Glutathione imparts stress tolerance against Alternaria brassicicola infection via miRNA mediated gene regulation

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Research note

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Abstract

Objective: Glutathione (GSH) is well-known to play a crucial role in imparting resistance against various pathogen invasions. Nevertheless, the role of GSH in regulating miRNA mediated defence response is yet to be explored. Results: To decipher the GSH mediated regulation of miRNA expression during necrotrophic infected Arabidopsis thaliana, wild-type Col-0 and AtECS1, the transgenic line exhibiting enhanced GSH content, was infected with necrotrophic pathogen Alternaria brassicicola. AtECS1 plants exhibited enhanced resistance as compared to wild-type. MiRNA next generation sequencing (NGS) was performed to compare the miRNA expression in Col-0 and AtECS1 leaves. Under control condition, differentially expressed 96 known miRNAs and 17 novel miRNAs viz. ath-miR8167f, ath-miR1886.3, ath-miR3932b-5p, etc were identified. Whereas, under infected condition, 73 known and 43 novel differentially expressed miRNAs viz. ath-miR5652, ath-miR160b, ath-miR865-5p, etc. were identified. Functional annotation and enrichment analysis revealed that several miRNAs that target defence-related genes like leucine rich repeat protein kinase, MYB transcription factors, TCP8, etc. were down regulated in the AtECS1 line which, in turn, relieves the repression of their target gene expression leading to resistance against infection.Together, the present investigation suggests that GSH plays a decisive role in modulating the miRNA mediated regulation of defence related genes during pathogen invasion.

Introduction

In plant defense signaling network, glutathione (GSH) is gaining massive importance in disease resistance by playing a central role in regulating the defence signaling network in plants. In the earlier studies, it was shown that over-expression of GSH biosynthesis genes and increased GSH level can induce the expression of different disease responsive genes thus imparts stress tolerance [1,2,3]. Earlier, it was shown that GSH deficiency mutant *pad2.1* was susceptible towards pathogen attack [3]. Exogenous application of GSH can also induce disease resistance in plants by activating NPR1 mediated SA signaling pathway, corroborating the regulatory role of GSH during biotic stress responses in plants [1,4]

MicroRNAs (miRNAs) are a group of regulatory molecules with 21–24 nucleotides length and help in gene regulation by sequence-specific cleavage or translational repression of their target gene [5]. Several families of miRNAs have been identified from different plant species during pathogen attack and their disease responsive role has been investigated [6,7,8]. However, GSH mediated regulation of any miRNAs during disease progression in plants is not studied so far.

Alternaria brassicicola is a devastating necrotrophic fungal pathogen that causes dark spot disease in Brassicaceae family [9]. However, the role of GSH in regulating miRNA mediated defense strategies in this pathosystem is still unexplored.

In this study, our aim is to dissect the role of different miRNAs during *A.brassicola* infection in *Arabidopsis* and their regulation by GSH. Our study identified several stress responsive miRNAs that are regulated by GSH during pathogen attack.

Materials And Methods

Plant material and growth condition and pathogen inoculation

The transgenic *A. thaliana* line (*AtECS1)* overexpressing *Le-*γ*ECS* gene (with enhanced GSH content) developed earlier [3], Columbia–0 (Col–0) served as wild-type. Plants were grown in Murashige and Skoog (MS) media and maintained in a growth chamber (22°C under 16 h light/8 h dark cycles)[10].

Spore suspension of *A. brassicicola* strain MTCC No. 2102, obtained from MTCC Chandigarh, India, was used to inoculate leaves following van Wees et al. 2003[11]. For mock treatment, 5 µL droplets of water was used. Infection was scores at 5dpi.

Trypan blue and DAB staining assay

To determine disease severity in Col–0 and *AtECS1* plant, 4weeks old Col–0 (CC), infected Col–0 (CI), *AtECS1* and infected *AtECS*1 (AI) plants were used for trypan blue staining [12]. Infected leaves were put into 1:1(vol/vol) ethanol and lactic acid–phenol–trypan blue solution for 45 min. Leaves were destained and mounted in 60% glycerol for observation.

