Transcriptome Analysis Identified Core Genes Involved in Maize Resistance to *Rhizoctonia Solani*

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Research Article

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Abstract

Background

Banded leaf and sheath blight (BLSB) caused by the necrotrophic fungus *Rhizoctonia solani* is a devastating disease on maize worldwide, especially in China and Southeast Asia. It is important to understand the interaction mechanism between maize and *R. solani* for control of invasion and expansion.

Results

In this study, the expression profile of maize infected by low virulence strain (LVS) and high virulence strain (HVS) of *R. solani* for 3 and 5 d was analyzed by RNA-sequencing. A total of 3015 and 1628 differentially expressed genes (DEGs) were identified under LVS and HVS infection, respectively. Meanwhile, these DEGs were classified by Gene Ontology (GO) for biological process analysis. Only defense-related GO terms were commonly enriched in LVS- and HVS-regulated genes. Furthermore, a core set of 388 up-regulated genes that are involved in maize response to *R. solani* infection were identified. Additionally, among the core genes, overexpressing *ZmNAC41* and *ZmBAK1* enhanced rice resistance to *R. solani*.

Conclusion

The results in this study provide additional insight into maize defense mechanisms against *R. solani*, and the core genes identified in this study will be important resources for improving BLSB resistance in the future.

Background

Maize (*Zea mays* L.) is a major cereal crop grown throughout the world for food, feed and fuel. However, maize production is frequently affected by biotic and abiotic stresses with devastating effects on yield and product quality. Among these stresses, banded leaf and sheath blight (BLSB) caused by *Rhizoctonia solani* is considered to be one of the emerging and serious diseases limiting maize production under climate change scenarios [1]. The dominant anastomosis group (AG) on maize is AG1-IA [2]. This pathogen usually causes necrotic spot on the leaf, sheath, and stem. Under high temperature and humidity conditions, it colonizes almost all the aboveground parts of the plants, including cobs and ears. Control of *R. solani* is a difficult problem in maize, and the traditional control methods are to use agricultural prevention measures and chemical fungicides to reduce the damage caused by *R. solani*. With the improvement of the awareness of food safety and environmental protection and requirements for high quality maize products, it is becoming increasingly important to cultivate disease-resistant varieties. However, most of the existing maize varieties are susceptible to *R. solani*, and there are few maize germplasms with high resistance, which greatly limits the study of resistance inheritance [3].

BLSB resistance is a quantitative trait regulated by multiple genes. To our knowledge, no major resistant gene has been mapped or cloned in maize. Up to now, many quantitative trait loci (QTLs) associated with BLSB resistance have been mapped on the maize genome. For example, 11 QTLs for resistance to BLSB were identified using the F_2 individuals derived from the cross of inbred lines R15 and 478 [3], while four BLSB resistance QTLs were mapped using the F_2 individuals derived from the cross of inbred lines CML429 and DM9 [4]. However, very few candidate genes were cloned in these QTLs.

Defense-related (*DR*) genes are a kind of genes that can be rapidly induced by pathogen infection and elicitors [5, 6]. These genes are regarded as important resources for disease resistance, especially for those diseases lacking major resistant genes. Currently, a large number of *DR* genes for resistance to *R. solani* have been functionally characterized in rice and other plants, such as *Os2H16* [7], *OsWRKY80*-*OsWRKY4* [8], *OsASR2* [9], *ScSnakin-1* [10], *OsBSR2* [11], *OsSWEET14* [12], *BdWRKY38* [13] and *TaWAK7D* [14]. However, only *ZmPR5* [15], *ZmWRKY79* [16], *ZmNBS25* [17] and *ZmFBL41* [18] were identified to function in maize resistance to *R. solani*. Therefore, identifying more *DR* genes is very important for genetic improvement of BLSB resistance.

Global gene expression analysis can not only be used to screen differentially expressed genes (DEGs), but also an important means to explore the molecular mechanisms of plant-pathogen interaction. RNA-sequencing (RNA-seq) technology provides a more accurate measurement of transcription levels to identify major changes in gene expression, and numerous DEGs have been identified in different plant-pathogen interactions using this approach [19–21]. RNA-seq also has been successfully used in investigating transcriptional changes of plants during interaction with *R. solani* [22–24]. However, all these studies focused on the expression changes of host genes at the early stage of *R. solani* penetration. Analysis of maize transcriptional changes during *R. solani* expansion has not been reported yet. Because there is no major resistant gene conferring BLSB resistance, genes involved in restricting *R. solani* expansion also have high application value in resistance improvement.

