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Research article

Investigation on comparative transcriptome profiling of resistant and susceptible non-CMS maize genotypes during *Bipolaris maydis* race O infection

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ABSTRACT

Maydis leaf blight is a significant disease of maize caused by *Bipolaris maydis* race T, O and *C.* Molecular mechanisms regulating defense responses in non-CMS maize towards race O fungus are not fully known. In the present investigation, comparative transcriptome profiling was conducted on a highly resistant maize genotype SC-7-2-1-2-6-1 against a standard susceptible variety CM 119 at 48 h post inoculation (h PI) along with non-infected control. mRNA sequencing generated 38.4 Gb data, where 9349602 reads were mapped uniquely in SC-7, whereas 2714725 reads were mapped uniquely in CM-119. In inoculated SC-7, the total number of differentially expressed genes (DEGs) against control was 1413, where 1011 were up-regulated, and 402 were down-regulated. In susceptible inoculated genotype CM 119, the number of DEGs against control was 2902, where 1703 were up-, and 1199 were down-regulated. DEGs between inoculated resistant and susceptible genotypes were 10745, where 5343 were up-, and 5402 were down-regulated. The RNA-seq data were validated using RT-qPCR. The key findings are that SC-7 poses a robust plant signaling system mainly induced by oxidation–reduction process and calcium-mediated signaling. It regulates its fitness-related genes efficiently, viz., aldolase 2 gene, isopropanoid, phyto hormones, P450 cytochrome, amino acid synthesis, nitrogen assimilation genes etc. These findings showed more transcriptional changes in the SC-7 genotype, which contains many defence-related genes. They can be explored in future crop development programmes to combat multiple maize diseases. The current finding provides information to elucidate molecular and cellular processes occurring in maize during *B. maydis* race O infection.

1. Introduction

Maydis leaf blight (MLB) also known as southern corn leaf blight (SCLB) ranks among the most significant foliar diseases affecting maize, caused by the fungal pathogen *Bipolaris maydis* Nisikado & Miyake (Teleomorph *Cochliobolus heterotrophus*). It exists principally as race O and, to a lesser extent, as race T. Typically, it causes tan, elliptical to rectangular lesions on the leaves [1] and the

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undersurface of foliage. MLB pathogen quickly spreads from crop debris and windborne spores [2]. In 1970, an epidemic of Southern Corn Leaf Blight (SCLB) ravaged the USA, primarily due to the extensive monoculture of susceptible alleles in the production of cytoplasmic male sterile (CMS) hybrid genotypes [3]. MLB disease can cause crop losses of 9.7%–11.7%, particularly in Asia, including India, in non-CMS maize (Non male sterile cytoplasm) [4]. So far, seven resistance genes in maize have been reported for multiple disease resistance in maize. Among them, two qualitative genes (genes which govern distinguishable traits and follow Mendelian inheritance), hm1 and Rp1-D, encode an NADPH-dependent HC-toxin reductase conferring resistance against *Cochliobolus carbonum* and common rust, respectively [5]. Additionally, other genes in maize, such as ZmFBL41, have been identified for their role in resistance against foliar disease banded leaf and sheath blight (BLSB) [6] Five quantitative genes (Rcg1, Rxo1, ZmWAK, Htn1, and ZmTrxh) have been identified, which confer resistance to various diseases across different plants. Specifically, Rcg1 and Rxo1 provide resistance to anthracnose stalk rot in maize, ZmWAK contributes to resistance against bacterial streak disease in rice, Htn1 confers resistance to head smut in maize, and ZmTrxh plays a role in resistance to Northern corn leaf blight and sugarcane mosaic virus in maize [7–9].

For race *O*, there are fewer known facts at the molecular level associated with disease resistance, which is crucial for developing appropriate control strategies. The response of maize to fungal attacks is a complex phenomenon. Therefore, here RNA-seq is applied for studying resistance mechanisms of highly resistant genotype SC-7-2-1-2-6-1 (SC-7) to understand the facts that make it robust against *B. maydis* because its basis of resistance at the molecular level is still unknown. This is probably the first transcriptome investigation of non-CMS maize lines in response to the MLB pathogen. Though we recently revealed the transcriptome of the fungal pathogen in another study [10], the present study unfurls the facts of the response of differential non-CMS-maize host. CM 119 is a highly susceptible genotype against *B. maydis* race O and has been used as a standard susceptible check, also the race "O" was recently re-confirmed [11].

The best strategy for addressing MLB is varietal resistance, as maize crops with non-CMS cytoplasm are resistant to race "T". race "T" can be managed by removing CMS-T from cultivars with high agronomic value. In India, a wide variety of maize genotypes serve as primary hosts for race "O", resulting in significant losses. So far, we only know that *C. heterostrophus's* rhm recessive gene confers race "O" resistance [12] and a very recent study shows that the interaction between RppC and AvrRppC NLR effectors is responsible for conferring resistance to southern corn rust in maize [13]. Defence mechanisms of non-CMS maize towards *B. maydis* race O fungus is not yet fully known, unlike race T, where there are reports of resistance genes in CMS lines. A monogenic recessive resistance mechanism was discovered for race T in Nigerian breeding stock in the late 1960s. The genetic locus was named *rhm1* for resistance to *Helminthosporium maydis* [14]. Many transcriptome profiles of maize have been analyzed to identify the genes constituting the expression networks underlying the physiological process for CMS maize lines, including CMS C and S [15] but none for non-CMS lines. A complete transcriptomics study reveals systemic symptom development in maize inbred lines during *Bipolaris zeicola* [16].

In this context, our study focuses on a comprehensive transcriptome investigation conducted on two maize genotypes: SC-7 (highly resistant) and CM 119 (highly susceptible) during infection with *B. maydis* race O. The selection of these genotypes is deliberate, with SC-7 demonstrating remarkable resistance under field conditions against MLB disease. It was subsequently registered (INGR 07025) under the Plant Germplasm Registration committee of ICAR in 2007. Both SC-7 and CM 119 originate from the same parental background and were chosen based on phenotypic selection for disease resistance. We specifically focused our study on the 48-h post-inoculation time point, known for its significance in the development stages of the pathogen, including the contact phase, penetration phase, incubation period, and symptom appearance. At this stage, extensive fungal colonization of the host occurs. This research aims to identify differentially expressed genes (DEGs) and unique genes linked to host defense in both genotypes.

2. Material and methods

2.1. Layout of the experiment, genotype selection and inoculation

Inbred lines of maize, SC-7-2-1-2-6-1 (SC-7) and CM 119, highly resistant and susceptible to maydis leaf blight (MLB) disease, respectively, were obtained from the Maize pathology lab of the division of plant pathology, ICAR-Indian agricultural research institute, New Delhi, India [17]. SC-7 (Registration number INGR 07025) was developed through collaboration between the maize pathology unit, and the directorate of maize research (DMR) [17]. The resistant SC-7 plants were selfed for five generations starting in 1994 to establish MLB resistance [[18,19]]. CM 119, identified as a susceptible check in the All-Indian Coordinated Maize Improvement Programme (AICMIP), has been consistently used as a standard in maize genotype screening programs for MLB disease resistance since its identification by the DMR [17]. The seeds of both inbred lines were sown in the net house of the division, and phenotypic selection was employed to identify resistant and susceptible characters [[18,19]]. Inoculation with *B. maydis* using sorghum grains culture was performed on 35-day-old plants, following a standardized method given by Payak and Sharma (1983). This established method ensures a consistent spore load of 10^{3-4} per 5 g inoculum powder of sorghum grains. Inoculum, obtained from sorghum powder, was uniformly spread within the whorls of both the resistant and susceptible inbred lines to maintain a consistent concentration of spores, thus ensuring the reliability and natural consistency of the inoculum used in the study [20]. Concurrently, seeds of both maize lines were sown in a maize field in 2017, 2018 and 2019 to confirm MLB disease establishment under artificial epiphytotic conditions, adhering to standard maize cultivation practices [17].

We also scored disease for two *kharif* seasons (July–October) using a 0–9 scale. The disease on CM 119 was rated 8 (very heavy infection, lesions abundant scattered on lower and middle leaves and spreading up to the flag leaf), and SC-7 rated 2 (slight infection, a few lesions scattered on two lower leaves) under field conditions.