DAB staining was performed according to Daudi et al. (2012) [13]. Control and infected leaves were put into DAB solution(1mg/ml) for overnight and then leaves were placed in bleaching solution (ethanol: acetic acid: glycerol = 3:1:1) for 5 min in boiling water bath and placed in fresh bleaching solution for 30mins and mounted for microscopic observation.

Small RNA library preparation and sequencing

Leaf from control and infected plants were used for the preparation of small RNA libraries. Total RNA was isolated using TRiZol reagent (Invitrogen, USA). The quality and quantity of RNA were analyzed using a Bioanalyser 2100 (Agilent Technologies) and small RNA library prepared using a small RNA Sample preparation Kit (Illumina Technologies). The small RNA libraries were sequenced using a HiseqIllumina 1.5. The sequence data obtained as FASTQ files were assessed for qualitative analysis using FastQC version 3 and Fastx-toolkit, version 0.0.13.

Data mining and identification of miRNAs

The raw sequences generated were trimmed for removal of adaptor/primer contamination and poly(A) tails using an miRDeep adaptor filter. The pre-processed data were cleaned off including low quality reads. The unique reads (17–23 nucleotide) from two samples were retained for genome mapping. The filtered reads were screened against non-coding RNA sequences, tRNA, rRNA, and chloroplast sequences found in the *Arabidopsis* genome database. The reads homologous to these sequences were discarded.

The miRDeep2 module was used to identify known and novel miRNAs in the high-throughput sequencing data.

Target gene prediction of novel miRNAs

EdgeR (R package, version 3.8.3) software was used to analyse the differential gene expression. The target gene was predicted using the psRNATarget server (http://plantgrn.noble.org/psRNATarget/) with default parameter [14,15] and penalty score ≤2.5.

Validation of miRNA expression and their corresponding target genes by qRT–PCR

Several putative novel and known miRNAs and their corresponding target genes were selected for qRT–PCR analysis. Isolation of miRNA was carried out using manufacturer's protocol (Favorgen, Taiwan). The stem–loop and forward primers for the selected miRNAs were designed using miRNA primer designer software. Experiments were performed in three biological replicas.

Gene expression analysis of the predicted target genes were performed by qRT-PCR analysis in three biological replicas. The constitutively expressed elongation factor 1α gene was used as the reference gene. The primer pairs used is listed in Table S1.

Functional enrichment and annotation

Functional enrichment of differentially expressed miRNAs and their target genes was analyzed by singular enrichment analysis (SEA) with the agriGO tool [16]. SEA analysis computes GO term enrichment in the selected set of genes by comparing it to the reference set (in this case, TAIR genome locus TAIR10_2017). Fisher test and Benjamini-Yekutieli method is used here.

Results

Morphological analysis after A. brassicicola infection

A. brassicicola infected leaves showed dark necrotic spots. The Col–0 plants exhibited more susceptibility to infection compared to *AtECS1* plants suggesting that GSH plays a crucial role in imparting resistance in the *AtECS1* plants against *A.brassicicola* infection.

Cell viability assessment and H_2O_2 detection after A. brassicicola infection

Trypan blue staining of Col–0 and *AtECS1* plants infected with *A. brassicicola* revealed significantly more cell death in infected Col–0 than *AtECS1* at 5dpi (Fig. 1a) In DAB staining assay, the H_2O_2 accumulation in infected Col–0 leaves was also significantly higher than infected *AtECS1* which once again indicates the role of GSH in making the plant more resistant against *A. brassicicola* infection (Fig. 1b).

