The dual interplay of RAV5 in activating nitrate reductases and repressing catalase activity to improve disease resistance in cassava

Yu Yan1,†, Peng Wang1,†, Yunxie Wei1,†, Yujing Bai1, Yi Lu1, Hongqiu Zeng1, Guoyin Liu1, Russel J. Reiter2, Chaozu He1 and Haitao Shi1,*

1Hainan Key Laboratory for Sustainable Utilization of Tropical Bioresources, College of Tropical Crops, Hainan University, Haikou, China
2Department of Anatomy and Cell System, UT Health San Antonio, San Antonio, TX, USA

Introduction

Nitric oxide (NO) is an important signalling molecule in several plant processes, including seed germination, root growth and development, flowering transition, stomatal movement and senescence (Besson-Bard et al., 2008; Neill et al., 2003). In addition, NO regulates plant defence responses to biotic and abiotic stresses (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2014; Fancy et al., 2016; Mur et al., 2006; Siddiqui et al., 2011), modulating defensive hormones and defensive-related genes expression (Bellin et al., 2013; Trapet et al., 2015; Yu et al., 2014). For example, the NO donor, sodium nitroprusside (SNP) reduces reactive oxygen species (ROS) induced by copper, while NO scavengers cause the opposite results by decreasing Cu-induced glutathione accumulation (GSH) and ascorbic acid (AsA) (Hu et al., 2015). NO treatment activates the activities of related enzymes and multiple metabolites, thereby improving disease resistance and preventing pathogen invasion in peach (Li et al., 2017). In tobacco, NO inhibits the activity of the bacterial effector HopAI1 by S-nitrosylation during the hypersensitive response (HR) to restore mitogen-activated protein kinase (MAPK) signalling, indicating the participation of NO-dependent S-nitrosylation in plant immunity (Ling et al., 2017).

Both NO and hydrogen peroxide (H2O2) are key signalling regulators, and the crosstalk between them plays important roles in the activation of plant immune response (Gupta et al., 2020; Keshavarz-Tohid et al., 2016; Qiao et al., 2015). In soya bean cells, the integration of NO and H2O2 is essential for plant hypersensitive disease resistance response (Delledonne et al., 2001). In potato, conditional activation of SIPK induces NO and H2O2 to improve disease resistance to both biotrophic and necrotrophic pathogens, indicating the synergistic function of NO and ROS in plant defence response (Yoshioka et al., 2009). In tomato, the crosstalk between NO and H2O2 is involved in plant defence response to root-knot nematodes (Leonetti et al., 2011). In wheat, NO and H2O2 are jointly involved in plant disease resistance against Puccinia Triticina (Qiao et al., 2015). In bean plants, NO induces H2O2 generation and regulates redox state, and this interaction is involved in basal and induced resistance against Rhizoctonia solani and Pseudomonas fluorescens, respectively (Keshavarz-Tohid et al., 2016). In Arabidopsis, S-nitrosylation of NADPH oxidase-AtRBOHD by NO regulates ROS accumulation and cell death in plant immunity, indicating the novel link between NO and ROS signalling (Yun et al., 2011, 2016). H2O2 is essential for plant immunity (Zhou et al., 2011a). AtrobhF knockout mutant with deficient NADPH oxidase activity and lower H2O2 shows decreased resistance against bacterial infection (Torres et al., 2013). On the contrary, exogenous H2O2 treatment enhances disease resistance against bacterial wilt in tomato (Hong et al., 2013), and redox rhythm could improve the circadian clock for immune response (Zhou et al., 2015a). Catalase (CAT) as the major H2O2-scavenging enzyme is also widely involved in plant immunity. Overexpression of CAT1 decreases disease resistance in tobacco, while knockdown of CAT

Summary

Cassava bacterial blight (CBB) caused by Xanthomonas axonopodis pv. manihotis (Xam) seriously affects cassava yield. Nitrate reductase (NR) plays an important role in plant nitrogen metabolism in plants. However, the in vivo role of NR and the corresponding signalling pathway remain unclear in cassava. In this study, we isolated MeNR1/2 and revealed their novel upstream transcription factor MeRAV5. We also identified MeCatalase1 (MeCAT1) as the interacting protein of MeRAV5. In addition, we investigated the role of MeCatalase1 and MeRAV5-MeNR1/2 module in cassava defence response. MeNRs positively regulates cassava disease resistance against CBB through modutation of nitric oxide (NO) and extensive transcriptional reprogramming especially in mitogen-activated protein kinase (MAPK) signalling. Notably, MeRAV5 positively regulates cassava disease resistance through the coordination of NO and hydrogen peroxide (H2O2) level. On the one hand, MeRAV5 directly activates the transcripts of MeNRs and NO level by binding to CAACA motif in the promoters of MeNRs. On the other hand, MeRAV5 interacts with MeCAT1 to inhibit its activity, so as to negatively regulate endogenous H2O2 level. This study highlights the precise coordination of NR activity and CAT activity by MeRAV5 through directly activating MeNRs and interacting with MeCAT1 in plant immunity.
improves disease resistance in tobacco (Mittler et al., 1999). In Arabidopsis, cat2 knockout lines confer improved disease resistance to Pseudomonas syringae (Chao et al., 2010), but show decreased disease resistance to Botrytis cinerea (Yuan et al., 2017). In addition, ATCAT2 mediates the crosstalk between salicylic acid (SA)-repressed auxin and jasmonic acid (JA) accumulation (Yuan et al., 2017).