In this study, gene expression profile of maize infected by different virulent strains of *R. solani* for 3 and 5 d was investigated by RNA-seq. A total of 388 core genes involved in maize resistance to *R. solani* were identified. Among these genes, differentially expressed transcription factors (TFs), hormone-related genes and pathogenesis-related (*PR*) genes were collected and analyzed. Furthermore, we identified and validated two novel genes involved in *R. solani* resistance. Taken together, these results provide a comprehensive insight into the resistant mechanisms of maize against *R. solani* expansion and potential gene resources for the improvement of BLSB resistance.

Results

Comparative analysis of B73 infected by different virulent *R. solani* strains

To compare the different responses of maize to *R. solani*, the maize inbred line B73 was inoculated with high virulent strain (HVS) and low virulent strain (LVS) of *R. solani*, respectively. At 5 d post inoculation

(dpi), B73 exhibited longer lesion (5.57 ± 0.25 cm) infected by HVS than by LVS (3.97 ± 0.34 cm) (Fig. 1a). Because *R. solani* was the same pathogen that caused BLSB on maize and sheath blight (ShB) on rice, and it would be much easier and faster to do the gene function research using rice transformation than maize transformation. The pathogenicity of HVS and LVS on the rice cultivar Zhonghua 11 (ZH11) was also investigated. After inoculation for 5 d, HVS caused longer lesion (5.03 ± 0.31 cm) than LVS (3.85 ± 0.23 cm) (Fig. 1b). These results indicated that the two strains showed the same difference in pathogenicity on maize and rice. Furthermore, the biomass of *R. solani* was measured by histological staining and qPCR. No significant difference in hyphae amount was observed at 1 dpi. At 3 and 5 dpi, sheaths infected by HVS showed more hyphae than by LVS (Fig. 1c, d). Because there were no major resistance genes for BLSB, we were more interested in genes associated with restriction of hyphae expanding instead of penetration. Time points of 3 and 5 dpi were selected for the transcriptome analysis.

Identification of DEGs in B73 inoculated with LVS and HVS

To explore the transcriptional changes of maize under *R. solani* attack, sheaths of B73 plants were inoculated with LVS and HVS, respectively. Un-inoculated sheaths were used as control. For each *R. solani* strain, the sheath samples from 3 and 5 dpi were pooled together as one biological replicate. Finally, the sheath samples with three biological replicates were collected for RNA-seq. Nine libraries were sequenced, and more than 54 million raw reads were obtained with an average of six million in each library. The percentage of clean reads ranged from 97.96% to 98.76%. More than 78% of clean reads were mapped to the reference genome (Table S1). The raw data was deposited in the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) under the accession number PRJNA739273.

Next, DEGs were identified in LVS- and HVS-infected B73 compared to un-inoculated B73 with the threshold of $|\text{fold change}| \geq 2$ and $\text{FDR} \leq 0.001$. In LVS- and HVS-infected B73, 1545 and 1166 DEGs were up-regulated, and 1470 and 462 were down-regulated, respectively (Fig. 2a). More DEGs were regulated by LVS infection than by HVS. Venn diagram showed that 49.8% of the up-regulated DEGs were specifically expressed in LVS_DEGs, while 72.8% of the down-regulated DEGs were specifically expressed in LVS_DEGs. In HVS_DEGs, only 33.5% and 13.2% of up- and down-regulated DEGs were specifically expressed, respectively (Fig. 2b).

Characterization of DEGs based on biological process

To understand the potential functions of DEGs, Gene Ontology (GO) enrichment was performed to classify the important biological processes and signaling pathways. For LVS_DEGs, “mitotic cell cycle”, “defense response”, “cell wall organization”, “response to stimulus”, “response to biotic stimulus”, “polysaccharide metabolism”, “response to hormone”, “phosphorylation”, “phosphorus metabolism”, “response to oxygen”, “nitrogen metabolism”, “response to stress”, “immune response” and “response to bacterium” were significantly enriched (Fig. 3a, Table S2), while “response to stimulus”, “mitotic cell cycle”, “chromosome organization”, “response to stress”, “defense response”, “response to abiotic

stimulus", "monocarboxylic acid metabolism", "polysaccharide metabolism", "response to biotic stimulus", "nitrogen metabolism", "lipid metabolism", "response to fungus", "cell wall organization", "phosphorus metabolism" and "salicylic acid metabolism" were enriched in HVS_DEGs (Fig. 3b, Table S2). Interestingly, GO term "response to stimulus" has the most DEGs both in LVS_DEGs and HVS_DEGs, and only defense-related GO terms, such as "response to stimulus", "defense response", "cell wall organization", "response to stress", "response to biotic stimulus" and "response to hormone", were commonly enriched in LVS_DEGs and HVS_DEGs. So we speculated that a set of common DEGs might constitute a major part of the immune transcriptional reprogramming engine.