2.2. RNA extraction and illumina GAIIx sequencing

Two replicate samples of the inoculated resistant and susceptible genotypes, as well as one sample of their respective controls, were subjected to transcriptome sequencing. Samples were collected and preserved in liquid nitrogen to send for sequencing. Total RNA was extracted from 1 g of leaves from CM119 and SC-7 plants 48 h post-inoculation, along with their non-inoculated controls, using the RNeasy plant mini kit (Qiagen) following the manufacturer's instructions. The total RNA of each sample was quantified and qualified by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), NanoDrop (Thermo Fisher Scientific Inc.) and 1% agarose gel. One microgram of total RNA with a RIN value above seven was used for library preparation. Next-generation sequencing library preparations were constructed as instructed in the manufacturer's protocol (NEBNext® UltraTM RNA Library Prep Kit for Illumina®).

2.3. Differentially expressed genes

DESEq2 V1.21.17 with the replicate package was run for DEG identification by keeping parametric fit Padj <0.05. A False Discovery Rate (FDR) of 0.05 and fold change (log2) > 2 were set as thresholds for DEG calling, as previously described [21], and a p-value <0.05 were set. The list of all DEGs is provided (Additional file 12: Table S11, Additional file 13: S12, Additional file 14: Table S13: Additional file 15: Table S14) to allow any other DEG sub-setting based on different FDR or fold changes.

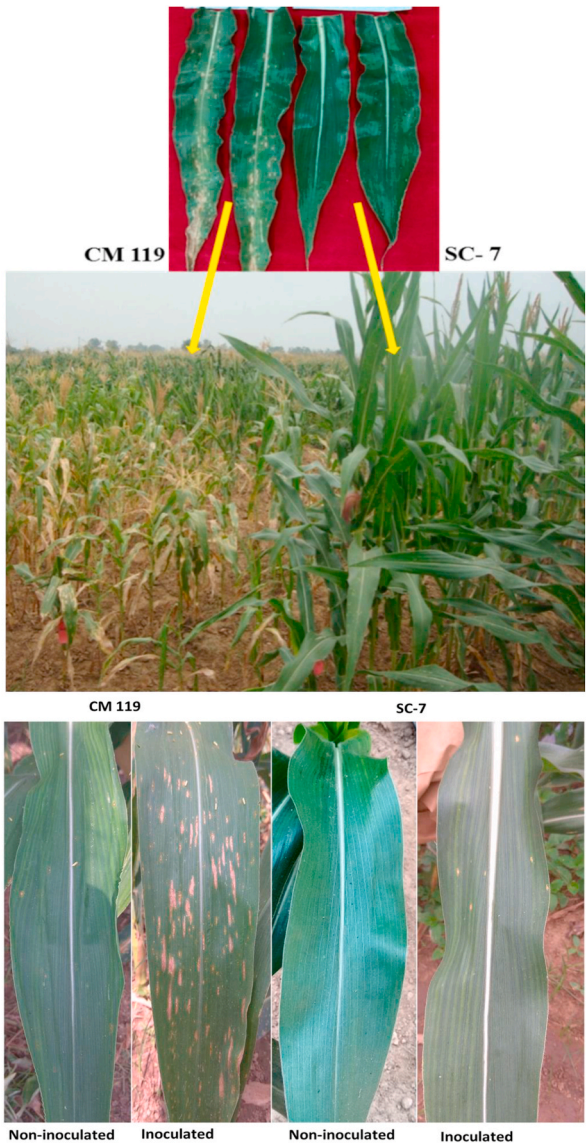


Fig. 1. Symptoms of maydis leaf blight on susceptible genotype CM119 (Score 8) and resistant genotype SC-7 (Score 2) under field condition.

2.4. GO enrichment and KEGG analyses

GO enrichment analyses were conducted with topGO, an R-bioconductor package for enrichment analysis version 2.28.0 and P-value: 0.001 with classic fisher ordering, ranks = topgoFisher. In addition, the bioconductor package Cluster Profiler version 3.10.0 was used to generate relevant KEGG pathway pictures incorporating color-coded expression values (Padj< 0.05) [22].

2.5. Summary of data processing

For mapping sequences, appropriate HISAT2 is chosen for mapping of reference genome (Zea_mays.B73_RefGen_v4.dna.toplevel.fa) and filter the reads were cleaned as FASTQC. Appropriate parameters were set, such as the most extended intron length. Only filtered reads are used to analyze the mapping status of RNA seq data to the reference genome (Zea_mays.B73_RefGen_v4.dna.toplevel.fa). Clean reads, the TMR (Total Mapped Reads or Fragments) was larger than 65%, and MMR (Multiple Mapped Reads or Fragments) was approx. 10%.

2.6. cDNA synthesis qRT-PCR for expression of selected genes

RNA isolation and cDNA synthesis (using kit thermofisher scientific SuperScript IV First-Strand Synthesis System) was performed to validate the RNA-seq data using qRT-PCR for fourteen selected up-and down regulated genes. Actin gene and NTC (non-template control) were used as an internal control. Relative gene expression levels were expressed as the number of cycles (Ct) required for amplification to reach a threshold fixed in the exponential phase of the PCR reaction. The gene expression level was normalized as that of housekeeping genes for each repetition of samples in every run to provide ΔCt value. The Mean of ΔCt values for each target gene was then normalized to the expression of treated samples with control samples to find ΔΔCt20. Comparing relative gene expression among all treatments was determined according to the 2-ΔΔCt method regarding fold changes using the formula below. Each of the samples and an NTC were used in triplicate.

$$\text{Formula, } \Delta\Delta CT = (CT.\text{Target} - CT.\ \text{Actin})\text{Timex} - (CT.\text{Target} - CT.\ \text{Actin})\text{Time 0}$$

Time x = Any time point, Time 0 = 1 × expression of the target gene normalized to β-actin. Here, Time x = 24, 48, 72 and 96 h, Time 0 = at 0 h for susceptible (SC00) and resistant control (RC00).

3. Results

3.1. Symptoms development and observation

The MLB disease reaction pattern of SC-7-2-1-2-6-1 and the standard susceptible check CM 119 (the same line used in the present study) was reproved under artificial field inoculations during 1999, 2000 and 2001 at IARI by Ref. [23]. They presented the detail of the line SC-7-2-1-2-6-1 as per DUS testing guidelines and registered (Reg. No. INGR 07025) under the Plant Germplasm Registration Committee of Indian Council of Agricultural Research (ICAR) on May 14, 2007 as one of the most useful resistance sources for MLB disease. Symptoms were observed on both genotypes, but on CM 119, more prominent and typical blight symptoms were developed (Figs. 1 and 2). We also made a dual comparison, by comparing these data with absolute control of the SC-7 (non-inoculated SC-7 genotype) at the same points so that we can reduce genotype background noise. We found many genes in SC-7 that were differentially expressed during infection compared to non-infected SC-7 and infected CM 119.

The presence of the pathogen on both host plants was confirmed by scanning electron microscopy (SEM) at 48 PI (symptom period). The abundance of mycelia was observed on CM 119 even under low magnifications, against scanty mycelia on SC-7 (Fig. 2) observed under higher magnifications. This confirms the difference in the pathogen's proliferation, infectivity or preference towards two types

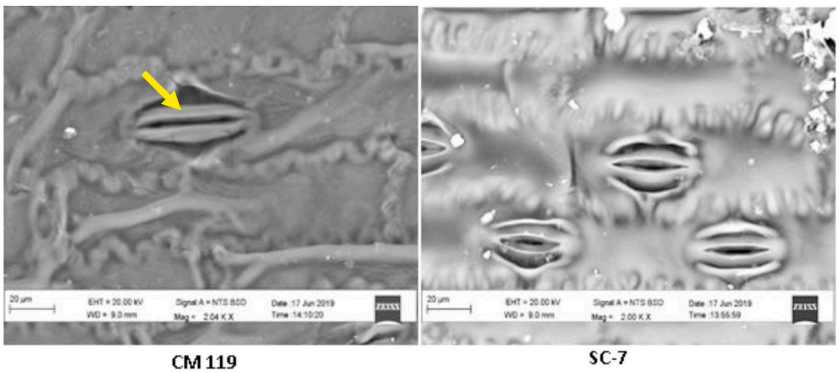


Fig. 2. Fungal mat of *Bipolaris maydis* on leaves of susceptible (CM 119) and resistant (SC 7) genotypes of maize at 48 h post inoculation (PI).

of genotypes. The phenotyping and SEM study in CM 119 and SC-7 supported selecting these two genotypes for transcriptome study.