NO synthesis in plants is divided into the oxidative pathway and the reductive pathway (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2014). The oxidative pathway involves mammalian L-arginine-dependent NO synthase (NOS)-like activity (Vitor et al., 2013). Although similar NOS activity has been detected in many plant organs (Cueto et al., 1996; Fossner et al., 2000; Ribeiro et al., 1999; Tun et al., 2001), no typical NOS enzyme has been found in higher plants (Hancock, 2012; Jeandroz et al., 2016). Polyamines and hydroxylamines may also be involved in the oxidative pathway of NO synthesis in plant cells (Rumer et al., 2009; Tun et al., 2006; Wimalasekera et al., 2011). The reductive pathway involves nitrate reductase (NR), which is composed of two subunits of three repair groups, namely FAD, haem b557, and molybdenum cofactor (Moco) (Campbell, 2001). NR reduces NO$_3^-$ to NO$_2^-$, and then nitrite reductase (NIR) reduces NO$_2^-$ to NO (Chamizo-Ampudia et al., 2017; Roszer, 2014). NR can also oxidize NO to nitrate through haemoglobin to remove NO (Chamizo-Ampudia et al., 2017). Thus, NR is a main regulator of NO homeostasis in plants (Chamizo-Ampudia et al., 2017). As an important regulatory enzyme and the rate-limiting enzyme in nitrogen metabolism (Campbell, 1988), NR is also involved in plant defence responses (Vitor et al., 2013). Arabidopsis thaliana NR double-deficient mutant nia1nia2 with lower NO content is sensitive to Pseudomonas syringae through regulating the expression of lignin biosynthesis-related gene cinnamyl alcohol dehydrogenase 1 (CAD1) and other defence-related genes, suggesting that AtNRs are essential for the NO-mediated plant immunity (Vitor et al., 2013).

The apetala2/ethylene response factor (AP2/ERF) is a large transcription factor family in plants (Wei et al., 2018). RAV is a subfamily of the AP2/ERF family with an AP2/ERF domain and a B3 domain, which binds to CAACCA motif and CACCTG, respectively (Hu et al., 2004; MAPK, 2002; Xu et al., 2011). RAV plays an important role in plant development and stress responses (Feng et al., 2005; Zhuang et al., 2011). The inhibition of AtRAV1 by brassinolide (BR) leads to early flowering, while overexpression of AtRAV1 inhibits lateral root and rosette leaf development, indicating that RAV1 as a primary BR response gene negatively regulates plant growth and development (Hu et al., 2004). Overexpression of ZmRAV1 activates the expression of ROS scavenging-related genes and some stress-related genes, resulting in improved salt tolerance and osmotic stress tolerance (Min et al., 2014). FarRAV1 enhances anthocyanin accumulation in strawberry fruit by activating the key activator of anthocyanin biosynthesis-FaMYB10 and directly activating the promoter of anthocyanin synthesis-related gene (Tavares et al., 2019). In addition, plant RAVs also involve in plant disease resistance. In tomato, SIRAV2 binds to the promoter of SIERFS to regulate the expression of pathogenesis-related genes so as to enhance the resistance to bacterial wilt (Li et al., 2011). In cassava, MeRAV1 and MeRAV2 positively regulate cassava disease resistance by directly activating endogenous melatonin biosynthesis (Wei et al., 2018). However, the interacting proteins of plant RAVs and their direct downstream genes remain elusive in plants.

Herein, the in vivo role of NR in plant immune response against Xanthomonas axonopodis pv. manihotis (Xam) and the corresponding upstream signalling in cassava are revealed. In addition, the genetic evidence of MeRAVs-MeNR1/2 and MeRAV5 & MeCatalse1 (MeCAT1) in the coordination of NO and H$_2$O$_2$ level in cassava defence resistance is highlighted.

**Results**

**Identification and subcellular localization of MeNR1 and MeNR2**

To isolate and identify the encoding sequence of NR in cassava, the CDS of AtNRs and OsNRs were used to blast in cassava database, and two sequences with high homology with AtNRs and OsNRs, named MeNR1 and MeNR2, respectively, were obtained. To explore the relationship among MeNRs and AtNRs, OsNRs and other NRs, we constructed the phylogenetic tree and found that MeNRs have high homology with these NRs (Figure 1a). In addition, MeNRs showed conserved domains of NR, including cytochrome b5-like haem, oxidoreductase NAD-binding domain, oxidoreductase NAD-binding domain and MO-co oxidoreductase dimerization domain (Figure 1b). To verify whether MeNRs have NR activity, the His-tag fusion MeNR1/2 proteins were successfully induced and purified, and then, the specific band of MeNR1/2 proteins was further confirmed by SDS-PAGE and Western blot (Figure 1c). The purified MeNR1/2 proteins were used for enzyme activity analysis by determining the transformation of NO$_3^-$ to NO$_2^-$ using different concentration of purified proteins, the enzyme kinetics of MeNR1/2 proteins was obtained (Figure 1d, e). Based on the linear relationship by Lineweaver–Burk, the substrate affinity ($K_m$) and maximum reaction rate ($V_{max}$) of the purified MeNR1 and MeNR2 proteins were calculated, respectively (Figure 1d,e). These results indicated that MeNRs indeed have NR activity. To identify the subcellular localization of MeNR1 and MeNR2, we transformed these recombinant vectors 35S::GFP-MeNRs into cassava protoplasts. Similar to the wide localization of 35S::GFP in plant cells, we found that both 35S::GFP-MeNR1 and 35S::GFP-MeNR2 were located in the nucleus and cytoplasm (Figure 1f).

