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An NBS-LRR-encoding gene *CsRPM1* confers resistance to the fungus *Colletotrichum camelliae* in tea plant

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Abstract

Nucleotide binding site, leucine-rich repeat (NBS-LRR) proteins are the main types of disease resistance proteins in plants, which can perceive plant pathogens. Anthracnose, caused by the fungus *Colletotrichum camelliae*, is one of the most severe diseases in tea plant. Here, we identified an NBS-LRR-encoding gene, *CsRPM1*, probably conferring resistance of tea plant to *C. camelliae*. Phylogenetic analysis showed that CsRPM1 was clustered with RPM1 in *Arabidopsis* and grouped into CC-NBS-LRR (CNL). It contained a signal peptide, a NB-ARC domain, and multiple LRR motifs. RNA-seq and qRT-PCR analysis showed that the transcript level of *CsRPM1* was significantly up-regulated after inoculation with *C. camelliae*. Transiently silencing of *CsRPM1* in tea leaves comprised the resistance to *C. camelliae*, indicating that *CsRPM1* was required for plant defense against the pathogen. The subcellular localization showed that CsRPM1 protein was localized in the nucleus, cytoplasm, and cell membrane. Furthermore, the promoter region of *CsRPM1* gene contained MeJA-responsive elements, and the expression of *CsRPM1* was induced by exogenous methyl jasmonate, suggesting that *CsRPM1* gene may be closely related to JA signaling pathway. A total of 17 transcription factors might be responsible for the expression of *CsRPM1*. Our data indicates that *CsRPM1* is required for disease resistance to *C. camelliae* in tea plant. The characterization of this disease resistance gene sheds light on NLR protein function in tea plant and may facilitate breeding to control the severe anthracnose.

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Introduction

Tea plant (*Camellia sinensis*) is a widely cultivated perennial evergreen plant, which is used to produce health-beneficial tea beverages[1]. However, tea plant frequently suffers from pathogens due to the warm and humid growing environment, which leads to serious losses in yield and decreased quality in tea[2,3]. *Colletotrichum camelliae*, as one of the dominant *Colletotrichum* species causing anthracnose in *Ca. sinensis*, caused large necrotic lesions in tea leaves and then resulted in the increased production losses[4]. In response to pathogen attacks, plants have evolved exquisite and effective defense systems[5]. The first tier of plant immune system is pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI), *via* the recognition of PAMPs by plant pathogen- or pattern-recognition receptors (PRRs)[6]. Pathogens can synthesize effectors and deliver them into the plant cell to counteract PTI, while plants can recognize the effectors *via* disease resistance proteins (R proteins) and implement effector-triggered immunity (ETI)[6,7]. The majority of R proteins contain central nucleotide-binding site (NBS) and Apaf-1, R-protein, and CED-4 homology (ARC) subdomains, and leucine-rich repeats (LRRs) domain, called NBS-LRR or NLR receptors[8].

Plants use NBS-LRR proteins to perceive fungal pathogens by direct or indirect recognition of fungal effectors, triggering ETI and developing defense responses against pathogens[9,10]. For example, the NBS-LRR proteins RGA4 and RGA5 in rice physically bind to two effectors ACR-CO39 and ACR-Pia of fungal pathogen *Magnaporthe oryzae*, resulting in resistance induction and the hypersensitive response (HR)[11,12]. Over the years, the function of *NLR* genes in host plants has been reported. In tomato, one resistance (R) gene *Sm* encoding an NBS-LRR protein confers resistance to gray leaf spot disease caused by *Stemphylium lycopersici*[13]. In barley, virus-induced gene silencing of an NBS-LRR gene *Rpg5* resulted in a compatible reaction with a normally incompatible stem rust pathogen *Puccinia graminis*, indicating the important role of *Rpg5* as the stem rust resistance gene[14]. An NLR protein YrU1 elicits effective ETI after recognition of the effectors derived from the stripe rust fungus *Puccinia striiformis* f. sp. *Tritici* in bread wheat[10,15]. Soybean *Rps11* is an *NLR* gene conferring broad-spectrum resistance to *Phytophthora sojae* causing root and stem rot[16]. In apple, three NLR proteins MdRNL1, MdRNL2, and MdRNL3 contribute to the resistance to apple leaf spot caused by *Alternaria alternate* f. sp. *mali*[8]. Rice *OsRLR1* gene encoding an NBS-LRR protein mediates resistance to the rice blast fungus *M. oryzae* through interaction with the transcription factor OsWRKY19[17]. Although the role of *NLR* genes in the resistance against fungal infection has been widely reported in many plants, few studies reported the contribution of *NLR* genes to disease defense in tea plant.