3.2. Differentially expressed genes (DEGs) in non-CMS maize and their association with host defence and symptom development

RNA was isolated from the leaves of non-inoculated (control) susceptible (SC), resistant (RC) and inoculated plants of both CM 119 (SI) and SC-7 (RI) at 48 h PI, where both showed similar growth rates during the sampling period. Two biological replicates were sequenced for each genotype (SC-7 vs CM 119), non-inoculated and inoculated with *B. maydis* at 48 h PI. Illumina filtered raw reads generated from the Illumina HiSeq 2500 passed filter call. Subsequently, adapters identified by fast QC and low-quality regions were filtered out by cut adapt application [24]. Finally, reads were filtered, and clean reads were obtained for each biological sample (Additional file 1: Table S1) and (mapped with Hisat2 version 2.2.1) mapped to reference maize genome sequence (Zea_mays. B73_RefGen_v4.dna.toplevel.fa).

Read counts were generated from Bam alignment files with HTSeq software [25]. In addition, data normalization and call of differentially expressed genes (DEGs) were implemented with edge version V3.24.3. Pearson correlation coefficients for normalized expression values of samples and filtered reads are shown in Additional file 2: Fig. S1. All the biological samples showed correlation coefficients above 0.8, which indicates good reproducibility between biological replicates. Sequence submitted to SRA database with tentative accession number SRX976797, SRX9767976, SRX9767975, SRX9767974, SRX9767973, SRX9767972.

In resistant genotype SC-7, the total number of DEGs was 1413, of which 1011 were upregulated and 402 were down-regulated. In susceptible CM 119, the numbers of DEGs were 2902, where 1703 were upregulated and 1199 were downregulated. DEGs between resistant and susceptible genotypes were 10745, with 5402 downregulated and 5343 upregulated (Fig. 3). Commonly expressed genes were 1694 in both genotypes under both inoculated and control conditions. Log FC and P values are shown in Fig. 4(a–c).

Modulated genes for both genotypes at susceptible and resistant conditions were compared. The result denoted numerically high DEGs in RI vs SI > SI vs SC > RI vs RC. In addition, it indicated the response of both genotypes to pathogen infection and between both genotypes' response towards the infection trend. DEGs and GO Enrichment analysis was performed using TopGO version 2.28.0. The criteria used to describe genes in the following sections were based on the higher fold changes (FC), defence related genes reported for other host-pathogen systems and genes exclusively expressed high in both resistant and susceptible genotypes.

3.3. Upregulated DEGs and their involvement in plant defence

3.3.1. H2APutative histone

H2A.4 gene (Zm00001d006213) was highly upregulated (LogFC = 13.21036256) in resistant inoculated (RI) plant compared to susceptible inoculated (SI) plant. In contrast, it was slightly downregulated in resistant inoculated then resistant control (RC) and neutral between susceptible inoculated and susceptible control (SC) (Fig. 5).

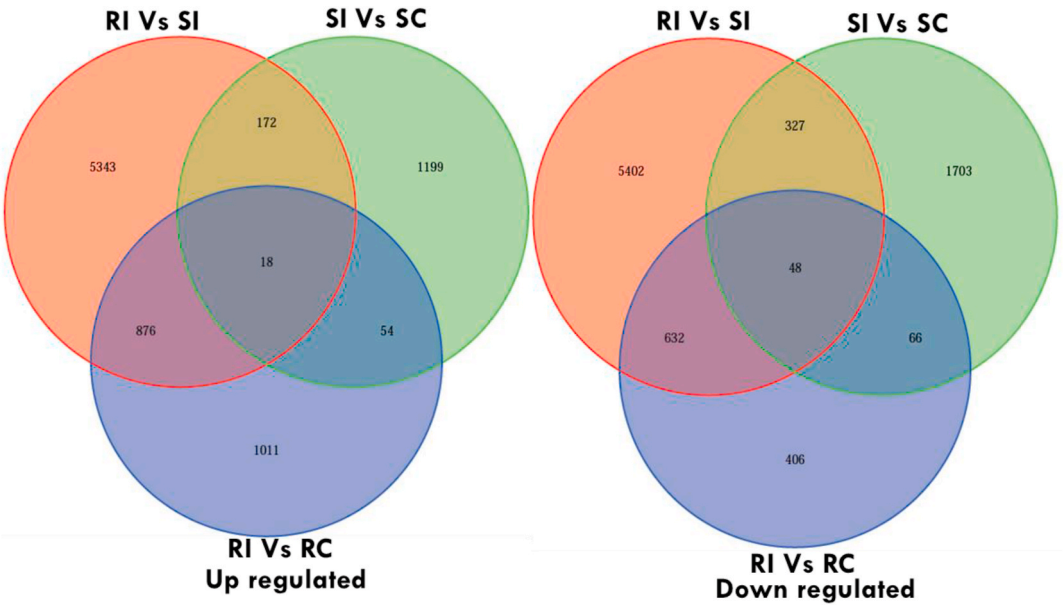


Fig. 3. Venn diagrams of DEGs modulated by maydis leaf blight disease. Venn diagrams represent DEGs in resistant (SC 7) and susceptible (CM 119) maize genotypes after 48 h of inoculation with *Bipolaris maydis* and their corresponding control. (RI = Resistant inoculated, RC= Resistant control, SI = susceptible inoculated, SC = susceptible control).

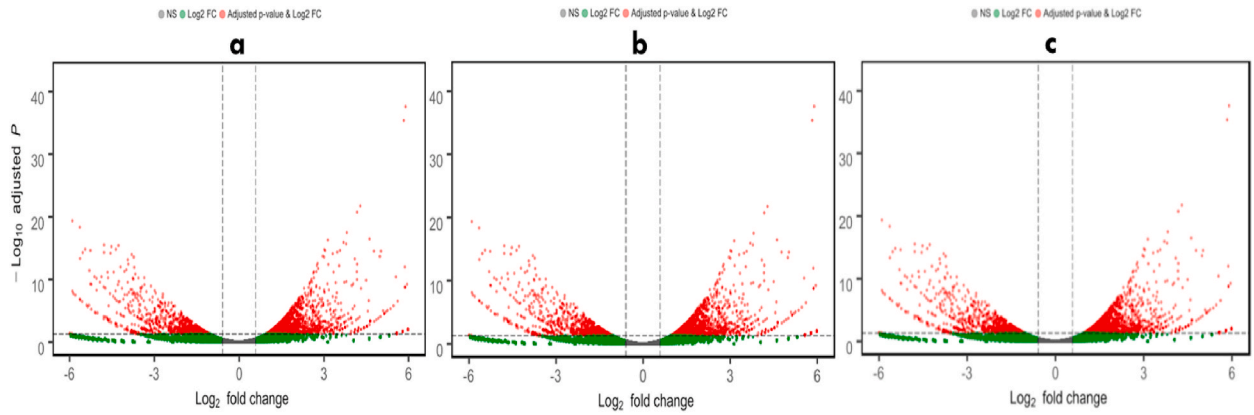


Fig. 4. Mean expression versus log fold change plots (MA-plots). Transcriptional changes are presented in SC-7 and CM 119 at 48 h PI and in control condition. Normalized P values are plotted versus Log2 fold changes. Genes with an FDR <0.05 are plotted. RI Vs RC (a), RI Vs SI(b) and SI Vs SC(c), respectively. (RI = Resistant inoculated, RC= Resistant control, SI = susceptible inoculated, SC = susceptible control).