**MeNRs positively regulate cassava disease resistance to Xam through NO**

Previous studies have shown that NR is related to plant defence responses (Vitor et al., 2013; Zhou et al., 2015b). Herein, quantitative real-time (qRT) PCR found that both the transcripts of MeNR1 and MeNR2 and MeNR activity were significantly increased during Xam infection, especially at 3 h postinfection (hpi) (Figure 2a,b). To explore their in vivo roles, we constructed MeNR-overexpressed cassava and MeNR-silenced cassava by virus-induced gene silencing (VIGS) (Figure 2c, d, f). Reverse transcription–PCR was first used to confirm the overexpression of MeNRs and the successful spread of TRV1 and TRV2 in cassava leaves (Figure 2c). Then qRT-PCR was used to verify the overexpression and silencing of MeNRs in cassava leaves (Figure 2d, f). The expression level of MeNR2 was about twofold of the expression level of MeNR1 (Figure 2f), indicating that there might be gene redundancy between MeNR1 and MeNR2 and MeNR2 might be dominant in cassava. Therefore, although the silencing of MeNR2 caused about twofold induction of MeNR1 gene, the silencing of MeNR2 might also have in vivo roles, and about twofold induction of MeNR1 might be plant response to the
silencing of MeNR2. By the statistical analysis of bacterial number after Xam infection, we found that overexpression of MeNRs in cassava decreased bacterial number than that observed in mock samples, while silencing MeNRs in cassava increased bacterial number; especially, MeNR2-silenced cassava leaves showed much more bacterial number than MeNR1-silenced cassava leaves (Figure 2e, g). These results suggest that MeNR1 and MeNR2 positively regulate cassava disease resistance to Xam, and MeNR2 might play critical roles in cassava immunity rather than MeNR1. Quantification of endogenous NO level by the specific 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM) staining showed that MeNRs-overexpressed cassava leaves had higher NO content than the control cassava leaves (Figure 2h, i), while the MeNR-silenced cassava leaves had lower NO content than the control cassava leaves (Figure 2j, k). Notably, MeNR2-silenced cassava leaves displayed lower NO content than MeNR1-silenced cassava leaves, consistent with more bacterial number. In addition, the results using NO assay kit further verified the DAF-FM stained NO level (Figure S1), confirming the positive regulation of MeNR1 and MeNR2 on endogenous NO. The significant

Figure 1 Identification and subcellular localization of MeNR1 and MeNR2. (a) The phylogenetic tree of MeNRs, AtNRs, AtNRs, OsNRs, CsNRs, CcNRs, HbNR, JcNR and RcNR based on their amino acid sequences. (b) The conserved domains of MeNR1 and MeNR2. (c) SDS-PAGE and Western blot analysis of induced and purified MeNR1 and MeNR2. (d, e) Enzyme kinetics of purified MeNR1 (d) and MeNR2 (e). All experiments included three biological replicates, and all data are expressed as means and SDs. (f) Subcellular localization of MeNR1 and MeNR2. The GFP signals were visualized using a confocal laser scanning microscope, and 1 mg/mL DAPI was used to stain cell nuclei. Bar = 25 μm.

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effects of both silencing of MeNR1 or MeNR2 and co-silencing of them on endogenous NO level and disease resistance indicated that both MeNR1 and MeNR2 are essential for NO and disease resistance. The gene redundancy and dose effect of them on NO also suggested that they might cooperatively regulate cassava immunity.

Consistently, exogenous treatment with the NO donor (SNP) increased disease resistance to Xam with lower bacterial number, while exogenous treatment of the NO scavenger (2-(4-carboxyphenyl)-4, 4, 5-tetramethylimidazoline-1-oxyl-3-oxide, c-PTIO) showed the opposite results (Figure S2). To investigate whether NO is involved in MeNR-mediated disease resistance, we treated the MeNR-overexpressed cassava leaves with 200 μM c-PTIO and infected Xam, and found that c-PTIO could significantly alleviate the effect of MeNR1/2 overexpression on bacterial number (Figure S3). These results indicate that NO is essential for MeNR-mediated disease resistance.

**Figure 2** MeNR1 and MeNR2 positively regulate cassava disease resistance to Xam and NO accumulation. (a) The transcript levels of MeNR1 and MeNR2 in response to Xam. (b) The NR activity of cassava in response to Xam. (c) RT-PCR showing the expression of MeNR1 and MeNR2 in MeNR-overexpressed cassava and TRV1/2 in MeNR-silenced cassava, respectively. MeEF1a was used as the reference gene. (d) The relative transcript levels of MeNR1 and MeNR2 in MeNR-overexpressed cassava and the vector control leaves. (e) Bacterial number in MeNR-overexpressed cassava and in the vector control leaves. (f) The relative transcript levels of MeNR1 and MeNR2 in MeNR-silenced cassava and the pTRV control leaves. The relative transcript level of MeNR1 in the pTRV control leaves was normalized to 1.0. (g) Bacterial number in MeNR-silenced cassava and the control plant leaves (vector). The cassava leaf discs stained with 5 μM DAF-FM DA for 20 minutes. Bar = 50 μm. (h) Quantification of NO accumulation as relative greyscale value by DAF-FM staining. (i) Quantification of NO accumulation as relative greyscale value by DAF-FM staining. All experiments included three biological replicates, and all data are expressed as means and SDs. Asterisks symbols (•) represent significant differences at P < 0.05.

**MeNRs mediate extensive transcriptional reprogramming**

To provide new insights into MeNR-mediated disease resistance, RNA-sequencing (RNA-seq) was performed and 194 differentially expressed genes (DEGs) were identified in MeNR-silenced cassava leaves under both mock and Xam infection conditions (Figure 3a, Table S3). As shown in the Venn diagram, there were 2432 DEGs (1180 down-regulated and 1252 up-regulated DEGs) in the MeNR-silenced cassava/pTRV2-silenced cassava under control conditions, and 2221 DEGs (1047 down-regulated and 1174 up-regulated DEGs) in the MeNR-silenced cassava/pTRV2-silenced cassava after Xam infection (Figure 3b). Although MeNR1 silencing, MeNR2 silencing and MeNR1/2 co-silencing had significantly specific DEGs, the common DEGs, especially 6 DEGs, involved in plant defence pathway were chosen for further analysis due to the
common effect of MeNR1 silencing, MeNR2 silencing and MeNR1/2 co-silencing on disease resistance (Figure 3c). Firstly, the transcript levels of the 6 DEGs were verified by qRT-PCR (Figure S4A). The linear diagram between the qRT-PCR and the RNA-seq showed the results of RNA-seq were accurate (Figure S4B). Consistently, these 6 DEGs were significantly up-regulated in MeNR1- and MeNR2-overexpressed cassava leaves (Figure S4C). In addition, NO donor could significantly up-regulated the transcript levels of the 6 DEGs, while NO scavenger treatment could significantly down-regulated the transcript levels of the 6 DEGs, indicating these genes were also regulated by NO (Figure S4D). The positive regulation of 6 DEGs in MAPK cascade and downstream pathogenesis-related genes (MePR1 and MePR4) by MeNR1/2 might contribute to MeNR-mediated disease resistance.