Identification of core defense-related genes during maize and *R. solani* interaction

To prove the above hypothesis, we focused on the 625 DEGs commonly expressed both in LVS_DEGs and HVS_DEGs (Fig. 2b, Table S3). Among these DEGs, 388 genes were up-regulated and 237 were down-regulated (Fig. 2b). Meanwhile, hierarchical clustering of the fold change of the 625 DEGs showed that they were divided into two groups: genes in the larger group were up-regulated and genes in the small group were down-regulated both by LVS and HVS (Fig. 4a). Then, the expression patterns of several DEGs were verified by quantitative Real-Time PCR (qRT-PCR). As shown in Fig. S1, the expression patterns of 10 DEGs (including 5 up-regulated and 5 down-regulated) were consistent with the RNA-seq analysis, indicating that the RNA-seq results were reliable. GO (biological process) enrichment of the 388 up-regulated genes and 237 down-regulated genes was performed, respectively. Defense-related GO terms were significantly enriched in the up-regulated genes, including "defense response", "salicylic acid biosynthesis", "phenol biosynthesis" and "response to stress" (Fig. 4b, Table S4), suggesting that the 388 up-regulated genes were important components in maize resistance to *R. solani*. However, no defense-related GO terms were significantly enriched in the down-regulated genes (Fig. 4c, Table S4). Taken together, these results indicated that most of the 388 up-regulated genes might be associated with maize immunity and are potential candidates for further functional studies. Therefore, the 388 genes were considered to be the core of maize immune genes.

Identification of differentially expressed TFs in the core gene list

TFs usually play positive or negative roles in plant defense response. In the core gene list, 26 genes encoded TFs, including six *ZF*, five *bHLH*, two *WRKY*, two *bZIP*, two *MYB*, two *AP2/EREBP*, two *HSF*, one *NAC*, one *TCP*, one *JMJ*, one *VQ* and one *GRAS* (Fig. 5a, Table S5). These TFs were further classified into two clusters in a hierarchical clustering based on the fold change. Most of the TFs (20 TFs) were moderately induced, while the minority TFs (6 TFs) were highly induced by *R. solani* (Fig. 5b). Four of them have been reported to be involved in maize defense response. For example, *ZmbZIP111* was up-regulated during the induced systemic resistance activation by *Trichoderma atroviride* [25], while *ZmNAC41* was transcriptionally induced by the hemibiotrophic ascomycete fungus *Colletotrichum graminicola* [26]. *ZmJMJ22* was identified to confer resistance to maize rough dwarf disease [27]. *ZmbHLH124* played an important role in the maize resistance-specific response to *Puccinia sorghi* [28].

These functionally characterized TFs implied that the core gene list contains important regulators associated with maize immunity.

TFs bind to the *cis*-elements in the promoter region to regulate target gene expression. Therefore, the TF-binding motifs in the promoters of the 388 core genes were investigated. Promoter analysis showed that binding sites of WRKY, bHLH, ZF, HSF, MYB, AP2/EREBP, bZIP, NAC and TCP TFs were found in the core genes (Table S6). Noticeably, the binding motifs for WRKY, bHLH, ZF, HSF, MYB and AP2/EREBP TFs were mostly identified among these core genes, and WRKY12, bHLH3, AZF1, HSFB2A, MYB105 and RAV1 were represented in these TFs (Fig. 5c, Table S6). In plants, these TFs play various roles in plant development and response to biotic and abiotic stresses. For example, overexpressing *BrWRKY12* enhanced *Arabidopsis* and Chinese cabbage resistance to *Pectobacterium carotovorum* [29]. AtbHLH3 acts as a transcription repressor to regulate jasmonate (JA)-mediated defense and development [30]. AtAZF1 negatively regulates abscisic acid (ABA)-responsive and auxin-inducible genes under abiotic stress [31]. CaHSFB2A positively regulates plant immunity against *Ralstonia solanacearum* by forming transcriptional cascade with CaWRKY6 and CaWRKY40 [32]. AtMYB105 was reported as a negative regulator of flowering time in *Arabidopsis* [33]. Overexpressing *CARAV1* enhanced *Arabidopsis* resistance to *Pseudomonas syringae* pv. *tomato* DC3000 [34]. These results suggested that these identified TFs might regulate maize resistance to *R. solani* by targeting the core genes.