In our previous studies, 400 and 303 *CsNLRs* genes have been identified from the genomes of *Ca. sinensis* var. *sinensis* (CSS)

and *Ca. sinensis* var. *assamica* (CSA), respectively. Based on the N-terminal domains, they were classified into two major groups, coiled-coil-containing NLRs (CNLs) and Toll/interleukin-1 receptor-containing NLRs (TNLs)[18]. The expression of these *CsNLRs* was induced by *Colletotrichum*, abiotic stresses, and exogenous methyl jasmonate (MeJA) by RNA-Seq analysis[18]. However, the role of *CsNLRs* in disease resistance have not been functionally validated. Here we identified one *CsNLR* gene, *CsRPM1*, whose expression was significantly induced by *C. camelliae*. Phylogenetic tree revealed that CsRPM1 was homologous to RPM1 in *Arabidopsis*, and clustered with CNL. It encodes a typical NBS-LRR protein located in the nucleus, cytoplasm, and cell membrane. Further functional analysis by transient expression confirmed that this gene confers resistance to *C. camelliae* in tea plant. The transcript level of *CsRPM1* was induced by exogenous MeJA, and may be regulated by several transcription factors.

Materials and methods

Plant materials and treatments

Five-year-old tea plant (*Ca. sinensis* cv. *Longjing43*) (LJ43) seedlings were grown under natural conditions. To analyze the expression of *CsRPM1* at different stages of infection with *C. camelliae*, the tea leaves inoculated by *C. camelliae* strain LS_19 were sampled at 0, 3, 6, 12, 24, 48, 72, and 96 h post inoculation. To determine the expression of *CsRPM1* under the induction of MeJA, tea leaves treated with exogenous 150 μM MeJA and then inoculated with *C. camelliae* strain LS_19 were sampled at 0, 24, 48, and 72 h after treatment. The collected samples were stored at -80°C for further experiments.

RNA-Seq analysis

The RNA-Seq expression data-set used to analyze the expression of *CsNLRs* in this study was obtained from our previous study, which was transcriptome of tea leaves in response to *C. camelliae*[19]. Differentially expressed genes (DEGs) were defined as their expression presented a > 1.5-fold change[18]. The heatmap was constructed by TBtools software[20].

qRT-PCR analysis

Total RNA was extracted from collected samples using FastPure® Plant Total RNA Isolation Kit (Polysaccharides& Polyphenolics-rich) (Vazyme Biotech Co., Ltd, China). cDNA was then synthesized using HiScript II 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme Biotech Co., Ltd, China) according to the manufacturer's instructions. qRT-PCR assays were performed with ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd, China) using the Bio-Rad CFX96™ Real-Time System (USA). *CsPTB1* gene encoding polypyrimidine tract-binding protein was used as reference[21]. Primer pair (forward: 5'-TCTCCTTCGTCGCTTGTC-3' and reverse: 5'-ATAGGGTCTTCTGTTAGTCTGG-3') was used to amplify *CsRPM1* for qRT-PCR. The experiment with at least three replicates was independently repeated three times.

Transient gene suppression of *CsRPM1*

To transiently inhibit the expression of *CsRPM1* in LJ43, antisense oligodeoxynucleotide (asODN) was used. Candidate sequences of asODN were designed using Soligo (http://sfold.wadsworth.org/cgi-bin/index.pl) with *CsRPM1* gene sequence as the input. The sense oligonucleotides (sODN) were used as the control. 10 sODNs-asODNs pairs were selected for synthesizing to ensure and improve the interference effect[22]. The synthesized 10 pairs of sODN and asODN were adjusted to the concentration of 30 μM with ddH_2O and then injected into the tea leaves respectively. After incubation, the injected leaves were inoculated with the 5-mm mycelial plugs cut from *C. camelliae* strain LS_19 and then sampled at 12, 24, 48, and 72 h post inoculation. At each point in time, the lesions on tea leaves were observed and measured. The leaves samples were then harvested and stored at -80°C for further analyses. The experiment with at least three replicates was independently repeated three times.