MeRAV5 directly regulates the transcripts of MeNR1 and MeNR2

To identify the upstream transcription factors of MeNRs, we analysed the promoter of MeNR1 and MeNR2 and found the common exist area of the related ABI3/VP1 (RAV) binding motif CAACA. Then, we detected the expression levels of MeNR1 and MeNR2 in MeRAV-overexpressed cassava leaves and the MeRAV-silenced cassava leaves, and found that the transcripts for MeNR1 and MeNR2 were regulated by MeRAVs (Figure S5). Compared with other MeRAVs, the correlation coefficient in gene expression between MeNR1/2 and MeRAV5 was the highest, so MeRAV5 was chosen as the target for further analysis. We speculated that the MeRAV5 might bind to the promoter of MeNRs in vivo and activate their expression. To test this speculation, the CDS of

Figure 3 RNA-seq of DEGs in MeNRs-silenced cassava leaves under mock and Xam infection. (a) Heat map of 194 DEGs in MeNRs-silenced cassava leaves under mock and Xam infection. The relative FPKM value of each gene in comparison with the vector under mock conditions is shown in the heat map. (b) Venn diagram showing all DEGs among MeNR-silenced cassava vs. control under mock and Xam infection. (c) The expression pattern of DEGs involved in plant defence pathway. The accession numbers of the genes: MeFLS2 (Manes.16G000800), MeBAK1 (Manes.03G148000), MeMKS1 (Manes.02G141700), MeWRKY33 (Manes.06G095600), MeMPK3 (Manes.04G026700) and MeWRKY22 (Manes.05G029500). RNA-seq included three biological replicates, and all data are expressed as means and SDs. Asterisks symbols (*) represent significant differences at \( P < 0.05 \).
MeRAV5 fused with GFP tag were transformed into the cassava leaf protoplasts. As shown in Figure 4a, MeRAV5 was mainly co-localized in the nucleus with DAPI staining and also showed some fluorescence in the cytoplasm. Overexpression of MeRAV5 could largely activate the luciferase (LUC) activity of mini35S-5 × CAACA::LUC but not mini35S-5 × mCAACA::LUC in leaf protoplasts (figure 4b). Further chromatin immunoprecipitation (ChIP)-PCR assay showed that overexpression of MeRAV5 greatly enhanced the enrichment of the MeNR promoter with CAACA motif (Figure 4c). We further investigated the regulation of the MeNR promoter by MeRAV5 using the dual-LUC assay, the results showed that overexpression of MeRAV5 enhanced the activity of MeNR promoter (Figure 4d). These results suggest that MeRAV5 directly regulates the MeNR1 and MeNR2 transcripts.

**MeRAV5 improves cassava disease resistance to Xam**

To explore whether MeRAV5 regulates disease resistance to Xam in cassava, we constructed MeRAV5-overexpressed cassava and MeRAV5-silenced cassava. The expression and relative transcripts of MeRAV5 in MeRAV5-overexpressed cassava and MeRAV5-silenced cassava leaves were verified by both reverse transcription-PCR and qRT-PCR (Figure 5a, b, d). The bacterial number showed that MeRAV5 positively regulated cassava disease resistance to Xam, with less bacterial number in MeRAV5-overexpressed cassava leaves (Figure 5c) but more bacterial number in MeRAV5-silenced cassava leaves (Figure 5e). Consistently, MeRAV5 also positively regulated endogenous NO level (Figure 5f). Moreover, overexpression of MeRAV5 in cassava exhibited a higher ROS photon count than mock during 22 highly conserved amino acids in N terminal of flagellin (flg22) treatment, while silencing MeRAV5 in cassava showed a lower photon count than mock during flg22 treatment (Figure 5j-k). In addition, diaminobenzidine (DAB) staining of endogenous H2O2 level documented that MeRAV5-overexpressed cassava leaves displayed higher H2O2 level than mock leaves, while MeRAV5-silenced cassava leaves exhibited lower H2O2 level than mock leaves (Figure 5h–n).

**MeRAV5 regulates cassava disease resistance to Xam through MeNRs**

To explore the relationship between the disease resistance of MeRAV5 and MeNRs, we silenced MeNRs in the MeRAV5-overexpressed cassava (Figure S6A-D) and investigated disease resistance and NO accumulation. The expression and relative transcripts of MeNRs and MeRAV5 were verified by both reverse transcription-PCR and qRT-PCR (Figure S6A-D). We found that silencing MeNRs restored the effects of MeRAV5 overexpression on disease resistance by quantification of bacterial number (Figure 6a) and the endogenous NO level in cassava leaves (Figure 6b-c). In addition, we overexpressed MeNRs in the MeRAV5-silenced cassava (Figure S7A-D) and investigated disease resistance and NO accumulation. The expression and relative
transcripts of MeNRs and MeRAV5 were verified by both reverse transcription-PCR and qRT-PCR (Figure S7A-D). We found that overexpression of MeNRs restored the consequences of MeRAV5 silencing on disease resistance by quantification of bacterial number (Figure 6d) and the endogenous NO level in cassava leaves (Figure 6e, f).