Identification of hormone signaling-related and *PR* genes in response to *R. solani* infection

Previous studies indicated that salicylic acid (SA), JA and ABA signaling pathways are involved in *R. solani* resistance [7,35,36], so hormone signaling-related DEGs were examined in the core gene list. A total of 28 genes were identified, including six SA-related, six JA-related and 16 ABA-related genes (Table S7). In the SA pathway, four SA synthesis-related genes, *calmodulin-binding protein 60g* (*ZmCBD60g*), *SAR DEFICIENT1* (*ZmSARD1*) and *phenylalanine ammonia lyase* (*ZmPAL*) were highly induced by both LVS and HVS. Also, transcription levels of SA catabolism gene *salicylate/benzoate carboxyl methyltransferase* (*ZmBSMT*) and SA-induced gene *anthocyanin acyltransferase 1* (*ZmAAT1*) were obviously increased when infected by *R. solani* (Fig. 6a). For the JA pathway, JA synthesis-related gene *acyl-activating enzyme 11* (*ZmAAE11*), JA transport gene *NRT1/PTR 5.2* (*ZmNPF5.2*) and four JA-induced genes *3-ketoacyl-CoA synthase* (*ZmKCS*) and *terpene synthase 2* (*ZmTPS2*) were significantly induced by *R. solani* (Fig. 6b). In the ABA pathway, ABA synthesis gene *nine-cis-epoxycarotenoid dioxygenase 6* (*ZmNCED6*) and ABA homeostasis gene *beta-glucosidase* (*ZmBGL*) were significantly induced under *R. solani* infection. Two *TF* genes in ABA signaling, *abscisic stress-ripening protein 6* (*ZmASR6*) and *ZmASR10*, were also identified to be up-regulated by *R. solani*. Meanwhile, expression levels of some regulatory and ABA-responsive genes, including *SNF1-related protein kinase 1* (*ZmSnRK1*), *glucan endo-1,3-beta-glucosidase* (*ZmGNs*), *syntaxin-related protein* (*ZmSYR*), *phosphatase 2C 13* (*ZmPP2C13*), *dehydrin 1* (*ZmDHN1*), *alpha-expansin 2* (*ZmEXPA2*), *beta-expansin 4* (*ZmEXPB4*) and *tonoplast intrinsic protein 4* (*ZmTIP4*), were significantly increased post inoculation with *R. solani* (Fig. 6c).

PR genes are markers in plant defense response and overexpressing some *PR* genes could enhance plants resistance to *R. solani* [37,38]. A total of 12 up-regulated *PR* genes were identified in the core gene list, including one *ZmPR1* gene, two *ZmPR2* genes, three *ZmPR4* genes, one *ZmPR8* gene, three *ZmPR9* genes and two *ZmPR10* genes (Fig. 6d, Table S8). In addition to these hormone-related and *PR* genes, 87 other genes have been characterized to function in plant defense response (Table S9).

ZmNAC41* and *ZmBAK1* positively regulate rice resistance to *R. solani

Among the 388 core genes, the *NAC transcription factor 41* (*ZmNAC41*, GRMZM2G439903) gene and *BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1* (*ZmBAK1*, GRMZM2G015933) gene exhibited very high transcription levels when infected by *R. solani*. To determine the role of *ZmNAC41* and *ZmBAK1* in *R. solani* resistance, we overexpressed these two genes in the rice cultivar ZH11 and obtained 23 *ZmNAC41* overexpression (OE) and 25 *ZmBAK1* OE lines, respectively. In T₁ generation, three OE lines of the two genes were selected and sheaths were inoculated with *R. solani*. At 5 dpi, the *ZmNAC41* and *ZmBAK1* OE lines were more resistant to *R. solani* than ZH11, with average 3.66 cm and 3.72 cm reduction in lesion length, respectively (Fig. 7a, b). Meanwhile, the expression of *OsPR1a*, *OsPR1b* and *OsPR10* was also detected in OE lines post inoculation with *R. solani*. Compared to ZH11, these *PR* genes were more strongly induced in the OE lines (Fig. 7c, d). Taken together, these results indicated that *ZmNAC41* and *ZmBAK1* positively regulate rice resistance to *R. solani*.