Subcellular localization of CsRPM1

The *CsRPM1* coding sequence lacking the termination codon was amplified with the primers (forward: 5'-CGAGCTCGGTACCCGGGGATCCATGGCCTTGGCTGCCGTGGG-3' and reverse: 5'-CCTTGCTCACCATGGTGTCGACAGTCAATCCTGTGGAACGAG-3'), and then fused with the vector pCAMBIA2300-35S-eGFP using CloneExpress II One Step Cloning Kit (Vazyme Biotech Co., Ltd, China). The fused vector and control vector (pCAMBIA2300-35S-eGFP) were then introduced into *Agrobacterium tumefaciens* GV3101 using the liquid nitrogen quick-freezing method respectively. The activated *A. tumefaciens* containing the vectors (OD_{600} = 0.5) were infiltrated into 5-week-old *Nicotiana benthamiana* leaves (expressing nuclear marker-RFP)[23]. After two days in the dark, the GFP fluorescence signal was observed using laser confocal scanning microscopy (Zeiss LSM 780, Germany). Excitation wavelengths of RFP and GFP were 532 and 488 nm respectively.

Screening of candidate transcription factors (TFs) regulating *CsRPM1* expression

TeaCoN website (http://teacon.wchoda.com/) was used for candidate TFs screening with CsRPM1 as the query[24].

Statistical analysis

Data were the mean ± standard deviation of three biological replicates. The mean and standard errors were respectively calculated using the MEAN and STDEV function in Excel 2019. Statistically significant differences were determined with one-way ANOVA analysis (*, $p < 0.05$; **, $p < 0.01$) *via* SPSS Statistics 18 software.

Results

Identification and expression pattern of *CsRPM1* after inoculation with *C. camelliae*

To identify and analyze the expression pattern of DEGs in LJ43 inoculated with *C. camelliae*, we performed RNA-Seq analysis. Results from Venn diagram identified 12 DEGs at different stages of infection (12, 24, and 72 h) (Fig. 1a). Notably, among the 12 DEGs, there was an NLR-encoding gene (TEA004876) that was significantly up-regulated at 72 h compared to the control group (Fig. 1b). TEA004876 was predicted as the disease resistance protein RPM1, and could be induced under various biotic and abiotic stresses (http://tpdbtmp.shengxin.ren:81/analyses_search_locus.html?id=TEA004876). It was named *CsRPM1*, and selected for further characterization. Phylogenetic analysis showed that CsRPM1 was clustered with RPM1 belonging to CNL (CC-NBS-LRR) group (Fig. 1c). *CsRPM1* gene in LJ43 encodes an NBS-LRR protein, containing a signal peptide, a NB-ARC domain, and multiple LRR motifs (Fig. 1d).