Identification of differentially expressed miRNA

Sequencing of the CC, AC, CI and AI leaf libraries generated a total of 507877, 391126, 179265 and 500809 sequence reads respectively provided in Table S2. After removal of low quality sequence reads and adaptor contaminants, distinct sequences obtained were perfectly matched with the *Arabidopsis* genome. The differences in size compared with the total small RNA population were determined on the basis of total abundance reads. The read count of mature sequences of known and novel miRNAs of CC, AC, CI and AI have been included in Table S3,S4 and MA-Plot diagram of the differential expression of miRNA in Col–0 and *AtECS1* are given in Fig. 2. The mature sequences of novel miRNA found in all the 4 set are included in Table S5. A total of 96 differentially expressed known and 17 novel miRNAs were identified in case of CC vs AC and in case of CI vs AI, the total number of differentially expressed known miRNAs was 73 while the number of differentially expressed novel miRNAs identified was 43 provided in Table S6.

Validation of selected differentially expressed miRNAs and their target genes

Few miRNAs like miR5645f, miR858a, miR3434–5p, miRTCP8 expression data obtained from NGS analysis were confirmed through qRT-PCR analysis for several selected genes viz. *AGD2, myb65, LRRK, TCP8* resp. and the results indicated that the miRNAs were significantly differentially regulated in accordance with NGS results (Fig. 3).

Functional enrichment and annotation

As observed by singular enrichment analysis by agriGo, the most significantly enhanced biological process for CC vs AC was regulation of transcription, followed by innate immune response, cellular response to stress etc. In case of known miRNAs and in case of novel miRNAs, the most significantly enhanced biological process was defense response followed by response to stress (Table S7). In case of CI vs AI most significant enhanced biological process was response to stimulus, metabolic process, transcription regulatory activity, immune system process ect. (Table S8).

Discussion

A.brassicicola infection has detrimental effects on plants. However, plant responses against this infection is very less explored. *Esa1,* an *Arabidopsis* mutant with enhanced susceptibility to *A.brassicicola* showed

a distorted induction of defence responses by generating ROS [17]. Deficiency in phytoalexin production also showed enhanced susceptibility of *planta* to *A.brassicicola [*18]. Thus, previous studies on the *A.brassicicola-Arabidopsis* pathosystem can be explored further.

Additionally, the enhanced level of GSH imparts significant tolerance to environmental stress [19,20,21,22,23,24] and *Nicotiana tabacum* overexpressing *γ-ECS,* exhibited biotic stress tolerance likely through NPR1-dependent salicylic acid-mediated pathway [1].Present study showed that the *A.thaliana* overexpressing *γ-ECS* gene viz. *AtECS1* showed increased resistance to *A.brassicicola* infection depicting the important role of glutathione in conferring resistance to *planta* during biotic stress.

The discovery of miRNAs has led to a paradigm shift in understanding the post-transcriptional regulation of a gene in plants. Recent studies revealed that many miRNAs could be responsive to ethylene and play significant roles in plant growth and development [25,26]. However, the direct role of miRNAs in controlling dynamic molecules like GSH is poorly understood. Present study focuses on the identification of miRNAs under altered GSH content in *A.Brassicicola* infected *A. thaliana.*

To identify the GSH responsive miRNAs under biotic stress, we constructed 4 miRNA leaf library from *A.brassicicola* infected *A.thaliana* exhibiting altered GSH content. Deep sequencing of miRNAs revealed 96 known and 17 novel ones in CC and AC. Whereas, 73 known and 43 novel miRNAs in CI and AI. In control samples, various known miRNAs playing diverse roles in biological processes like stress-responsive miRNAs like miR858a[27], hormonal signalling pathways like miR5652, miR396a–3p [15,28] have been identified. In infected samples, various biotic stress responsive miRNAs like miR3932b–5p, miR5645f [29] have also been identified. Most of the miRNAs were found to be down-regulated in AC and AI with respect to CC and CI.

Furthermore, 17 putative novel miRNAs were identified in CC vs AC. Out of this 17 novel miRNAs, 8 miRNAs were up-regulated and 9 were down-regulated. In CI vs AI samples, out of 43 putative novel miRNAs identified, 16 were down-regulated and the rest were up-regulated.