Discussion

BLSB is an important maize disease that severely threatens yield and quality. Although some *DR* genes have been identified in maize and other plants [7–18], these genes still cannot meet the needs of BLSB resistance breeding. Meanwhile, the defense mechanisms of maize against *R. solani* are not very clear. In this study, we performed RNA-seq to explore the transcriptional changes in maize at the *R. solani* expansion stage. Thousands of DEGs were identified under LVS and HVS infection. Noticeably, less DEGs were regulated by HVS (1166 up-regulated and 462 down-regulated) than by LVS (1545 up-regulated and 1470 down-regulated) (Fig. 2), suggesting that HVS might suppress partial resistance that antagonizes LVS. A set of DEGs commonly regulated by LVS and HVS was identified and most of them were involved in plant defense response. Therefore, these genes were considered core genes in maize-*R. solani* interaction.

Among the identified core genes, a total of 26 TFs encoding genes were induced by *R. solani* (Fig. 5a, Table S5). In addition to *ZmbZIP111*, *ZmNAC41*, *ZmJM22* and *ZmbHLH124*, which have been functionally characterized in maize defense response [25–28], other types of TFs, such as ZF, WRKY, MYB, AP2/EREBP and VQ have been also reported to function in plant immunity to *R. solani*. For example, GhZFP1 enhances *R. solani* resistance by interacting with CZIRD21A and GZIPR5 [39]. OsWRKY80, OsWRKY4, BdWRKY38 and ZmWRKY79 positively regulates plants resistance to *R. solani* [8, 13, 16]. Overexpressing *OsMYB-R1* in rice showed endurance against *R. solani* [40]. A cell-wall-degrading enzyme-induced AP2/EREBP TF, OsAP2/ERF152, leads to resistance to *R. solani* by activating OsMPK3/6

[41], while another AP2/EREBP TF, NbOPBP1, confers rice resistance to *R. solani* [42]. VQ TFs usually interact with WRKY to regulate plant defense response [43], however, their roles in *R. solani* resistance have not been illustrated. Previous and our studies showed that VQ could be highly induced by *R. solani* [44], suggesting that VQ TFs may also have the *R. solani* resistant function. Taken together, these results demonstrated that the identified TFs in this study may have potential disease resistance to *R. solani*, and their functions should be further investigated.

SA and JA are typical phytohormones involved in plant defense response to pathogens, and previous studies showed that SA functioned in resistance to biotrophic pathogens, while JA was associated with resistance to necrotrophic pathogens [45]. Current evidence indicated that SA- and JA-mediated defense response is mutually antagonistic [46]. *R. solani* is a necrotrophic pathogen, while some findings suggested a hemi-biotrophic nature of *R. solani* [36]. Therefore, the mechanisms of resistance against *R. solani* induced by SA and JA are more complex. In this study, 12 up-regulated genes of the core genes were identified to be related to SA and JA signaling pathways (Fig. 6a, b, Table S7). In SA signaling pathway, SA synthesis-related genes were mostly found. CBP60g and SARD1 are two master TFs in plant immunity [47]. During pathogen attack, SA biosynthetic genes such as *ISOCHORISMATE SYNTHASE 1* (*ICS1*), *ENHANCED DISEASE SUSCEPTIBILITY 5* (*EDS5*) and *AVRPPHB SUSCEPTIBLE 3* (*PBS3*) were coordinately activated by CBP60g and SARD1 [48, 49]. PAL is an important enzyme in SA synthesis [50] and it mediates plant defense response by regulating SA accumulation [51, 52].

AAE activates JA biosynthetic precursors as one of the key JA biosynthetic enzymes [53]. In this study, *ZmAAE11* was identified to be up-regulated by *R. solani*. In *Medicago truncatula*, knocking-down *MtAAE3* increased the plant susceptibility to *Sclerotinia sclerotiorum* [54]. NPF proteins were originally identified as nitrate or di/tri-peptide transporters [55]. Recent studies revealed that this transporter family also can transport JA [56]. *ZmNPF5.2* was dramatically induced by *R. solani* in the RNA-seq data. However, there is no direct evidence suggesting resistant functions of this transporter family. Additionally, JA-induced genes *ZmKCS* and *ZmTPS2* were up-regulated post *R. solani* inoculation. KCS was reported to positively regulate abiotic stress tolerance and defense against herbivore wounding [57, 58], while in *M. truncatula*, silencing *MtTPS10* resulted in a susceptibility phenotype under *Aphanomyces euteiches* infection [59].