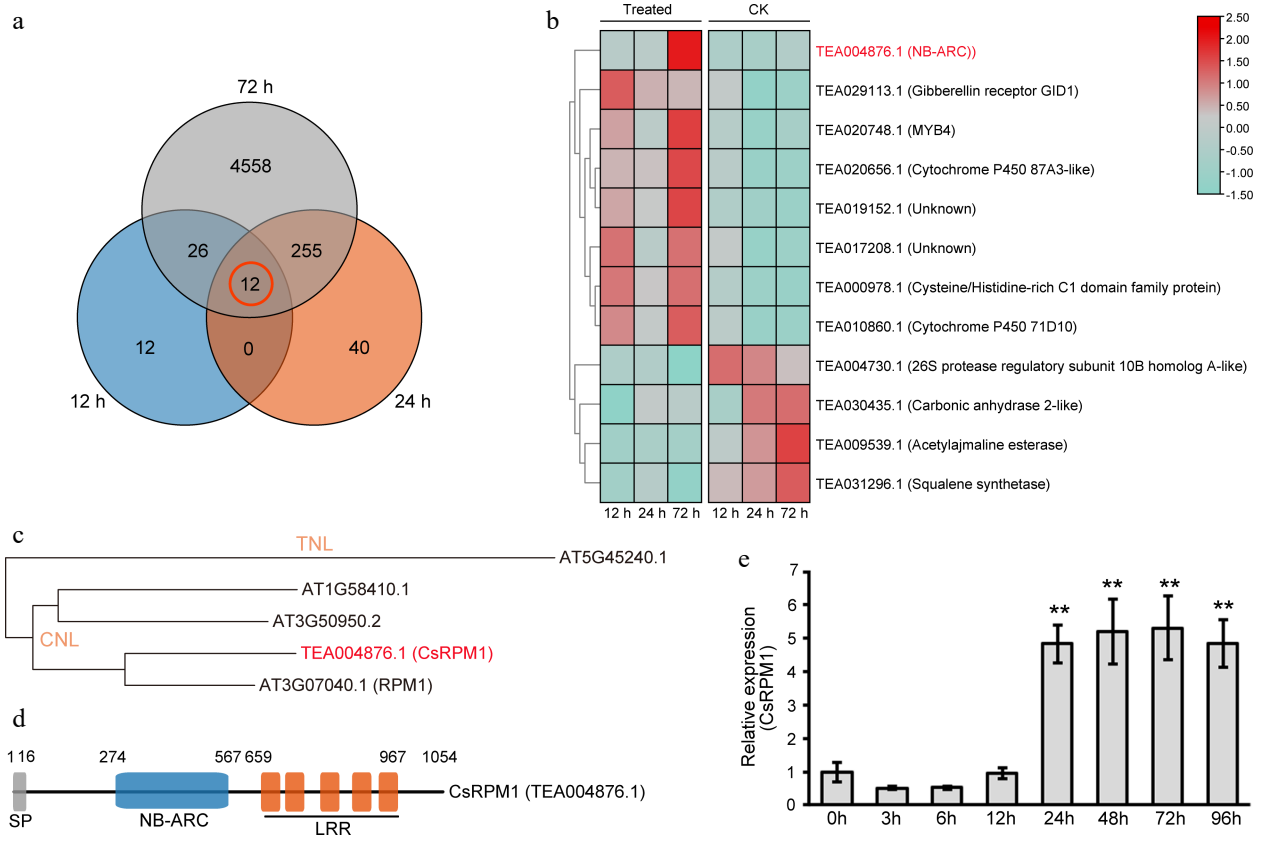

Fig. 1 Identification and expression pattern of *CsRPM1* in tea plant leaves inoculated with *C. camelliae*. (a) Venn diagram of DEGs in tea plant leaves inoculated with *C. camelliae* at 12, 24, and 72 h. (b) Heat maps of the expression of 12 DEGs in LJ43 at 12, 24, and 72 h after inoculation with ddH_2O (Control) or *C. camelliae* (Treated). (c) Phylogenetic tree constructed *via* the TNL and CNL genes. (d) Domain map of CsRPM1 protein. The domain prediction of CsRPM1 was performed with SMART analysis service. (e) The expression pattern of *CsRPM1* in tea plant leaves during different stages of infection with *C. camelliae* by qRT-PCR analysis. **, $p < 0.01$.

The expression pattern of *CsRPM1* at different stages of infection with *C. camelliae* was further confirmed by qRT-PCR analysis. The result showed that the expression of *CsRPM1* was significantly up-regulated at 24, 48, 72 h, and 96 h after inoculation (Fig. 1e), suggesting that *CsRPM1* can be induced by the infection with *C. camelliae* and probably involved in disease resistance in tea plant.

Silencing of *CsRPM1* impairs host resistance to *C. camelliae*

To further valid the role of *CsRPM1* in disease resistance in tea plant, asODN was used to transiently inhibit the expression of *CsRPM1* in LJ43. The transcript of *CsRPM1* was significantly down-regulated at 12 and 72 h post injection with 30 μM asODN compare to the control group (transformed with sODN) (Fig. 2a). At 72 h, the necrotic lesions caused by *C. camelliae* on the asODN group were significantly larger than those on the sODN control group (Fig. 2b, c). The results indicated that silencing of *CsRPM1* facilitates the infection by *C. camelliae* and impairs the resistance of tea plant to *C. camelliae*.

Subcellular localization of CsRPM1

For examining the localization of CsRPM1 protein, we constructed the fused vectors expressing CsRPM1-GFP under the 35S promoter. When CsRPM1-GFP and nuclear marker-RFP were co-expressed in the *N. benthamiana* leaves, the green and red fluorescent protein signals were superimposed (Fig. 3). Besides, GFP signals were also observed in plasma membrane