3.3.2. SNARE 12 protein

SNARE (Zm00001d005751) is a specialized class of proteins present in eukaryotes. SNAREs are membrane-anchored proteins that contain α-helical heptad repeats and a characteristic central amino acid within the SNARE motif. It was upregulated (LogFC = 12.92496178) in RI exclusively and neutral in RC. SNARE proteins are associated with cytokinesis, shoot gravitropism, pathogen defense, symbiosis, and abiotic stress responses [26]. Upregulated enriched GO terms for this gene in the RI plant were Cytoplasm (GO:0005737), Golgi apparatus GO:0005794, and cis-Golgi network GO:0005801.

3.3.3. Peroxisome hydroxyl acid oxidase (HAOX/GOX)

HAOX/GOX (Zm00001d023759) is a photorespiratory enzyme which is strongly associated with photosynthesis regulation. In MLB disease, photosynthesis regulation is essential since it is connected to symptom development. This gene was upregulated (LogFC = 13.04753174) in SC-7 in inoculated conditions. Upregulated enriched GO term for this gene in RI plant metabolic process (GO:0008152) and (GO:0055114) obsolete oxidation-reduction process.

3.3.4. Thioredoxin H-type (Trx)

Trx genes are associated with the redox pathway and play an essential role in redox homeostasis at the cellular level. GE of Trx-H type (Zm00001d035390) upregulated in RI (LogFC = 14.20234519) compared to SI and neutral when compared with resistant control (RC). Enriched GO term is obsolete cell (GO: 0005623).

3.3.5. NADPH quinone oxidoreductase 1

This enzyme act as quinone reductase (Zm00001d047441) involved in conjugation reactions of hydroquinones in detoxification pathways (Uniprot). This gene was upregulated (LogFC = 10.20220144) in RI compared to SI.

3.3.6. Long chain base biosynthesis protein 1

LCB 1 is associated with lipid metabolism with serine palmitoyl transferase as a catalytic core component. It is the first enzyme in Sphingolipid Biosynthesis. *LCB 1*(Zm00001d007424) was up regulated (LogFC = 13.80579975) in RI. The expression recorded neutral in SI and SC. Associated GO terms are metabolic process (GO: 0008152) and biosynthetic process (GO: 0009058).

3.3.6. Cysteine proteinases

Cysteine proteases (Zm00001d022036) are classes of enzymes which degrade proteins. These proteins are produced as inactive precursors with a signal peptide for protein secretion and an auto-inhibitory prodomain to prevent unwanted protein degradation [27]. Cysteine proteinases superfamily protein (LogFC = 11.36795493) was upregulated in the RI plant and neutral in RC. It was also observed as neutral in SI and SC. Enriched GO term hydrolase activity (GO: 0016787), Catalytic activity (GO:0003824), cysteine-type peptidase activity (GO:0008234), peptidase activity (GO:0008233).

3.3.7. Probable low-specificity L-threonine aldolase 2

Threonine aldolase converts threonine to glycine and acetaldehyde. It is associated with the biosynthesis of the amino acids pathway (KEGG). A similar gene (Zm00001d029237) in RI overexpressed (LogFC = 9.309011656). Carboxylic acid metabolic process (GO: 0019752), nitrogen compound metabolic process (GO:0006807), cellular amino acid metabolic process (GO:0006520), and cellular metabolic process (GO:004423) were among the enriched GO terms.

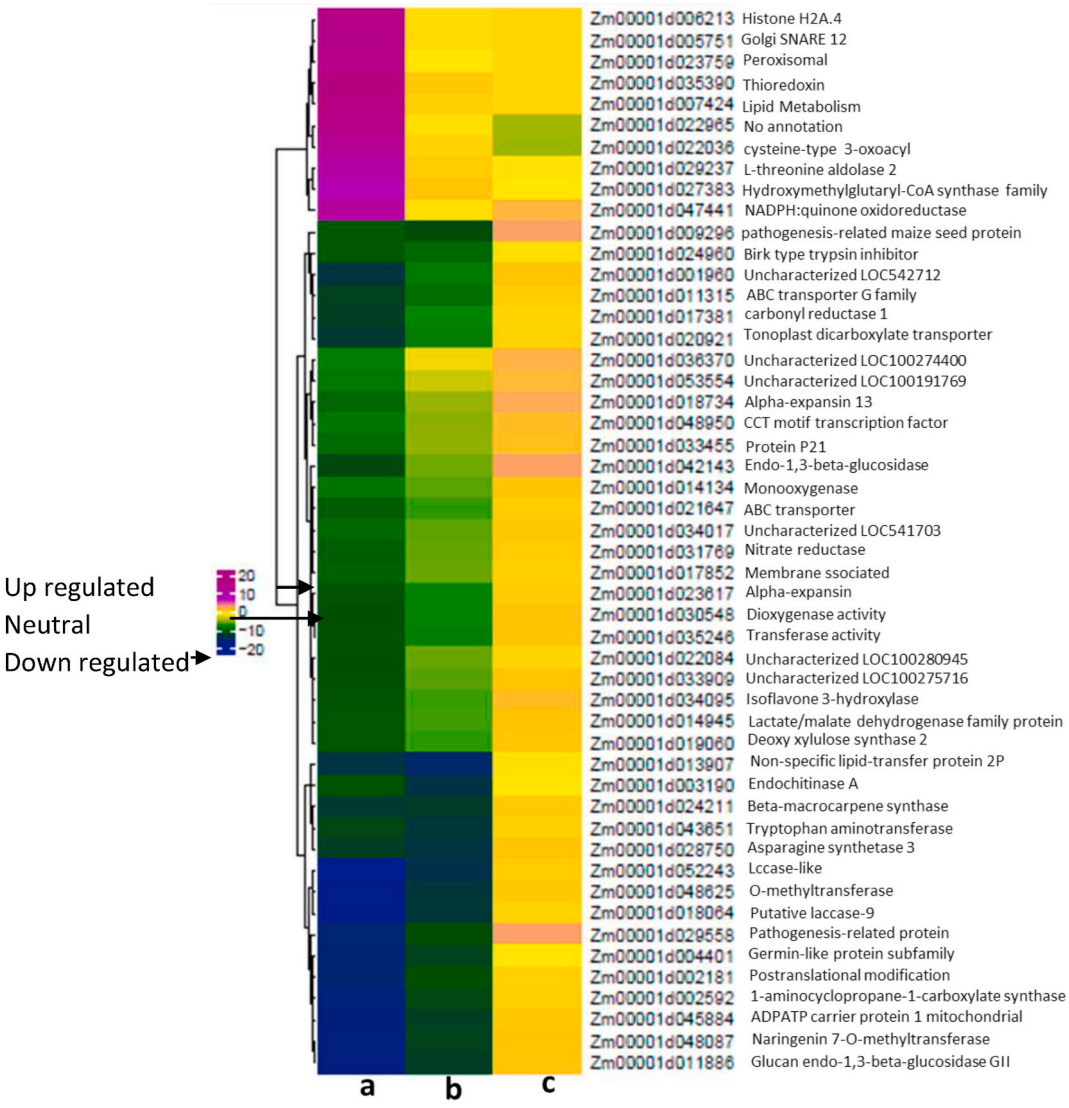


Fig. 5. Heat map of differentially expressed genes in inoculated resistant (SC-7) RI and susceptible (CM 119) SI maize genotypes and their corresponding controls RC, SC, respectively (a = RI Vs SI, b = RI Vs RC, c = SI Vs SC). The maize genes displayed in the heat map is based on the highly differential gene expression (up and down regulation) after pathogen inoculation at 48 h obtained during whole transcriptional analysis, further relation with disease resistance is mentioned below.

3.3.8. Hydroxy methyl glutaryl-CoA synthase (HMGR)

HMGR (Zm00001d027383) is an essential enzyme in synthesizing the mevalonate pathway for the synthesis of isoprenoid biosynthesis. This enzyme was upregulated (7.492318281) in RI and RC. Lipid metabolic process (GO: 0006629), biosynthetic process (GO: 0009058), isoprenoid biosynthetic process (GO: 0008299), lipid biosynthetic process (GO: 0008610), cellular metabolic process (GO: 0044237) were enriched GO terms.