ABA is well known for its involvement in response to abiotic stress, and its functions in defense response have been also illustrated [60, 61]. In this study, 16 ABA-related genes were identified among the core genes (Fig. 6c, Table S7). NCED is the key ABA biosynthetic enzyme that catalyzes the oxidative cleavage of *cis*-epoxycarotenoid [62]. Here, *ZmNCED6* was identified to be up-regulated by *R. solani*. However, the roles of NCED in plant defense response have not been reported yet. Our previous studies showed that *DR* gene *Os2H16* regulated *R. solani* resistance via ABA signaling pathway [7], and the TF *OsASR2* bound to the GT-1 *cis*-element to regulate *Os2H16* expression [9]. In this study, two TF genes, *ZmASR6* and *ZmASR10*, were induced by *R. solani*, implying their potential involvement in *R. solani* resistance. Additionally, some ABA regulatory genes which have been functionally characterized in plant immunity, such as *SnRK*, *SYR*, *PP2C* and *expansin*, were also identified in the core genes.

PR proteins are synthesized in response to pathogen attack and limit the growth of pathogens. According to the molecular mass, isoelectric point, localization and biological activity, *PR* genes can be classified into 17 families [63]. In this study, 12 *PR* genes among the core genes were up-regulated post inoculation with *R. solani*, including one *ZmPR1* gene, two *ZmPR2* genes, three *ZmPR4* genes, one *ZmPR8* gene, three *ZmPR9* genes and two *ZmPR10* genes (Fig. 6d, Table S8).

PR1 genes are generally considered as marker genes for systemic acquired resistance (SAR) and SA plays an important role in the signal transduction of SAR [64]. Several *PR1* promoters contain SA-inducible *cis*-elements [65]. Previous studies showed that overexpressing *PR1a* increased tolerance to oomycete pathogens in transgenic tobacco [66], and some PR1 proteins had an antimicrobial activity against *Phytophthora infestans* [67], indicating the promising resistant function of *PR1* genes to oomycetes. Here, one *ZmPR1* gene was significantly up-regulated by *R. solani*, implying that it may result in resistance in maize against *R. solani*.

PR2 genes are predicted to encode beta-1,3-glucanase, which hydrolyzes beta-1,3-glycosidic bonds in beta-1,3-glucans, and have been proved to degrade the fungal cell walls [68]. The PR2 proteins may also degrade endogenous substrates to generate elicitors and induce defense response [63]. In this study, the transcription levels of two *ZmPR2* genes were obviously increased challenged by *R. solani*, suggesting that these two genes may regulate *R. solani* resistance through the above pathways.

PR4 protein family consists of two classes of proteins. Class I PR4 proteins contain a conserved cysteine-rich chitin-binding domain at the N-terminal, while Class II PR4 proteins lack the chitin-binding domain [69]. Of the three identified *ZmPR4* genes, two genes (*GRMZM2G145461* and *GRMZM2G358153*) belong to Class I and the other one (*GRMZM2G465226*) belongs to Class II. *PR4* genes are also considered as JA pathway markers [70]. Studies showed that knocking-out *VvPR4b* increased susceptibility to *Plasmopara viticola* [71] and OsPR4b protein exhibited *in vitro* antifungal activity against *R. solani* [72].

PR8 genes encode chitinases that have well-understood antifungal activity due to their hydrolytic action [73, 74]. Plant chitinases are widely distributed and structurally heterogeneous enzymes, and they mainly function in plant-fungus interaction. In the process of preventing fungal pathogen infection, plants secrete chitinases to digest chitin in the fungal cell wall, produce pathogen-associated molecular patterns and finally lead to immune recognition [75]. In this study, one *ZmPR8* gene was significantly up-regulated by *R. solani*, suggesting its possible roles in blocking the invasion or expansion process by degrading the cell wall of *R. solani*.