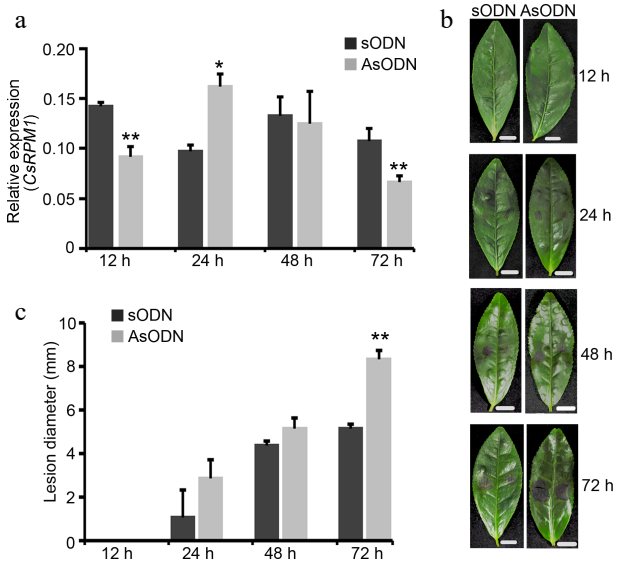

Fig. 2 Silencing of *CsRPM1* impairs host resistance to *C. camelliae*. (a) Expression level of *CsRPM1* gene at different stages after injection with 30 μM asODN or sODN. *, $p < 0.05$; **, $p < 0.01$. (b) Symptoms in detached tea leaves that were injected with 30 μM asODN or sODN caused by *C. camelliae* strain LS_19. Bar = 0.5 cm. (c) Bar chart showing statistical analysis of the sizes of necrotic lesions in (b). Error bars represent standard deviation. **, $p < 0.01$.

and cytoplasm under the confocal microscope (Fig. 2d). Above all, CsRPM1 localized in the nucleus, cytoplasm, and cell membrane.

MeJA can induce the expression of *CsRPM1*

When analyzing the *cis*-regulatory elements presenting in the 2 kb upstream region of *CsRPM1* that was predicted from LJ43 genomic sequences, we found that MeJA-responsive elements, and defense and stress-responsive elements are presented in the promoter of *CsRPM1* gene (Fig. 4a). The transcript level of *CsRPM1* was then determined after exogenous treatment with MeJA. qRT-PCR analysis showed that the level of *CsRPM1* transcripts was significantly up-regulated at 48 h after MeJA treatment compared with the control group (Fig. 4b). The results indicated that *CsRPM1* might be involved in the jasmonic acid (JA) signaling pathway.

Predication of candidate transcriptional factors regulating the *CsRPM1* expression

To further gain insight into the regulatory mechanism of *CsRPM1*, we predicted the transcriptional factors (TFs) regulating the *CsRPM1* expression through the TeaCoN website. A total of 17 DEGs annotated as involved in transcriptional regulation, including 15 up-regulated genes and two down-regulated genes, were identified (Fig. 5a, b). Especially, five genes, TEA030980 (encoding a late embryogenesis abundant protein), TEA031553 (encoding a multicopper oxidase), TEA031563 (encoding a multicopper oxidase), TEA013693 (encoding a calmodulin-binding family protein), and TEA018280 (encoding a VQ motif-containing protein), showed higher correlation with the expression of *CsRPM1* and were also significantly up-regulated at 72 h after inoculation with *C. camelliae* (Fig. 5b). The result suggested that they may be involved in the regulation of *CsRPM1* expression.

Discussion

As the most important gene families involved in disease resistance in plants, *NLR* genes are usually highly expressed following pathogens infection, and the transcript levels of *NLRs* are correlated with plant defense response against pathogens[9,13,25]. In apple, a TIR-NBS-LRR gene *MdTNL1* positively regulating Glomerella leaf spot (GLS) resistance was highly expressed in resistance apple cultivar 'Fuji' after inoculation with the GLS pathogen *C. fructicola*[26]. Powdery mildew resistance gene *Pm2b* in wheat encoding a CC-NBS-LRR protein was rapidly up-regulated after inoculating with *Blumeria graminis* f. sp. *tritici*[27]. Multiple *StTNL* genes encoding TIR-NBS-LRR proteins in potato showed strong and constant upregulation under fungal pathogen *Alternaria solani* treatment[28]. In tea plant, RNA-Seq have revealed that the expression of *CsNLRs* can be induced by anthracnose pathogens[18]. In this study, we identified 12 DEGs commonly expressed at 12 h, 24 h, and 72 h after inoculation with *C. camelliae* causing anthracnose by RNA-Seq analysis (Fig. 1a), suggesting their potential functions in disease resistance.