MeRAV5 physically interacts with MeCAT1 and inhibits its activity

To investigate the mechanism of MeRAV5-mediated disease resistance, we performed yeast two-hybrid to screen its interacting proteins in cassava cDNA library (Wei et al., 2020), and
identified some candidate proteins including MeCAT1 as potential interacting protein of MeRAV5. Further, yeast two-hybrid assay using the coding sequences of MeRAV5 and MeCAT1 confirmed their interaction in yeast (Figure 7a). Meanwhile, pull-down assay using the purified proteins also verified the interaction between MeRAV5 and MeCAT1 in vitro (Figure 7b). In addition, the co-localization of yellow fluorescence and peroxisome signal of px-ck (CD3-977) (Wei et al., 2020) in bimolecular fluorescence complementation (BiFC) further showed the interaction between MeRAV5 and MeCAT1 in peroxisome in Nicotiana benthamiana leaves (Figure 7c), which was consistent with the localization of them (Figure 7d). Notably, MeRAV5 was co-localized in both nucleus with DAPI (Figure 4a) and peroxisome (Figure 7d), while MeCAT1 was mainly localized in peroxisome (Figure 7d) (Wei et al., 2020). Using the purified proteins, we found that the purified MeRAV5 protein could significantly inhibit MeCAT1 activity in vitro (Figure 7e). Consistently, both MeRAV5 overexpression and silencing had no significant effects
on MeCATs’ transcripts (Figure 7f), while MeRAV5 negatively regulated CAT activity, with lower CAT activity in MeRAV5-overexpressed cassava but higher CAT activity in MeRAV5-silenced cassava (Figure 7g).

Modulation of MeCAT1 alleviates the effect of MeRAV5 expression on cassava disease resistance

In this study, the expression of MeCAT1 and CAT activity was significantly increased during Xam infection (Figure 8a,b). To explore the relationship between the disease resistance of MeRAV5 and MeCAT1, we overexpressed MeCAT1 in the MeRAV5-overexpressed cassava (Figure S8A-C) and investigated disease resistance. The expression and relative transcripts of MeCAT1 and MeRAV5 were verified by both reverse transcription-qPCR and qRT-qPCR (Figure S8A-C). We found that overexpressing MeCAT1 alleviated the effects of MeRAV5 overexpression on disease resistance by quantification of bacterial number (Figure 8c), CAT activity (Figure 8d) and the endogenous H2O2 level (Figure 8e,f). In addition, we silenced MeCAT1 in the MeRAV5-silenced cassava (Figure S9A-C) and investigated

![Figure 7](image_url)

**Figure 7** MeRAV5 physically interacts with MeCAT1 and inhibits its activity. (a) Yeast two-hybrid showing the interaction between MeRAV5 and MeCAT1. (b) Pull down showing the interaction between MeRAV5 and MeCAT1 in vitro and in vivo, respectively. For pull-down assay, HA-fused MeCAT1 was immunoprecipitated using anti-HA antibody, and His-fused MeRAV5 was detected using anti-His antibody. (c) BiFC showing the interaction between MeRAV5 and MeCAT1. Bar = 25 μm. (d) The subcellular localization of MeRAV5 and MeCAT1 in N. benthamiana leaves. The peroxisome marker px-ck signal (CFP) was used for co-localization in N. benthamiana leaves. Bar = 25 μm. (e) The effect of purified MeRAV5 protein on MeCAT1 activity in vitro. (f, g) The effect of MeRAV5 overexpression and silencing on MeCATs’ transcripts (f) and CAT activity (g) in cassava leaves. All experiments included three biological replicates, and all data are expressed as means and SDs. Asterisks symbols (*) represent significant differences at P < 0.05.
disease resistance and NO accumulation. The expression and relative transcripts of MeCAT1 and MeRAV5 were verified by both reverse transcription-PCR and qRT-PCR (Figure S9A-C). We found that MeCAT1 silencing alleviated the consequences of MeRAV5 silencing on disease resistance by quantification of bacterial number (Figure 8g), CAT activity (Figure 8h) and the endogenous H$_2$O$_2$ level (Figure 8i-j).

Discussion

Cassava (Manihot esculenta) is one of the most important tropical crops, which can be used as food- and industrial alcohol-produced energy plant (Mccallum et al., 2017). Cassava bacterial blight (CBB) caused by Xam is the most important bacterial disease in cassava, resulting in a serious yield loss (Lopez and Bernal, 2012). Previous studies have reported some cassava transcription factors including MeWhirlys (Liu et al., 2018) and MeRAV1/2 (Wei et al., 2018) as positive regulators of plant disease resistance to Xam. In this study, we found that MeRAV5 positively regulated cassava disease resistance through the coordination of NO and H$_2$O$_2$ level via MeCAT1 and MeRAV5-MeNR1/2 module. We have to note the localization of MeRAV5 in both nucleus and cytoplasm to reconcile the effect on regulating MeNR1/2 expression and simultaneously controlling MeCAT1 enzyme activity predominantly in the peroxisome. Besides the main localization in the nucleus as other transcription factors to directly regulating downstream genes, the remaining localization of MeRAV5 in the cytoplasm such as peroxisome might be also very important. Here, the dual localization of MeRAV5 might be essential in the interplay between NO and H$_2$O$_2$ levels.

NR plays important roles not only in nitrogen acquisition in plants, algae, yeasts and fungi, but also in plant immune response during plant-pathogen interaction (Chamizo-Ampudia et al., 2017). AtNR1/2-deficient double mutant nia1nia2 is sensitive to Pseudomonas syringae, Sclerotinia sclerotiorum (Percopied et al., 2010) and B. cinerea (Rasul et al., 2012). Consistently, MeNR1 and MeNR2 are also essential for multiple immune responses and disease resistance against CBB in cassava. In addition, the induction of gene expression and enzyme activity of MeNR1/2 by Xam infection, and the improved disease resistance are significant.