Among the 96 known miRNAs in control samples of *Arabidopsis,* approximately 199 targets were predicted and of the 17 novel miRNAs, almost 30 targets were predicted. Out of these predicted target genes, some were found to be functionally involved in defense response and in case of infected *A. thaliana* having altered GSH level, the number of target gene found for known miRNAs were 307 and target genes for potential novel miRNAs were 68.These 68 target genes were found to be involved in multiple physiological and metabolism processes (Table S6). The expression profile from NGS data was validated by qRT-PCR. Few miRNAs and their target genes used are miR5645f-AGD2, miR858a-myb65, miR3434–5p-LRRK, and miRTCP8-TCP8. Result revealed that the miRNAs were significantly down-regulated and their respective target genes were up-regulated in *AtECS1.*

In order to better understand the potential role of miRNAs in *A.thaliana* functional enrichment and annotation analysis was done by agriGO. Based on this analysis, many significant enriched GO terms were RNA biosynthetic process(GO:0032774), transcriptional regulation(GO:0006355) in control samples

of *Arabidopsis.* The significant GO terms in infected category were regulation of transcription(GO:0006355), regulation of response to biotic stimulus(GO:0009607), response to stress(GO:0006950), primary metabolic processes(GO:0044238), response to stimulus(GO:0050896) etc.

Hence, in presence of biotic stress, the miRNAs that were found to be down-regulated in response to GSH induced the expression of its respective target genes mainly *LRRK, myb65, AGD2 and TCP8* which are involved defence response and developmental processes. Cumulatively, our findings suggest that under elevated GSH level in plants during pathogen attack, miRNAs regulate the stress-related genes and help the plant in combating stress.

Limitations

MicroRNA family are species specific and also stress specific so as our finding deals with *Alternaria brassicicola* infected *A. thaliana,* identification of miRNA in *Alternaria brassicicola* infected condition on others plant species should be evaluated carefully.

Additional Files

Table S1. The primers of miRNAs and target genes used for qRT-PCR verification.

Table S2. Processed data read count

Table S3. Total Known miRNAs read Count

Table S4. Total Novel miRNAs read Count

Table S5. All Novel miRNA and sequence

Table S6 A) Differentially expressed miRNA and their regulation under AtECS1 Control (AC) vs Col–0 Control (CC) B) Differentially expressed miRNA and their regulation under AtECS1 Infected(AI)vsCol–0 Infected(CI) C) Target genes predicted for known miRNAs down regulated in AtECS1 plants under control condition (AC vs CC) D) Target genes predicted for novel miRNAs down regulated in AtECS1 plants under control condition (AC vs CC) E) Target genes predicted for known miRNAs down regulated in AtECS1 plants under infection condition (AI vs CI) F) Target genes predicted for Novel miRNAs down regulated in AtECS1 plants under infection condition (AI vs CI)

Table S7. Enriched GO terms of target gene predicted for down and up regulated miRNA in *AtECS1* Control vs Col–0 Control

Table S8. Enriched GO terms of target gene predicted for down regulated miRNA in AtECS1 infected vs Col–0 infected