Among the 12 DEGs, *CsRPM1* (TEA004876) encoding an NBS-LRR protein was predicted as the disease resistance protein RPM1. RPM1 is an NBS-LRR protein from *Arabidopsis* that confers resistance to bacterial pathogen *Pseudomonas syringae* expressing either two avirulence genes, *avrRpm1* or *avrB*[29]. The functional and potentially molecular homologs of RPM1 also exists in conceivably other crop species[30,31]. In wheat,

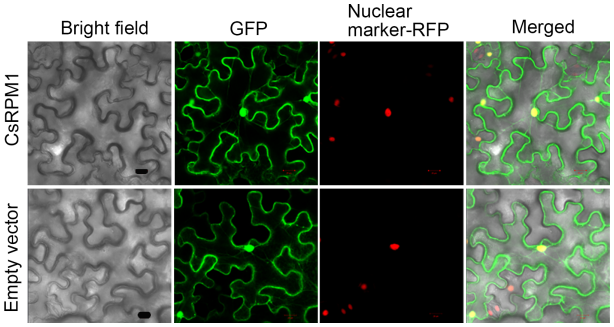

Fig. 3 Subcellular localization of CsRPM1 in the epidermal cells of *N. benthamiana* leaves. Tobacco leaves expressing nuclear marker-RFP were infiltrated with *A. tumefaciens* carrying the fused vectors expressing CsRPM1-GFP or empty vector and were observed under laser confocal scanning microscopy after 2 d in the dark. Bar = 50 μm.

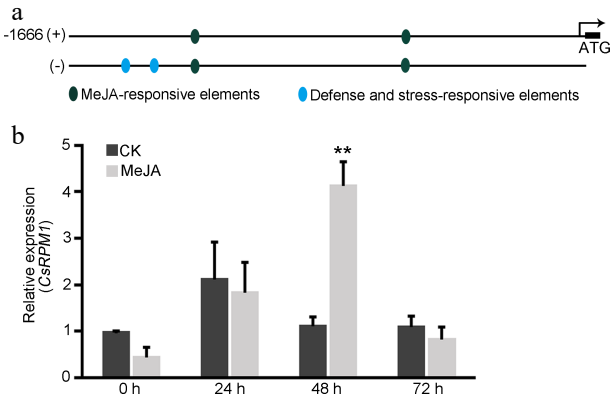

Fig. 4 MeJA can induce the expression of *CsRPM1*. (a) The predicted promoter elements of *CsRPM1* gene from the 2 kb upstream region to ATG. (b) qRT-PCR analysis of *CsRPM1* transcripts in tea leaves treated with exogenous 150 μM MeJA. Error bars represent standard deviation. **, $p < 0.01$.

TaRPM1 plays an important role in the resistance to infection by the powdery mildew pathogen *B. graminis* f. sp. *tritici*[32]. *PsoRPM1* from Xinjiang wild cherry plum (*Prunus sogdiana*) is a root-knot nematode resistance gene candidate[33]. In rice, RPM1-like resistance gene 1 *OsRLR1* mediates the defense response to the fungal pathogen *M. oryzae* and the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* through direction interaction with the transcription factor OsWRKY19[17]. In this study, the transcript level of *CsRPM1* was significantly upregulated after inoculation with *C. camelliae* (Fig. 1e), and silencing of *CsRPM1* facilitates the infection by *C. camelliae* and impairs the resistance of tea plant to *C. camelliae* (Fig. 2a–c). The results indicated that *CsRPM1* plays an important role in the resistance to the infection by *C. camelliae*. RPM1 interacts with other proteins, such as RIN4 and RIN13, which can regulate the RPM1-mediated disease resistance[34,35]. Therefore, based on the characterization of CsRPM1 function, we can identify proteins directly interacting with CsRPM1 in a yeast two-hybrid screen.