Figure 8  Modulation of MeCAT1 alleviates the effect of MeRAV5 expression on cassava disease resistance. (a) The transcript levels of MeCAT1 in response to Xam. (b) The CAT activity of cassava in response to Xam. (c) Overexpressing MeCAT1 reduces improved disease resistance of overexpressed MeRAV5 in cassava. Bacterial number in cassava leaves. (d) The CAT activity in cassava leaves. (e) DAB staining of H$_2$O$_2$ level in cassava leaves. Bar = 200 μm. (f) The relative greyscale value of DAB staining showing the endogenous H$_2$O$_2$ level in cassava leaves. (g) Silencing MeCAT1 rescues disease sensitivity of silenced MeRAV5 in cassava. Bacterial number in cassava leaves. (h) The CAT activity in cassava leaves. (i) DAB staining of H$_2$O$_2$ level in cassava leaves. Bar = 200 μm. (j) The relative greyscale value of DAB staining showing the endogenous H$_2$O$_2$ level in cassava leaves. All experiments included three biological replicates, and all data are expressed as means and SDs. Asterisks symbols (*) represent significant differences at $P < 0.05$. 

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resistance of MeNR1/2 overexpression indicate that MeNR1/2 play essential roles during cassava–Xam interaction. NO also plays a primary function in plant defence against bacterial pathogens (Delledonne et al., 1998). NO activates HR to improve resistance to tobacco mosaic virus (TMV) in tobacco (Asai and Yoshioka, 2009; Klessig et al., 2000; Kumar and Klessig, 2000). On the contrary, the NO scavenger (PTIO) or NO synthesis inhibitor (tungstate) reduces virus resistance in Arabidopsis (Zou et al., 2018). To date, the roles of NR in plant stress response and defence response are primarily dependent on NO (Hao et al., 2010; Jian et al., 2015; Zou et al., 2018). For example, glutamate treatment restores the amino acids but not the disease sensitivity of the nia1nia2 mutant in Arabidopsis (Oliveira et al., 2009), while NO fumigation and nitrate that produce NO rescues the disease sensitivity of nia1nia2 mutant (Modolo et al., 2005, 2006; Vitor et al., 2013), indicating that the disease susceptibility is caused by NO but not insufficient nitrogen absorption. In accordance with these studies, SNP and c-PTIO alleviated the effects of MeNR silencing and MeNR overexpression on cassava disease resistance to Xam, showing that NO is essential for MeNR1/2-mediated disease resistance.

RNA-seq with three biological repeats identified extensive transcriptional reprogramming by MeNR1 and MeNR2. Although so many DEGs were identified by MeNR1 silencing, MeNR2 silencing and MeNR1/2 co-silencing, the DEGs between them were remarkably different. According to gene ontology (GO) analysis of the common DEGs and the specific DEGs by MeNR1 silencing and MeNR2 silencing under mock and Xam infection conditions (Figure S1OA-F), the top 20 functional category of MeNR1-specific DEGs and MeNR2-specific DEGs was significantly different, indicating that they might have different functions besides the same functions. When MeNR1 and MeNR2 were co-silenced, other genes might also be differentially regulated and other unknown mechanisms might be modulated in response to more NR-deficient conditions, so the dose effect of NR and other unknown mechanisms might result in that the overexpressed DEGs were not so well. Based on the common significant effect of MeNR1 silencing, MeNR2 silencing and MeNR1/2 co-silencing on disease resistance, we focused on the common 194 DEGs overlapped between them under both mock and Xam infection conditions, especially 6 DEGs in the MAPK cascade. MAPK cascade is a conserved signalling module in all eukaryotes (Asai and Yoshioka, 2000; Kumar and Klessig, 2000). On the one hand, CAT that locates in peroxisome directly catalyses the decomposition of H$_2$O$_2$ into O$_2$ and H$_2$O, thereby regulating the expression of PR genes (Chinchilla et al., 2006, 2007). MAPK cascade is mainly consist of MEKK1-MKK4/MKK5-MPK3/MPK6 or MEKK1-MKK1/MPK2-MPK4 (Boller and Felix, 2009). Herein, MeNRs positively regulated the transcripts of MeFLS2, MeMK51, MeWRKY33, MeBAK1, MeMPK3 and MeWRKY22 in MAPK cascade and downstream MePR1/4, which further conferred improved immune responses.

Although the roles of plant NRs have been widely investigated in Arabidopsis (Vitor et al., 2013; Zhou et al., 2015b), the corresponding upstream signalling pathways remain elusive. Previous studies have shown that RAV plays a critical role in plant disease resistance. Overexpression of CaRAV1 in Arabidopsis confers improved disease resistance to Pseudomonas syringae through the activation of several pathogenesis-related (PR) genes (Sohn et al., 2006). SRAV2 improves tomato disease resistance to Pseudomonas solanacearum by regulating the expression of PR genes through SIERFS (Li et al., 2011). Previously, we reported that RAV1 and RAV2 activate melatonin biosynthesis through binding to the CAACA motif in the promoters of tryptophan decarboxylase 2 (TDC2), tryptamine 5-hydroxylase (TSH) and N-acetylserotonin methyltransferase (ASMT2) in cassava (Wei et al., 2018). In this study, we found that MeRAV5 directly activated MeNRs by binding to their promoters with CAACA motifs. Genetic experiments further revealed that MeNRs are essential for MeRAV5-mediated cassava disease resistance and immune response including both NO accumulation and 5 DEGs in MAPK cascade (Figure S11A-B). Therefore, transcriptional activation of MeNR1/2 and NO biosynthesis are essential for MeRAV5-mediated immune response in cassava.