3.3.9. Calcium binding proteins and calmodulin proteins

Calcium targets are involved in plant defence, Eg.calmodulin, a calcium-binding protein, Ca^{2+} protein kinase. In addition, calcium is involved in changes in defence related gene expression, phytoalexin accumulation and HR-related cell death [28]. In the present study, we observed upregulation of putative calcium-binding locus *viz.*, Zm00001d031921 (LogFC = 5.29326), Zm00001d053659 (LogFC = 3.146952), Zm00001d053659 (LogFC = 3.146952) in the resistant inoculated plant compared to the susceptible inoculated plant. On the other hand, it was neutral in its corresponding control and for calmodulin protein Zm00001d020722 (LogFC = 7.392307), Zm00001d035377 (LogFC = 12.26439). We also found the associated calmodulin locus, where five loci were upregulated, and two were down-regulated. For Ca^{2+}dependent protein kinase, seven loci were upregulated, and six were downregulated. Similarly, four calcium-binding protein loci were upregulated, and one locus was downregulated in the resistant inoculated RC plant compared to

the SC plant. Enriched GO terms are metal ion binding (GO: 0046872), molecule binding such as DNA/Protein/Ion binding (GO: 0005488), calcium ion binding (GO: 0005509), and protein binding (GO: 0005515).

3.3.10. Auxin response factor 4 (ARF)

Transcriptional factors bind specific DNA sequences, the auxin-responsive promoter element (AuxREs). It activates and represses, and modulates early auxin response. The expression of this gene (zm00001d001945) was observed up regulated (LogFC = 3.45743117) in RI compared to SI and non-significant RI compared to RC and its corresponding controls. It was further downregulated (LogFC = −5.533583523) in susceptible inoculated SI genotype compared to its control SC. Auxin-activated signaling pathway (GO:0009734), signal transduction (GO:0007165), response to hormone (GO:0009725), response to auxin (GO:0009733), cellular response to stimulus (GO:0051716) were GO enriched terms.

3.3.11. NB-ARC domain-containing disease resistance orthologous protein

NB-ARC domain signaling found in prokaryotes and eukaryotes related to regulations of cell death (ADP binding GO**:** 0043531). We found the presence of the NB-ARC domain (Zm00001d035377) in both resistant SC-7 and susceptible CM 119. In the RI plant, GE was quite over-expressed (LogFC = 12.68654). In other combinations, RC, SI and SC, it was non-significant. GO enriched term was protein binding (GO:0005515).

3.3.12. WRKY transcription family proteins

WRKY transcription factors are associated with the class of DNA-binding proteins. We found some of the associated loci in non-CMS maize, which were differentially expressed *viz*., Zm00001d044171, Zm00001d025669, Zm00001d020881 were upregulated in RI plant compared to SI. Interestingly, Zm00001d044171 was upregulated compared to RC (LogFC = 4.612981587) and downregulated in SI compared to SC (LogFC = −2.582515383). Zm00001d020881 was also upregulated in RI compared to RC (LogFC = 2.043531508). Another locus, Zm00001d038023 (LogFC = −2.83089), was downregulated in RI compared to SI.DNA binding (GO: 0003677), sequence-specific DNA binding (GO: 0043565), and nucleic acid binding (GO: 0003676) were enriched terms. We found some of the associated loci in non-CMS maize, which were differentially expressed *viz*., Zm00001d044171, Zm00001d025669, Zm00001d020881 were upregulated in RI plant compared to SI. Interestingly, Zm00001d044171 was upregulated compared to RC (LogFC = 4.612981587) and downregulated in SI compared to SC (LogFC = −2.582515383). Zm00001d020881 was also upregulated in RI compared to RC (LogFC = 2.043531508). Another locus, Zm00001d038023 (LogFC = −2.83089), was downregulated in RI compared to SI.DNA binding (GO: 0003677), sequence-specific DNA binding (GO: 0043565), and nucleic acid binding (GO: 0003676) were enriched terms.

3.3.13. Cytochrome P450 monooxygenases

Most of the putative cytochrome P450 monooxygenases genes (GO: 0004497) were upregulated, including Zm00001d002937 (LogFC = 12.29794). In addition, Zm00001d004389 (LogFC = 7.883378283) genes were highly upregulated in RI compared to SI. Zm00001d002937 was also slightly upregulated in RI compared to RC (LogFC = 1.120057).

Fifty-six genes were upregulated, and 94 genes were downregulated in RI compared to SI. Among the highest downregulated genes, P450s were Zm00001d003283 (LogFC = −6.310028746) in RI compared to SI and in SI compared to SC (LogFC = −4.912253071) andZm00001d005823 (LogFC = −4.912253071, −3.776863454) in RI versus SI and in RI versus RC, respectively. Enriched GO terms were monooxygenase activity (GO: 0004497), oxidoreductase activity (GO: 0016491) and catalytic activity (GO: 0003824).

3.4. Highly downregulated genes

3.4.1. Non-specific lipid-transfer protein 2P

Non-specific lipid-transfer proteins are basic plant proteins and are found plenty in plants. We found these proteins Zm00001d013907 highly downregulated in RI compared to SI (LogFC = −11.51974045). Interestingly these proteins were highly downregulated in RI compared to its control RC (LogFC = −13.14076115). In contrast, it recorded neutral in SI vs SC combination. Down-regulated GO term is lipid transport (GO:0006869).

3.4.2. (S)-beta-macrocarpene synthase-like enzyme

This enzyme is involved in the biosynthesis of the bicyclic sesquiterpene (S)-beta-macrocarpene in maize. Zm00001d024211 was downregulated in RI compared to SI (LogFC = −10.89587158). Compared to the resistant control, it was downregulated again (LogFC = −10.6104). When compared to SI and SC, there was no significant difference. Associated GO terms were lyase activity (GO: 0016829) and catalytic activity (GO: 0003824).

3.4.3. Tryptophan aminotransferase-related protein 4

This gene is involved in the tryptophan-dependent pathway of auxin biosynthesis. We found downregulation of the Zm00001d043651 gene (LogFC = −9.938509937) in RI vs SI comparison. Compared to the resistant control, it was highly down-regulated (LogFC = −11.23576686). However, in susceptible inoculated and control, it showed no significant difference. GO terms were lyase activity (GO: 0016829) and carbon-sulfurylase activity (GO: 0016846).

3.4.4. Asparagine synthetase 3

Asparagine synthetase Zm00001d028750 catalyzes ammonium assimilation into asparagine. Here we found downregulation of (LogFC = −10.49136098) of this gene in RI compared to SI. Resistant inoculated again showed down-regulation (LogFC = −11.41571473) with respect to its control RC. There was no difference between inoculated and control conditions in the susceptible genotype. Down-regulated GO terms are carboxylic acid metabolic process (GO: 0019752), cellular metabolic process (GO: 0044237) and nitrogen compound metabolic process (GO: 0006807).

3.4.5. O-methyltransferase ZRP4

O-methyltransferase ZRP4 is involved in the carboxylic acid metabolic process. Zm00001d048625 gene was highly downregulated (LogFC = −15.19531547) in RI compared to SI. However, it was still downregulated compared with its resistant control RC (LogFC = −11.26071703). A similar gene recorded a neutral difference between SI and SC. The GO term associated is O-methyltransferase activity (GO: 0008171).

3.4.6. Germin-like protein

GLPs are large and ubiquitous proteins present in plants. We found downregulation (LogFC = −13.81626313) of germin-like protein Zm00001d004401 gene expression in the RI plant compared to SI. It was also downregulated (LogFC = −10.10404322) compared to its control RC. In susceptible plants, both inoculated and control did not exhibit differences.

3.4.7. 1-aminocyclopropane-1-carboxylate synthase

1-aminocyclopropane-1-carboxylate synthase is related to ethylene biology. Locus associated with this gene Zm00001d002592 was highly downregulated (LogFC = −14.17399458) in RI maize compared to SI. It was again downregulated (LogFC = −9.903114638) compared to its resistant control RC; like most cases, there was no difference between the expression SI and SC. Metabolic process (GO:0008152), biosynthetic process (GO:0009058).