PR9 proteins are reported to have peroxidase activity and are involved in lignin biosynthesis, which act as the cell wall barrier against pathogens [76]. Peroxidase is also involved in the oxidation of isophenols to forms that are more toxic to pathogens [77]. The two identified *ZmPR9* genes in this study were enriched to the “cell wall organization” GO term (Table S4). Overexpressing rice peroxidase 114 gene enhance disease resistance to *Alternaria radicina* in transgenic carrot [78]. Hence, we predicted that up-regulation

of the two *ZmPR9* genes may limit the infiltration of *R. solani* toxins into cells and thereby the spread of infection.

PR10 proteins are classified as intracellular proteins and are closely related to a group of major food allergens, which belong to the Bet v 1 family [79]. Although several studies have reported that PR10 proteins have ribonuclease or antimicrobial activities [80, 81], their biological functions remain unclear. In this study, two *ZmPR10* genes were induced after *R. solani* inoculation, and one ZmPR10 protein encoded by *GRMZM2G112488* has been reported to possess broad spectrum antifungal activity against *Botrytis cinerea*, *S. sclerotiorum*, *Fusarium oxysporum*, *Verticillium dahlia* and *Alternaria solani* [82]. So their roles in resistance to *R. solani* need to be further confirmed.

Conclusions

We used RNA-seq to explore the transcriptional changes of maize at the expansion stage of different virulence strains of *R. solani*. Selection and GO enrichment analysis of DEGs identified a core gene list that played important roles in limiting the expansion of *R. solani* in maize tissues. The core gene list mainly included *TF* genes, *PR* genes, hormone-related genes and genes which have been characterized to function in plant defense response. Furthermore, the resistant function of two genes in the core gene list, *ZmNAC41* and *ZmBAK1*, was investigated in transgenic rice plants.

Methods

Plant materials and inoculation

Maize inbred line B73 and rice cultivar ZH11 were grown in a growth chamber at $28 \pm 2^\circ\text{C}$ with a 16/8 h light/dark cycle. *R. solani* strains were cultured on potato-dextrose-agar medium at 25°C . For the sequencing library construction, sheaths of B73 were inoculated with LVS and HVS, and harvested at 3 and 5 dpi for RNA extraction. For expression analysis of *PR* genes, sheaths of ZH11 and transgenic rice plants were inoculated with *R. solani* and harvested at 24 h post inoculation for RNA extraction.

RNA extraction and mRNA library construction

Total RNA was isolated from sheaths inoculated with LVS and HVS at 3 dpi and 5 dpi using TRI reagent (Sigma-Aldrich, USA). The RNA of 3 dpi and 5 dpi was mixed as one biological replicate (three biological replicate, respectively). The mRNA was purified using oligo (dT)-attached magnetic beads and then fragmented. The first- and second-strand cDNAs were synthesized and end repaired. The cDNA fragments with adaptors were amplified by PCR, and the products were purified by Ampure XP Beads. Quality and quantity of the library was assessed using the Agilent 2100 bioanalyzer. The qualified library was amplified on cBot to generate the cluster on the flowcell. The amplified flowcell was sequenced single end on the HiSeq4000 platform (BGI-Shenzhen, China).

RNA-seq data analysis

The sequencing data was filtered with SOAPmuke and clean reads were obtained. The clean reads were mapped to the maize reference genome (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/902/167/145/GCF_902167145.1_Zm-B73-REFERENCE-NAM-5.0/GCF_902167145.1_Zm-B73-REFERENCE-NAM-5.0_genomic.fna.gz) using HISAT2. Bowtie2 was applied to align the clean reads to the reference coding gene set, then gene expression was calculated by RSEM. The heatmap was drawn by MeV (v4.9.0) according to the gene expression. Differential expression analysis was performed using DESeq2 with Q value ≤ 0.05 . Gene ontology enrichment was performed by g:Profiler (<http://biit.cs.ut.ee/gprofiler/gost>) based on Hypergeometric test. The significant terms were corrected by Q value with a threshold (Q value ≤ 0.05) by Bonferroni.

