplementary-material
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