Seventeen DEGs including 15 up-regulated genes and two down-regulated genes were predicted to be involved in transcription regulation of *CsRPM1* (Fig. 5a, b). Especially, five genes (TEA030980, TEA031553, TEA031563, TEA013693, and TEA018280) were also significantly up-regulated at 72 h after inoculation with *C. camelliae* (Fig. 5b), showing similar expression patterns to *CsRPM1*. The results suggested that these

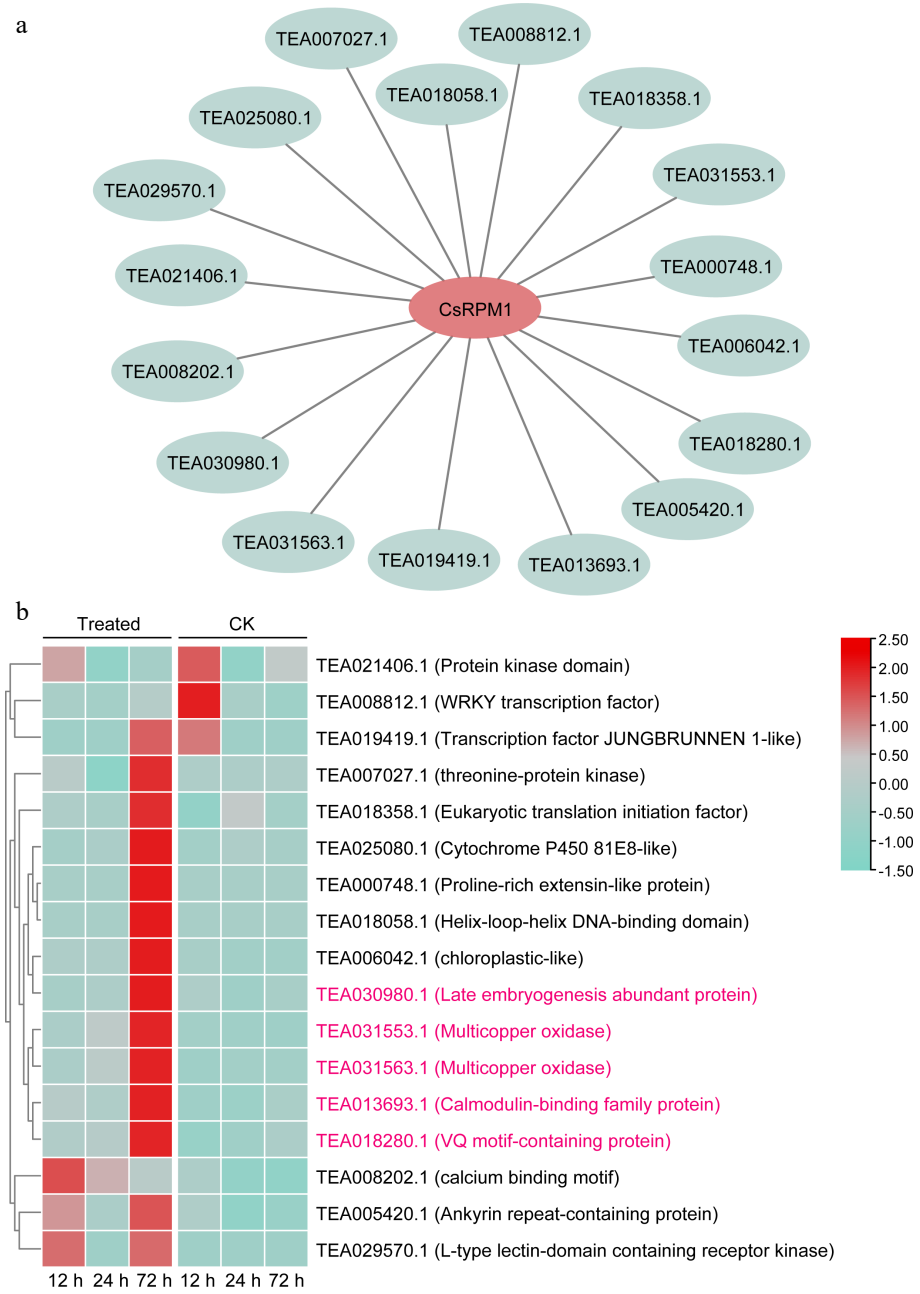

Fig. 5 Connection network between CsRPM1 and candidate TFs. (a) Predicted interaction relationship between CsRPM1 and TFs. (b) Heat map of the expression of candidate TFs in LJ43 at 12 h, 24 h, and 72 h after inoculation with ddH_2O (Control) or *C. camelliae* (Treated).