Additional file 9. Cover Letter

Abbreviations

GSH: Glutathione; miRNA/miR: MicroRNA; NGS: Next Generation Sequencing; Col–0: Columbia–0; *AtECS1: Arabidopsis thaliana ECS1;* ath-miR: *arabidopsis thaliana*-miR; PTI: Pathogen Associated Molecular Patterns Triggered Immunity; ETI: Effector Triggered Immunity; *pad2.1: phytoalexin deficient2.1;* NPR1: Non-expressor of pathogenesis-related genes 1; SA: Salicylic Acid; *Le-ECS: Lycopersicum esculentum-ECS;* MS: Murashige and Skoog; MTCC: The Microbial Type Culture Collection and Gene Bank; DAB: 3,3'-Diaminobenzidine; tRNA: transferRNA; rRNA: ribisomalRNA; qRT-PCR:quantitative Reverse Transcriptase-PCR; cDNA: complementaryDNA; SEA: Singular Enrichment Analysis; TAIR: The *Arabidopsis* Information Resource; H_2O_2:Hydrogen Peroxide; AGD2: Aberrant Growth and Death2; LRRK: Leucine Rich Repeat Kinase; JA: Jasmonic Acid; ABA: Abscisic Acid; dpi: days post infection; *Esa1:Enhanced susceptibility to Alternaria1;* SNF kinase: Sucrose Non Fermenting Kinase; MAPK15: Mitogen Activated Protein Kinase15

Declarations

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Availability of data and material

The data described in this data note can be freely and openly accessed on the Sequence Read Archive (SRA) with Project ID GSE134863 in NCBI database and are also available from the corresponding author on reasonable request.

Authors' contributions

RD and SC designed the experiments. RD carried out the major experimental work, PB and KM did further experiments and analyzed the data. AS help to analyse data. RD drafted the manuscript. SC supervised

the analysis and prepared the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