3.4.8. ADP-ATP carrier protein 1 mitochondrial

ADP-ATP carrier protein 1 mitochondrial (ANT1) is involved in mitochondrial oxidative phosphorylation in mitochondria. Here we found Zm00001d045884 which is ANT1 downregulated (LogFC = −14.34624368) in RI compared to SI. In comparison to RC, RI was again downregulated (LogFC = −10.4144903), whereas there was no change between SI and SC. Membrane (GO: 0016020), cytoplasm (GO: 0005737) mitochondrion (GO: 0005739).

3.4.9. Bowman-Birk type trypsin inhibitor

Zm00001d024960 is identified as a bowman-birk type trypsin inhibitor protein kind of serine protease inhibitor. We found downregulation (LogFC = −8.616960189) of this gene in RI compared to SI. This gene was also downregulated compared (LogFC = -7.816119523) to RC in RI. We did not find a significant difference between SI and SC when looking at the susceptible plant. The enriched GO term was extracellular region (GO:0005576).

3.4.10. PR proteins

Pathogenesis-related protein PRB1-2 (Zm00001d029558) and Glucan endo-1, 3-*beta*-glucosidase GII (Zm00001d011886) were downregulated (LogFC = −13.78063114 and −14.609132) respectively in RI compared to SI. Compared with the resistant control, RC showed downregulated (LogFC = −9.575008679 and −10.43316012), respectively, for PRB 2 and endo-1,3-*beta*-glucosidase. Comparison between SI and SC shows slight upregulation of PRB 2 (LogFC = 2.44789949) and neutral for endo-1,3-beta-glucosidase. glucan endo-1,3-beta-D-glucosidase activity (GO:0042973). There were more significant up-regulations observed in genes discussed in brief below (Additional file 3: Table S2, Additional file 4: Table S3, Additional file 5: Table S4).

Table 1
Important enriched GO terms of molecular function differentially expressed in inoculated resistant and susceptible maize and their corresponding control.

GO Term Molecular	Annotation	No. of time DEGs					
		RI vs SI		RI vs RC		SI vs SC	
		UP	DOWN	UP	DOWN	UP	DOWN
GO:0005515	Protein binding	296	228	112	19	77	95
GO:0016787	Hydrolase activity	301	345	97	77	60	106
GO:0003723	RNA binding	95	34	34	3	8	33
GO:0003677	DNA binding	116	144	47	23	26	53
GO:0016874	Ligase activity	59	58	14	12	14	22
GO:0016740	Transferase activity	259	394	73	81	109	89
GO:0005524	ATP binding	40	41	19	8	8	10
GO:0004497	Monooxygenase activity	56	94	21	35	47	13
GO:0022857	Transmembrane transporter activity	68	57	20	12	20	6
GO:0005509	Calcium ion binding	18	41	6	9	3	13
GO:0016829	Lyase activity	46	55	12	17	10	09

3.4.11. GO enrichment analyses for plant defence and susceptibility

In GO term analysis, genes were classified into three categories, i.e., biological process, molecular function and cellular component. The resistant genotype least downregulated differentially expressed genes fall under cellular function. Most of the differentially expressed genes were found in resistant vs susceptible comparison (Tables 1–3); GO enriched genes for each comparison are provided in Additional file 6: Table S5, Additional file 7: Table S6, Additional file 8: Table S7.

3.4.12. KEGG pathway

KEGG pathway enrichment analysis was performed on the up-and down-regulated genes for all the samples to compare and summarize the response of two maize genotypes to infection. In the resistant infected plant (RI), when we compared enrichment with resistant control (RC), 26 pathways were found down-regulated, and 31 pathways were up-regulated in the RI plant. (Figs. 5 and 6, S9).

3.4.13. qRT-PCR-based validation of highly expressed genes

To validate the RNA-Seq technique, fourteen DEGs were selected based on their expression patterns at 48 h PI for quantitative RT-PCR (qRT-PCR) by using the same RNA extracts for RNA-seq experiments (Fig. 7). The results of the selected DEGs showed that the qRT-PCR was consistent with the RNA-Seq results showing the similar expression pattern of up-and down-regulated genes by using both RNA-Seq and qRT-PCR analyses. Details of the primer sequence, qRT-PCR melt curve and fold change value of selected genes are provided in Additional file 9: Table S8, Additional file 10: Table S9., and Additional file 11: Table S10.

4. Discussion

To identify transcriptional mechanisms conferring non-CMS maize resistance, comparative RNA-seq transcriptome profiling was performed on infected maize genotypes at 48 h (disease period) post-inoculation (PI), and their corresponding non-infected control. SC-7, that is highly MLB-resistant genotype of maize, was evaluated against standard susceptible check CM 119; we found symptoms of MLB started appearing at 48 h PI, and symptoms were quite severe in the susceptible plant, which further increased over time and became more prominent after 96 h PI (symptom development period). This observation of inflectional stages resembled the study on the northern corn leaf spot caused by *B. zeicola* [29].

4.1. Important up regulated differentially expressed genes (DEGs) involved in plant defence, oxidative burst or reactive oxygen species and programmed cell death

Our investigation identified several genes implicated in the molecular defense mechanism of maize against *B. maydis*. Notably, the putative histone H2A, known for chromatin modification, exhibited elevated expression in the resistant genotype (SC-7) during pathogen attack. This histone modification might have heritable implications across generations [30]. Studies suggest that Histone protein modifications regulate gene expression in plants, activating defense-related genes during pathogen attacks. They establish epigenetic memory, priming plants for stronger defense responses, and interact with hormonal pathways to fine-tune defense mechanisms, aiding adaptation to biotic stress [31]. Additionally, Genes associated with oxidative burst were identified. Peroxisome Hydroxyl Acid Oxidase (HAOX/GOX), Thioredoxin H-type (Trx) genes and NADPH Quinone Oxidoreductase 1 were overexpressed in

Table 2
Important enriched GO terms of biological process differentially expressed in inoculated resistant and susceptible maize and their corresponding control.

GO term Biological	Annotation	No. of time expressed					
		RI vs SI		RI vs RC		SI vs SC	
		UP	DOWN	UP	DOWN	UP	DOWN
GO:0016310	Phosphorylation	295	335	81	38	31	140
GO:0006508	Proteolysis	86	37	19	11	21	14
GO:0006355	Regulation of transcription	71	74	30	22	14	33
GO:0008152	Metabolic process	1206	1275	391	222	258	409
GO:0055114	Oxidation-reduction process	73	96	28	24	31	11
GO:0016567	Protein ubiquitination	52	87	29	13	12	21
GO:0032259	Methylation	46	27	15	4	5	17
GO:0055085	Transmembrane transport	120	129	35	39	31	44
GO:0005975	Carbohydrate metabolic process	76	84	18	15	17	26
GO:0009734	Auxin-activated signaling pathway	24	3	9	1	1	7
GO:0006468	Protein phosphorylation	48	41	12	2	19	3
GO:0098869	Cellular oxidant detoxification	28	20	4	7	4	5
GO:1902600	Proton transmembrane transport	27	32	9	10	7	12
GO:0006950	Response to stress	78	77	33	10	27	24
GO:0006629	Lipid metabolic process	97	94	31	19	22	40
GO:0071805	Potassium ion transmembrane transport	16	10	5	4	4	2
GO:0007165	Signal transduction	69	37	28	3	9	28
GO:0008643	Carbohydrate transport	15	5	3	2	4	2

Table 3
Important enriched GO terms of cellular process differentially expressed in inoculated resistant and susceptible maize and their corresponding control.