Notably, we also found that MeRAV5 positively regulated H$_2$O$_2$ level during plant–pathogen interaction. ROS especially H$_2$O$_2$ is considered to be the key to induce programmed cell death, a process that is directly related to HR (Grant and Loake, 2000). In addition, ROS play an important role in the reprogramming of host transcriptome, which is necessary for the establishment of plant resistance (Vitor et al., 2013). To reveal how MeRAV5 regulates H$_2$O$_2$ level, we screened MeRAV5-interacting proteins through yeast two-hybrid and further confirmed the physical interaction between MeRAV5 and MeCAT1 in vitro and in planta. On the one hand, CAT that locates in peroxisome directly catalyses the decomposition of H$_2$O$_2$ into O$_2$ and H$_2$O, thereby regulating many stress responses (Gao et al., 2014; Wei et al., 2020; Xu et al., 2013). On the other hand, CAT interacts with other proteins to involve in various stress signalling pathways. For example, AtCAT2/3 interact with salt overly sensitive 2 (SOS2) to mediate salt stress resistance (Verslues et al., 2007). AtCAT2 interacts with JA biosynthetic enzymes AtACKX2/3 to regulate JA level and disease resistance to B. cinerea, and the suppression of AtCAT by SA regulates endogenous H$_2$O$_2$ level and further sulphhydration of tryptophan synthetase β subunit 1, so as to regulate auxin level and disease resistance to Pseudomonas syringae (Yuan et al., 2017). In addition, AtCAT2 interacts with calmodulin-binding protein 1 (AtIBP1) to affect pathogen defence response (Lv et al., 2019). Herein, MeRAV5 interacted with MeCAT1 to inhibit its activity, so as to negatively regulate endogenous H$_2$O$_2$ level and further improve disease resistance. Therefore, the activation of NO and H$_2$O$_2$ level during cassava–Xam interaction might be contributed to MeRAV5-mediated disease resistance in cassava (Delledonne et al., 2001; Vitor et al., 2013).

In summary, the above results suggest a proposed model documenting the involvement of MeRAV5-MeNR1/2-NO and MeRAV5 & MeCAT1-H$_2$O$_2$ in plant disease resistance in cassava (Figure 9). Xam infection induces the transcripts of MeRAV5 with further activation of MeNRs by the binding of MeRAV5 to their promoters in cassava. The induction of MeNRs improves cassava disease resistance through stimulating NO accumulation and transcriptional reprogramming, especially in MAPK signalling. In
addition, MeRAV5 interacts with MeCAT1 to inhibit its activity and increase endogenous H$_2$O$_2$ level during cassava-Xam interaction, resulting in improved disease resistance. This study highlights the precise coordination of NO and H$_2$O$_2$ level by MeCAT1-MeNR module-mediated plant disease response in cassava.

**Methods**

**Plant materials and growth conditions**

South China 124 (SC124) cassava variety was used in this study. The cassava stems were grown in Meteorite under 12-h light of 120–150 mmol quanta m$^{-2}$ s$^{-1}$ and 12-h dark, at 26–28 °C in glasshouse.

**Identification of MeNRs**

The sequences of AtNRs and OsNRs were downloaded from Arabidopsis Information Resource (TAIR) v10 (http://www.Arabidopsis.org) and Rice Genome Annotation Project (RGAP) v7 (http://rice.plantbiology.msu.edu). The sequences of AtNRs and OsNRs were used for blast in cassava database of Phytozome v10.3 (http://www.phytozome.net/cassava.php). Two cassava sequences with high homology with AtNRs and OsNRs and the conserved domain of NR were named MeNR1 and MeNR2. Based on previous research results, the sequences of HbNR, CcNR, JcNR, RcNR, OsNR and CsNRs were obtained from National Center for Biotechnology Information (NCBI), and then, the phylogenetic tree was obtained by MEGA 7.0.

**qRT-PCR**

RNA isolation and cDNA synthesis were performed using kits as previously described (Wei et al., 2017). The qRT-PCR was performed in LightCycler® 96 Real-Time PCR System (Roche, Basel, Switzerland) using cDNA and FastStart Essential DNA Green Master (Roche, 06924204001, Basel, Switzerland). The primers are listed in Supplemental Table S1. With at least three independent biological replicates, all gene transcripts were normalized to MeEF1a using the comparative $\Delta \Delta$CT method.

**Protein expression and purification**

To obtain the His-fused proteins, the coding sequences of the MeNR1, MeNR2 and MeRAV5 were cloned into pET28a vector, and the recombinant plasmids MeNR1-pET28a, MeNR2-pET28a and MeRAV5-pET28a were transformed into Escherichia coli BL21 (DE3). MeCAT1-pET28a has been described previously (Wei et al., 2020). The DE3 strain containing the recombinant plasmids was cultured at 37°C in 200 ml LB to induce protein expression. When the concentration of bacterial solution reached OD$_{600}$ about 0.6, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the bacterial solution at the final concentration of 1 mM IPTG, and then, the bacterial solution was cultured at 37 °C for 6 h. The collected bacterial solution was used for protein purification using His-tag Protein Purification Kit (Beyotime, P2226, China). Samples taken at 0 and 6 h were used for SDS-PAGE and Western blot.

**Subcellular localization**

The coding sequences of the MeNR1, MeNR2 and MeRAV5 were cloned into the pEGAD vector after DNA sequencing, to form the 35S::GFP-MeNR1, 35S::GFP-MeNR1 and 35S::GFP-MeRAV5 plasmids. MeCAT1-pEGAD has been described previously (Wei et al., 2020). These plasmids were transformed into cassava leaf protoplasts as previously described (Wei et al., 2017). The GFP signal and DAPI staining signal were determined using a confocal laser scanning microscope (TCS SP8, Leica, Heidelberg, Germany).