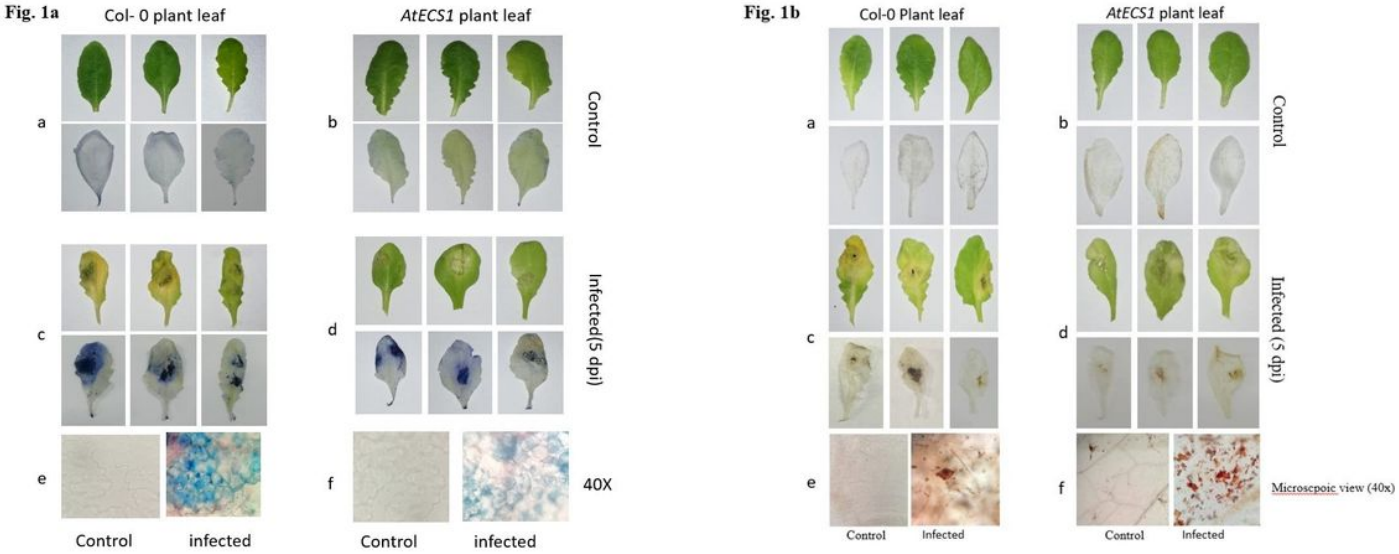

Figure 1

1a Trypan blue detection of necrotic cell in Col-0 and AtECS1 leaves after Alternaria brassicicola infection. a and b are controls (treated with ddH2O). No necrosis in leaf of Col-0 a and AtECS1 b at 5dpi.c and d at 5dpi with A.brassicicola. c Shows large necrotic area compare with d. e Microscopic view(40x) of control and infected leaf of Col-0. f microscopic view(40x) of control and infected leaf of AtECS1 1b Accumulation of H2O2in infected leaf by DAB staining in Col-0 and AtECS1. a and bare controls (treated with ddH2O).c and d at 5dpi with A.brassicicola. e microscopic view(40x) of Col-0 leaf (control and infected). f microscopic view(40x) ofAtECS1 leaf (control and infected)