GO term Cellular	Annotation	No. of time expressed					
		RI vs SI		RI vs RC		SI vs SC	
		UP	DOWN	UP	DOWN	UP	DOWN
GO:0016021	Integral component of membrane	459	515	114	94	96	130
GO:0005634	Nucleus	206	188	95	21	113	41
GO:0009507	Chloroplast	68	14	5	3	15	6
GO:0005840	Ribosome	65	67	7	3	20	14
GO:0005622	Intracellular	572	565	194	69	190	146
GO:0005623	Cellular function	629	611	208	75	213	159
GO:0005737	Thylakoid	256	258	6	25	76	50
GO:0016020	Membrane	572	637	143	117	129	171
GO:0019898	Extrinsic component of membrane	16	1	2	1	2	2
GO:0005886	Plasma membrane	30	30	6	5	7	19
GO:0009507	Chloroplast	68	14	5	3	15	6
GO:0005829	Cytosol	15	11	6	1	4	4

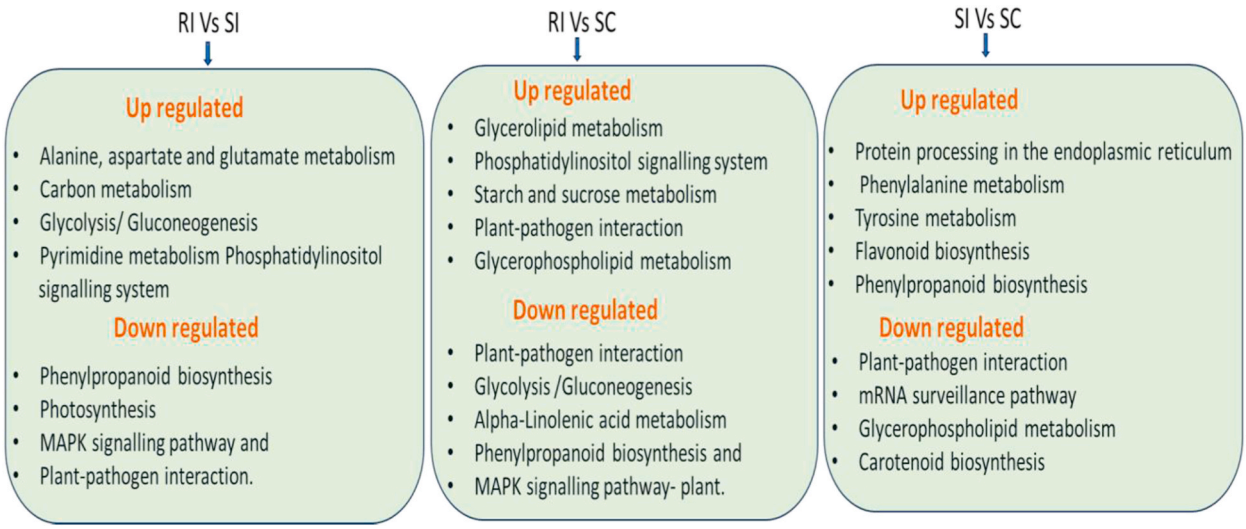
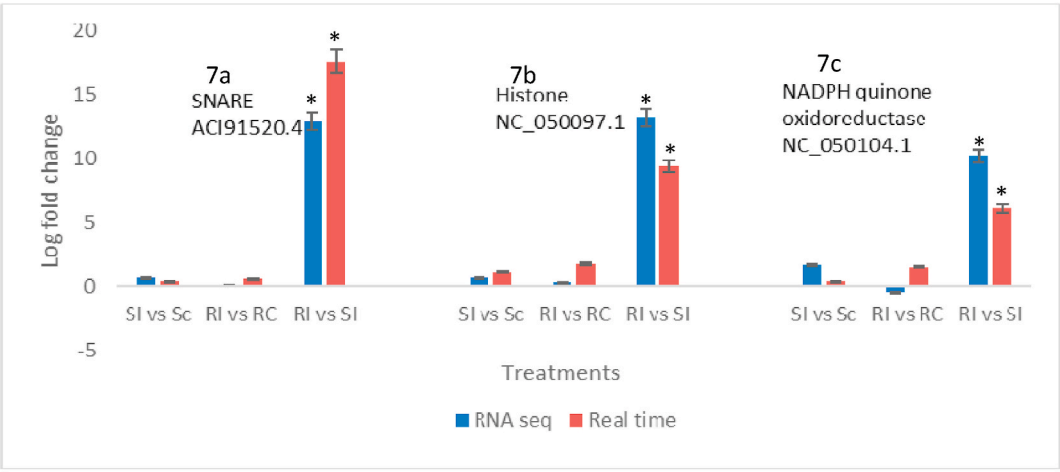


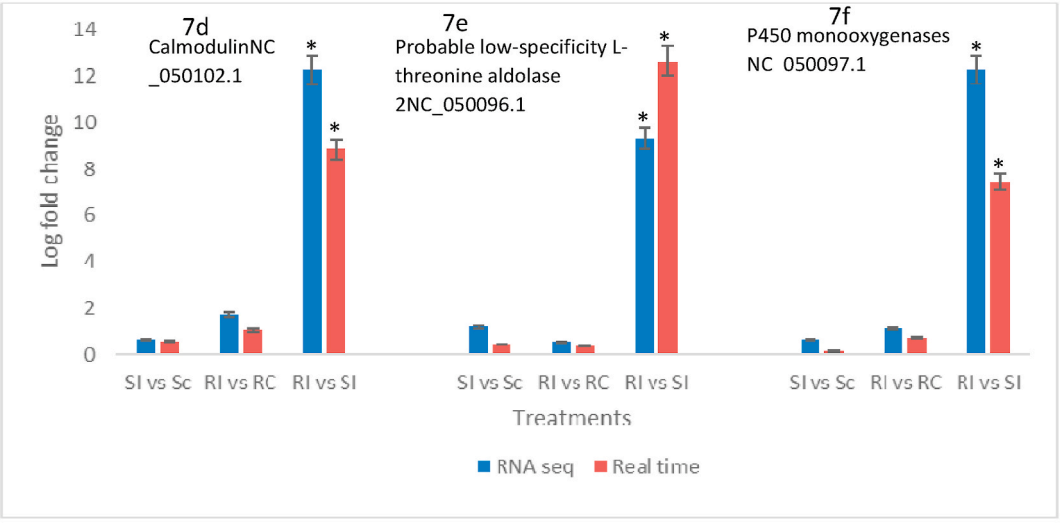
Fig. 6. Summary figure of KEGG pathway enrichment analysis of DEGs in maydis leaf blight susceptible and resistant genotypes of maize, which show up and down regulation of RI vs SI, RI vs RC, and SI vs SC.

resistant plants, indicating their involvement in redox maintenance post-infection [[32,33]]. Implying its potential role in disease resistance by modulating H_2O_2 levels as reported in several studies [[34–36]].

The observed escalated expression of SNARE protein in SC-7 and its absence in susceptible plants suggests its potential role in conferring disease resistance [37]. Studies in wheat have linked SNARE protein to disease resistance, particularly in restricting pathogen penetration [38]. Additionally, SNARE protein is implicated in membrane trafficking and cell signaling in plant immunity [39]. SNARE proteins regulate vesicle trafficking, fortifying cell barriers and restricting pathogen entry. They aid in signaling for defense responses and facilitate the delivery of defense-related molecules, contributing to enhanced disease resistance in plants [40].

On the other hands up regulated genes related to program cell death and senescence regulation such as LCB1 (Long chain base biosynthesis protein 1) regulates pathways associated with programmed cell death, a critical aspect of plant defense against pathogens. It plays a role in signaling cascades that control cell death processes, which can be triggered as a defense mechanism to limit pathogen spread and infection [[41,42]]. Its involvement in PCD pathways and membrane integrity maintenance contributes to effective defense responses, including host and non-host resistance mechanisms [43]. The upregulation of L-threonine aldolase 2 and HMGR in resistant genotypes may link to amino acid metabolism, stress response, and phytoalexin production [44–46]. Elevated expression of calcium-binding proteins and calmodulin in SC-7 signifies robust signaling involved in defense responses against pathogens [47]. Overexpression of ARF's, NB-ARC domain, and WRKY transcription factors in resistant plants suggests their roles in disease resistance mechanisms [[48–52]]. Upregulation of P450 genes in plants aids in synthesizing defense-related compounds and phytohormones, bolstering resistance against pathogens and environmental stresses [53]. Enhanced P450 expression facilitates the production of specialized metabolites, fortifying the plant's defense mechanisms against diverse threats. These enzymes play a crucial role in synthesizing compounds vital for plant resilience and adaptation to changing environmental conditions [54].