Validation of RNA-seq by qRT-PCR

Ten DEGs were randomly selected and expression levels were validated by qRT-PCR. Primers were designed in qPrimerDB (<https://biodb.swu.edu.cn/qprimerdb/>) (Table S10). cDNA was synthesized with a ReverTra Ace qPCR RT Master Mix with gDNA Remover Kit (Toyobo Life Science, Japan). qRT-PCR was performed with a KOD SYBR qPCR Mix Kit (Toyobo Life Science, Japan) on a qTOWER3G Touch thermal cycler (Analytikjena, Germany). *ZmActin* was used as internal control to normalize the results. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Vector construction and rice transformation

For generating *ZmNAC41* and *ZmBAK1* overexpression vectors, the cDNAs of the two gene were cloned into the *XcmI*-digested pCXUN vector. Primers were listed in Table S10. The constructs were transformed into *Agrobacterium tumefaciens* strain EHA105. For rice transformation, embryonic callus derived from mature embryos was infected by EHA105 strain.

Disease assays

Sheaths of ZH11 and transgenic rice plants were inoculated with *R. solani* for 5 d. The disease symptoms were taken photographs and the lesion length was measured on *R. solani*-infected sheaths. Three independent T₁ transgenic lines and 10 plants for each line were assessed.

Trypan blue staining

Sheaths of B73 were inoculated with LVS and HVS, and harvested at 1, 3 and 5 dpi. Then the sheath samples were incubated with lactophenol-trypan blue staining solution (0.05% trypan blue, 25% lactic acid, 25% water-saturated phenol, 50% ethanol) at 37°C for 12 h, and destained with 2.5 g/ml chloral hydrate. The hyphae were taken photographs under the microscope ECLIPSE 90i (Nikon, Japan) after washing.

Statistical analysis

Means and SD values were calculated using Microsoft Excel 2019. The two-tailed student's *t* test was used to calculate the significance between samples. All statistical analysis was performed using SPSS software version 19.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The sequencing raw data for all libraries presented in this study are openly available in NCBI with the accession PRJNA739273 at <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA739273>.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

N.L. conceived and designed research. H.C. and Z.Y. conducted experiments and analyzed data. S.S. performed vector construction and rice transformation. J.L. performed gene expression analysis. N.L. wrote the manuscript.

Authors' information

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Figures

Figure 1

Phenotypic investigation of maize B73 and rice ZH11 post inoculation with LVS and HVS. a Symptoms and lesion length of B73 at 5 dpi. b Symptoms and lesion length of ZH11 at 5 dpi. c Trypan blue staining of *R. solani* hyphae in B73 sheaths. Bars = 5 μ m. d Biomass of *R. solani* in B73 by qPCR. Values are means $\pm$ SD. Asterisks indicate $P < 0.05$ (*) in student's t test.

Figure 2

DEGs in B73 after LVS and HVS infection. a Number of DEGs in B73 inoculated with LVS and HVS. b Venn diagram of up-regulated and down-regulated DEGs in B73.

Figure 3

GO enrichment for biological process analysis of DEGs. a GO enrichment analysis of DEGs regulated by LVS. b GO enrichment analysis of DEGs regulated by HVS. The y-axis represents the negative log10-transformed Q value (cyan bars) and gene numbers (yellow dots).

Figure 4

Identification and biological process analysis of a core set of DEGs related to *R. solani* resistance. a Hierarchical clustering of the 625 overlapped DEGs between LVS and HVS. b GO enrichment for biological process analysis of up- and down-regulated DEGs of the 625 DEGs. The y-axis represents the negative log10-transformed Q value (cyan bars) and gene numbers (yellow dots).

Figure 5

Characterization of TFs and cis-elements in DEGs in maize response to *R. solani*. a Number of TFs in the 388 core genes. b Hierarchical clustering of the 26 TFs that were up-regulated by *R. solani*. c Sequence logo depiction of the key overrepresented TF DNA-binding motifs in the 750-bp promoters of the 388 core genes.

Figure 6

Heatmaps of relative expression levels of DEGs of particular functions. a DEGs in SA signaling pathway. b DEGs in JA signaling pathway. c DEGs in ABA signaling pathway. d Hierarchical clustering of PR genes.

Figure 7

Disease resistance of ZmNAC41 and ZmBAK1 transgenic rice plants. a Symptoms of ZH11 and transgenic rice plants at 5 dpi. b Statistics of lesion length on ZH11 and transgenic rice plants at 5 dpi. c PR genes expression in ZmNAC41-OE lines at 24 h post inoculation with *R. solani*. d PR genes expression in ZmBAK1-OE lines at 24 h post inoculation with *R. solani*. CK indicates no inoculation. Values are means $\pm$ SD. Asterisks indicate P < 0.05 (*) and P < 0.01 (**) in student's t test.

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