Fig. 2

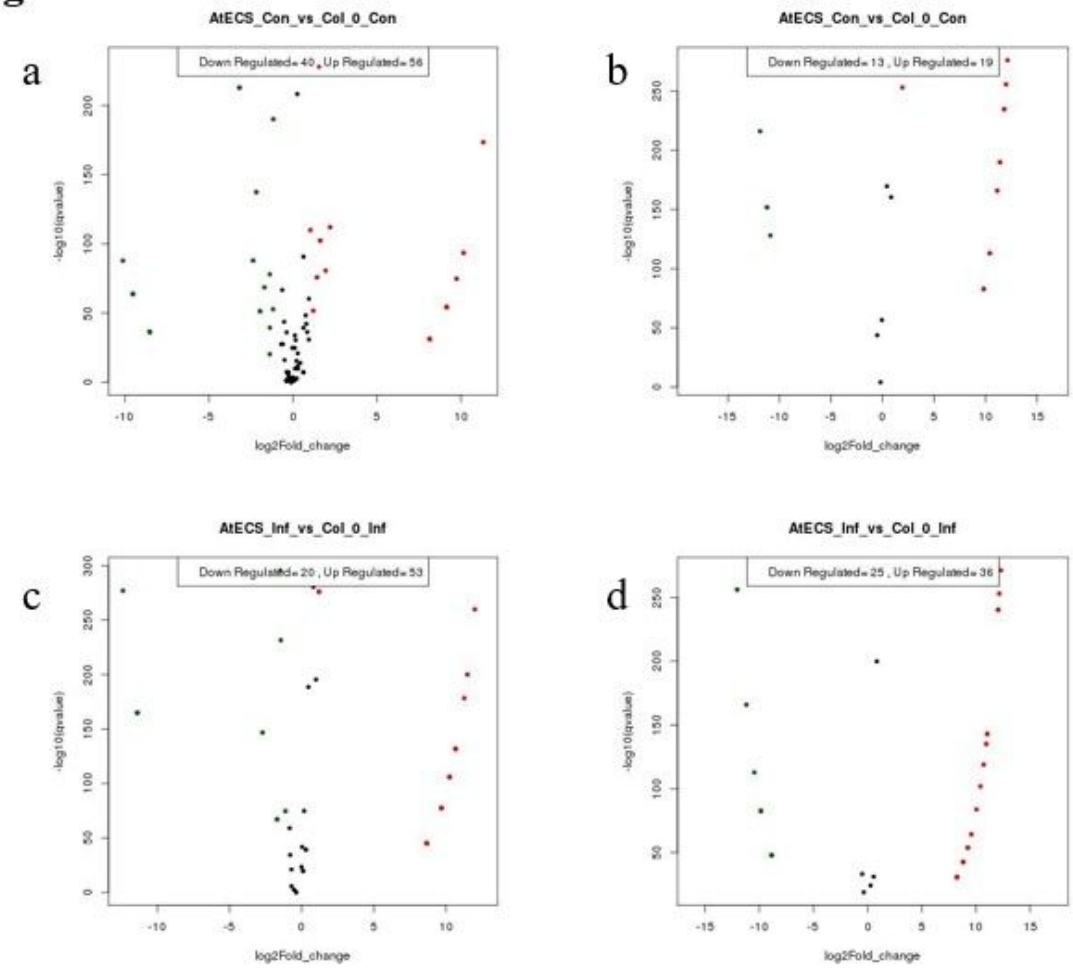

Figure 2

MA-Plot diagram of the differential expression of miRNA in Col-0 and AtECS1. In red and green miRNAs differentially expressed with padj<0.05; a Known miRNA (AtECS1 Control vs Col-0 Control). b Novel miRNA (AtECS1 Control vs Col-0 Control). c Known miRNA (AtECS1 Infected vs Col-0 Infected). d Novel miRNA (AtECS1 Infected vs Col-0 Infected)

Fig. 3

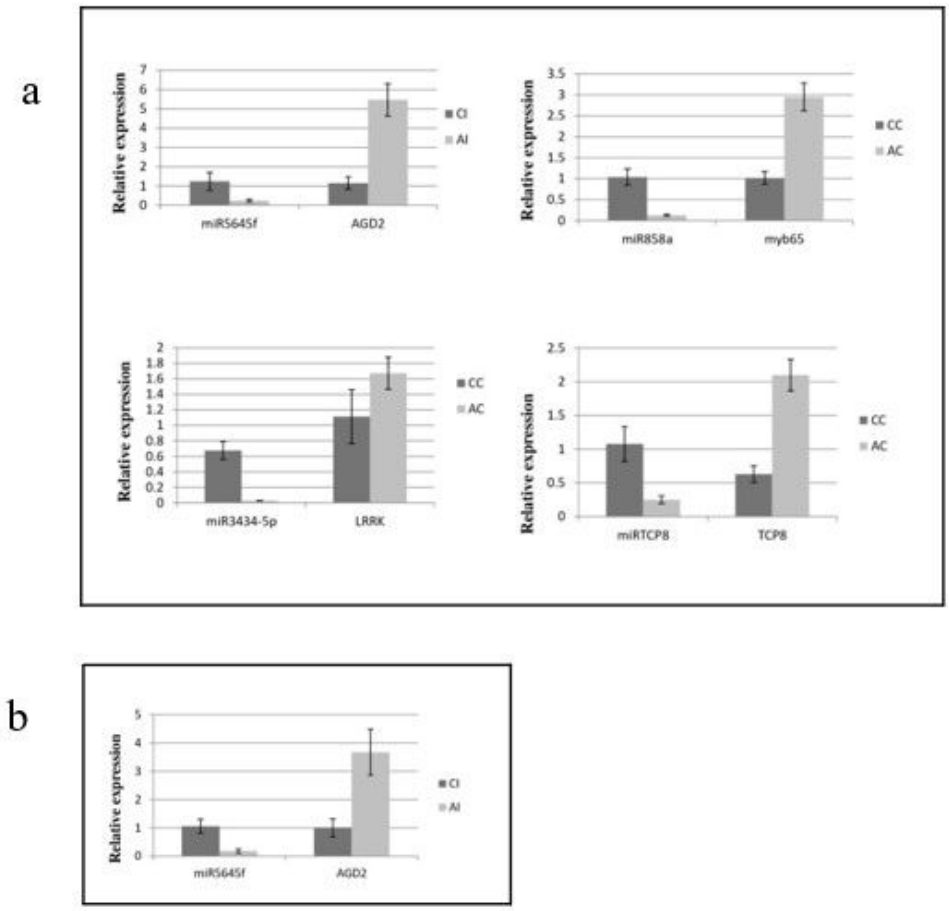

Figure 3

Validation of differentially expressed known and novel miRNA and their respective target gene by qRT-PCR. U6 RNA gene was use as reference gene for miRNA and EF-1 alpha for target gene. Data are mean ± SD from three biological replicates determines by Student t test. a miRNA and their respective target under AtECS1 Control vs Col-0 Control .b miRNA and their respective target under AtECS1 Infected vs Col-0 Infected

Supplementary Files

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- TableS2.xls