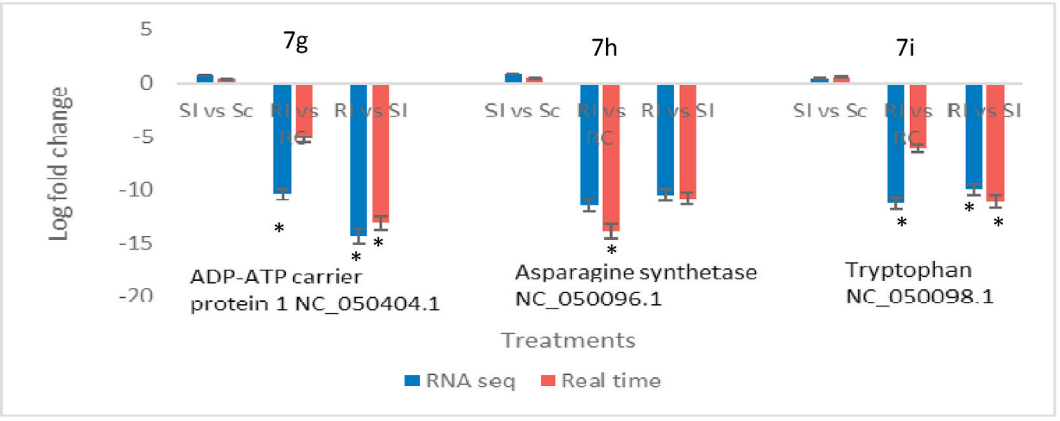


Fig. 7. qRT-PCR validation of the relative expression data of genes obtained in RNA-seq analysis showing consistency. Expression levels of selected transcripts are shown in dark blue (RNA-seq) and maroon (qRT-PCR). y-axis represents LogFC value. The X-axis shows comparisons of the results of the analysis. Error bars show standard deviations for assays. The '*' mark in graphs indicate that expression levels are significantly different between RNA-seq and qRT-PCR (unpaired *t*-test, $P < 0.05$). Accession numbers of genes are mentioned along with the gene name. Correlation coefficient (r value) between RNA-seq and qRT-PCR data, which yielded a value of 1.0.

Fig. 7. (*continued*).

4.1.1. Highly downregulated genes and their functions

Several down-regulated genes including Non-specific lipid-transfer protein 2P, (S)-beta-macrocarpene synthase-like enzyme, and tryptophan aminotransferase-related protein 4 (TrA), among others, are involved in diverse cytological processes, cell wall organization, and secondary metabolite biosynthesis pathways [[28,55,56]]. Their reduced expression in resistant maize plants during pathogen infection suggests potential alterations in biochemical pathways and diversion from essential regulatory processes [57]. The downregulation of these genes in resistant plants might influence defense responses by impacting secondary metabolite synthesis, possibly due to organ-specific gene expression discrepancies [[58,59]]. Moreover, the downregulation of pathogenesis-related proteins (PR proteins) PRB1-2 and Glucan endo-1,3-beta-glucosidase GII in resistant plants could be attributed to the necrotrophic nature of the pathogen *B. maydis* [60]. Differential expression of genes related to ethylene precursor synthesis implies altered ethylene content influencing plant defense response against pathogens [61]. Additionally, the identified genes like O-methyltransferase ZRP4 and glutathione S-transferase have implications in lignin biosynthesis and MLB resistance, suggesting the involvement of defense-related genes in SC-7 against various pathogens [[4,9,62–66]]. These gene expressions provide insights into SC-7's defense gene repertoire against diverse pathogens (Fig. 8).

5. Conclusions

In summary, this study presents the first comprehensive transcriptome analysis of non-CMS maize infected by *B. maydis* race O at the 48-h disease phase, shedding light on defense mechanisms. It deepens our understanding of key genes and pathways underlying resistance in the SC-7 genotype and susceptibility in CM-119. Comparing resistant infected (RI) plants with controls (RC) reveals how SC-7 effectively combats pathogenic invasion. Comparing RI with susceptible infected (SI) plants uncovers significant transcriptional differences, highlighting complex cellular, biochemical, and molecular defense responses. Notable pathways include glycerolipid metabolism, phosphatidylinositol signaling, and plant-pathogen interactions. SC-7 demonstrates a robust plant signaling system, primarily oxidation-reduction and calcium-mediated signaling. It efficiently regulates fitness-related genes, including aldolase 2, isopropanoid, phytohormones, and P450 cytochrome, as shown by DEG expression patterns. Multiple genes contribute to SC-7's resistance, with key DEGs including O-methyltransferase ZRP4, glutathione S-transferase, Thioredoxin H-type (Trx), and NADPH quinone oxidoreductase 1. These findings underscore SC-7's potential for future crop improvement programs.

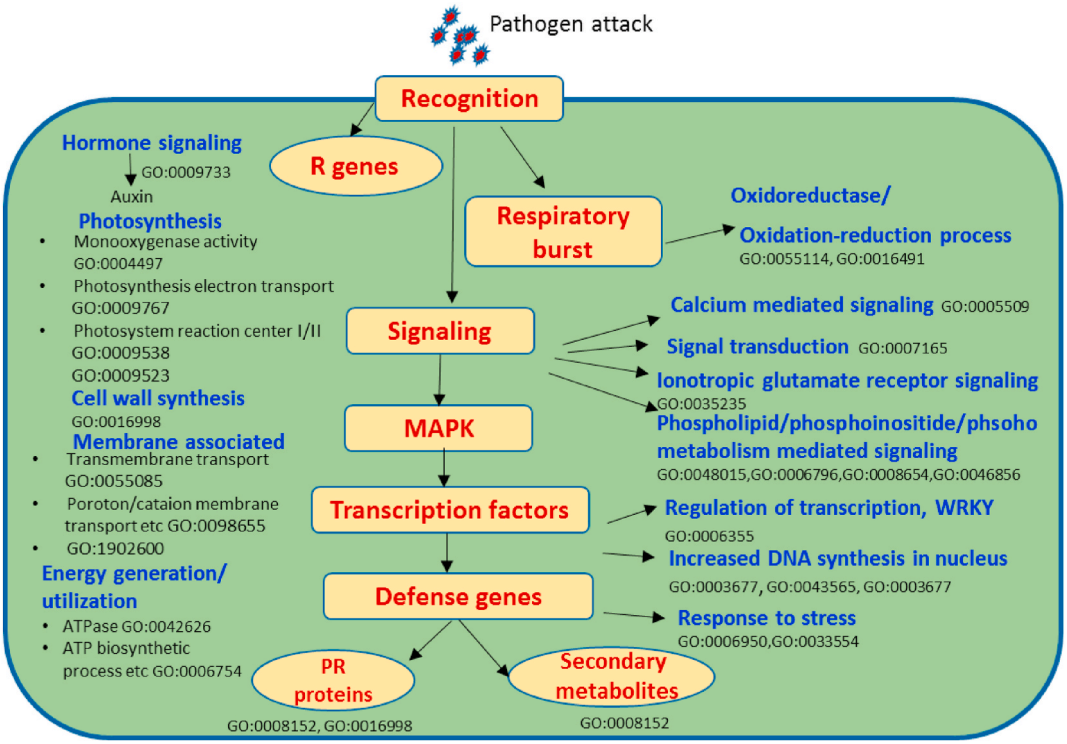


Fig. 8. Putative representation of possible activation of genes and pathways based on DEGs in present study, which makes SC-7 maize genotype excellent robust and resistance against its invading fungal pathogen *Bipolaris maydis* during early stages of infection. GO term and associated gene IDs are provided in additional file 13: S12.

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Availability of data and materials

The datasets analyzed during the current study are available in the repository of NCBI SRA database (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA689117) bio project IDPRJNA689117 with accession number SRX976797, SRX9767976, SRX9767975, SRX9767974, SRX9767973, and SRX9767972.All the other data, including primers and the source of all species studied here, can be found in the article itself and its supplementary data

CRediT authorship contribution statement

Shweta Meshram: Writing – original draft, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Robin Gogoi:** Writing – review & editing, Conceptualization. **Bishnu Maya Bashyal:** Writing – review & editing, Supervision, Investigation. **Pranab Kumar Mandal:** Writing – review & editing, Resources. **Firoz Hossain:** Supervision, Methodology. **Aundy Kumar:** Writing – review & editing, Supervision, Formal analysis.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as no potential competing interests:

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Appendix A. Supplementary data

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