genes may be involved in the regulation of *CsRPM1* expression. TEA030980 was predicted to encode a late embryogenesis abundant (LEA) protein. In tea plant, *LEA* gene family are identified and involved in response to abiotic stresses including drought, dehydration, osmotic, and cold[36–39]. However, over-expressing of wheat *TdLEA3* in *Arabidopsis* showed increased resistance to fungal infections by *Fusarium graminearum*, *Botrytis cinerea* and *Aspergillus niger*[40], suggesting its potential role in disease resistance. Multicopper oxidase encoded by TEA031553 or TEA031563 are ubiquitous enzymes that catalyze the oxidation of various substrates by reducing O_2 to H_2O, including laccases and several oxidases[41]. Calmodulin-binding protein encoded by TEA013693 may act as TF to regulate biotic and abiotic stress responses, especially in low temperature responses[42]. TEA018280 was predicted to encode a VQ (FxxhVQxhTG, h: hydrophobic amino acid, x: any amino acid) motif-containing protein, this family genes in tea plant are identified and found to play important roles in response to salt and drought stress[43]. Although all the five gene families are identified in tea plant, and transcriptome analysis revealed their expression pattern in response to abiotic stresses, their roles in disease resistance to *Colletotrichum* infection are little known. In future research, we will verify the interaction between CsRPM1 and the five genes by yeast one-hybrid, dual luciferase or electrophoretic mobility shift assays to reveal the regulatory mechanism of *CsRPM1* and their roles in disease resistance.

JAs are important phytohormones that regulate a wide range of physiological processes in plant growth and development, and especially the mediation of responses to various stresses[44]. After plants are infected by pathogens, JA and other

signaling pathways are activated, which leads to the activation of plant disease resistance[45]. For example, JA biosynthesis and signaling genes in rice can be effectively induced by the rice blast fungus *M. oryzae* at the warm temperature, which leads to enhanced blast resistance[46]. The JA signaling pathway is involved in PTI and ETI activation, thereby stimulating downstream transcription factors and initiating plant defense responses to pathogen infections[45]. NLRs as the key pathogen effectors activate ETI, many studies have shown that phytohormone pathways including JA signaling pathway are correlated with the resistance mediated by NLRs. In rice, Gene Ontology (GO) analysis revealed strong correlation of hormone pathways, especially salicylic acid (SA) and JA, with the resistance to brown planthopper (BPH) mediated by an *NLR* gene *BPH9*[47]. JA and its conjugate JA-Ile rapidly increased after BPH infestation[47]. In tea plant, JA content was also significantly increased in the diseased leaves infected by *C. camelliae* at 72 h post inoculation[19]. In this study, we have confirmed that the level of *CsRPM1* transcripts was significantly up-regulated at 48 h after MeJA treatment (Fig. 4b). These results imply that *CsRPM1* may combine with JA signaling pathway to regulate the disease resistance to *C. camelliae* in tea plant. In cucumber, the transcript levels of two NBS-LRR genes *CsRSF1* and *CsRSF2* were also upregulated after exogenous treatment with MeJA and correlated with plant defense response against powdery mildew caused by *Sphaerotheca fuliginea*[9]. The pepper NLR Tsw can recognize pathogen interference of phytohormone signaling as a counter-virulence strategy, thereby activating plant immunity[48]. Therefore, the underlying mechanism of CsRPM1 regulating the defense response to *C. camelliae* in combination with JA signaling pathway needs to be further explored.

Conclusions

NBS-LRR proteins play important roles in plant disease resistance. An NBS-LRR-encoding gene *CsRPM1* was identified from tea plant cultivar LJ43. RNA-Seq and qRT-PCR analysis showed that the transcript level of *CsRPM1* was up-regulated after inoculation with *C. camelliae* LS_19 strain. The transient silencing of *CsRPM1* in LJ43 leaves comprised the resistance to *C. camelliae*. The subcellular localization showed that CsRPM1 protein was localized in the nucleus, cytoplasm, and cell membrane. The expression of *CsRPM1* was induced by exogenous MeJA. We also predicated the candidate TFs regulating the *CsRPM1* expression. Our data indicates that *CsRPM1* is required for disease resistance to *C. camelliae* in tea plant. Further research on the mechanism of CsRPM1 regulating the defense response to *C. camelliae* in combination with JA signaling pathway and TFs will broaden our comprehension of NLRs in tea plant.

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Conflict of interest

The authors declare that they have no conflict of interest. Xinchao Wang is the Editorial Board member of *Beverage Plant Research* who was blinded from reviewing or making decisions on the manuscript. The article was subject to the journal's standard procedures, with peer-review handled independently of this Editorial Board member and his research groups.

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