**NO assay**

To detect endogenous NO in cassava leaves, the leaves were stained with 5 μM DAF-FM DA (Beyotime, S0019, China) for 20 min, and then washed twice with 50 mM PBS (pH = 7.4). The fluorescent signal was then observed under a fluorescence microscope (DM500B, Leica, Heidelberg, Germany), and the relative grey value was quantified by ImageJ software. In addition, the endogenous NO was also verified using NO assay kit (A12, Nanjing Jiancheng Bioengineering Institute, China) according to the instructions.

**NR activity and enzyme kinetics assay**

NR activity was measured using the NR activity detection kit (Solarbio, BC0080, China) according to the instructions. To analyse the enzyme kinetics of the purified recombinant MeNR protein, five concentrations of purified protein were used for enzyme activity, and the linear relationship was generated by Lineweaver–Burk. Then, $K_m$ and $V_{max}$ were obtained.

**Xam infiltration and bacterial number quantification**

The isolation, cultivation and infiltration of Xam into cassava have been described in Yan et al. (2018). For the bacterial number quantification, about five-week-old cassava leaves were infected by Xam and grown in a glasshouse. At each sampling point, 20 leaf discs were taken; these discs were immersed in 75% alcohol for 1 min, rinsed with sterile water and then ground with a grinding rod to form a homogenate. Thereafter, the homogenate was spotted on the LB plate according to the gradient dilution method, and the bacterial number after being grown at 28°C for one day was quantified.

**Transient expression in cassava leaves**

For the overexpressing vector construction, the coding sequences (CDS) of MeNR1, MeNR2 and MeRAV5 were cloned into the
p81121 vector; the primers for vector construction are listed in Supplemental Table S2. For VIGS vector construction, the partially coding sequence of the MeNR1, MeNR2 and MeRAV5 was cloned into pTRV2 (Liu et al., 2002); the primers for vector construction are listed in Supplemental Table S2. Then, the recombinant plasmids were transformed into Agrobacterium tumefaciens strain GV3101 after DNA sequencing. The Agrobacterium tumefaciens strain GV3101 containing recombinant plasmids was cultivated to OD600 = 0.6 at 28 °C. The culture was resuspended with 10 mM MgCl2, 10 mM MES and 20 mM acetylserine to OD600 = 1, and then injected into the back of the cassava leaves.

RNA-seq

The cassava leaves under mock and Xam infection were used for total RNA extraction. The total RNA was processed using the mRNA enrichment method, fragmented and reverse-transcribed to construct the cDNA library. The cDNA library was used for sequencing on BGISEQ-500 platform (BGI Genomics, Shenzhen, China). Three biological replicates were performed for RNA-seq.

Data were statistically analysed by SOAPnuke and filtered by trimmomatic. For gene annotation, diamond annotation was used to compare with PRGdb gene database, and the results were further annotated based on query coverage and identity. The DEGs (log2 fold change > 1) were used to construct the cDNA library. The cDNA library was used for sequencing on BGISEQ-500 platform (BGI Genomics, Shenzhen, China). Three biological replicates were performed for RNA-seq.

Sequencing on BGISEQ-500 platform (BGI Genomics, Shenzhen, China). Three biological replicates were performed for RNA-seq.

Statistical analysis

All experiments included three biological replicates, and all data were expressed as means and SDs. Asterisks symbols (*) represent significant differences at P < 0.05.

Accession numbers

All sequence data from this article can be found under accession numbers: MeRAV5 (Me.06G036900), MeNR1 (Me.0077300.1), MeNR2 (Me.0077800.1), MeCAT1 (Me.05G130500), MeCAT2 (Me.05G130700), MeCAT3 (Me.07G024500), MeCAT4 (Me.18G004500), MeCAT5 (Me.18G004400), MeCAT6 (Me.01G154400), MeCAT7 (Me.02G113300), MeUBQ10 (Me.07G019300), MeEF1a (Me.15G054800), MeFLS2 (Me.08G022800), MeUBQ10 (Me.07G019300), MeEF1a (Me.15G054800), MeFLS2 (Me.08G022800).
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Conflicts of interest

The authors declare no competing financial interests.

Author contributions

Shi H conceived and directed this study, designed the experiments, wrote and revised the manuscript. Yan Y wrote and revised the manuscript. Yan Y, Wang P, Wei Y, Bai Y, Lu Y, Zeng H and Liu G performed the experiments and analysed the data. Reiter R and He C revised the manuscript. All authors approved the final version of the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Relative NO level in cassava leaves using NO assay kit.

Figure S2 NO confers improved cassava disease resistance.

Figure S3 cPTIO rescues the improved disease resistance of overexpressing MeNRs.

Figure S4 Verification of RNA-seq results by qRT-PCR.

Figure S5 The transcript levels of MeNR1, MeNR2 and MeRAVs in cassava leaves.

Figure S6 The transcript levels of MeNR1, MeNR2 and MeRAV5 in cassava leaves in Figure 6A.

Figure S7 The transcript levels of MeNR1, MeNR2 and MeRAV5 in cassava leaves in Figure 6D.

Figure S8 The transcript levels of MeCAT1 and MeRAV5 in cassava leaves in Figure 8C.

Figure S9 The transcript levels of MeCAT1 and MeRAV5 in cassava leaves in Figure 8F.

Figure S10 GO analysis of the common DEGs and the specific DEGs by MeNR1 silencing and MeNR2 silencing.

Figure S11 The transcript levels of 6 DEGs in MAPK cascade in MeRAV5-overexpressed cassava leaves (A) and MeRAV5-silenced cassava leaves (B).

Table S1 The primers used for quantitative real-time PCR and RT-PCR.

Table S2 The primers used for vector construction.

Table S3 All DEGs in MeNRs-silenced cassava leaves under mock and Xam infection.