The long non-coding RNA GhlncRNA149.1 improves cotton defense response to aphid damage as a positive regulator

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Abstract

Long non-coding RNAs (lncRNAs) play crucial roles in plant various biological regulatory processes and plant defense response to all sorts of the biotic and abiotic stresses. However, studies on the functions and mechanisms of lncRNAs are still been scarely reported in plant defense response to the damage of phytophagous insects. Here, *GhlncRNA149.1*, a lncRNA from *Gossypium hirsutum*, was shown to be induced by *Aphis gossypii*, methyl jasmonate and salicylic acid. Overexpression of *GhlncRNA149.1* in cotton plants improved their defense response to the damage of *Aphis gossypii*, while silence of *GhlncRNA149.1* in cotton plants reduced their defense response to the damage of *Aphis gossypii*. The target gene *GhA01G0129* of *GhlncRNA149.1* was also induced by *Aphis gossypii*, methyl jasmonate, salicylic acid and it was highly expressed in cotton plants overexpressing *GhlncRNA149.1*, while lowly expressed in cotton plants silencing *GhlncRNA149.1*. The activity and expression of antioxidant enzymes superoxide dismutase(*SOD*), peroxidase(*POD*) and catalase(*CAT*) were up-regulated in transient overexpression *GhlncRNA149.1* cotton plants, and their expression levels were down-regulated in *GhlncRNA149.1* silenced cotton plants. In addition, the expression levels of Phenylalanine ammonialyase(*PAL*), Polyphenol oxidase(*PPO*), Enhanced disease susceptibility 1 (*EDS1*)and Nonexpressor of pathogenesis-related genes 1(*NPR1*) were up-regulated in cotton plants transiently overexpressing *GhlncRNA149.1*. These results collectively suggest that *GhlncRNA149.1* improve the plant defense response to aphid attack, and thus has potential for enforing cotton aphid prevention and control.

Key Message

Cotton aphid is a worldwide cotton pest. In order to promote the development of aphid resistant cotton, the aphid resistance of *GhlncRNA149.1* was studied.

Introduction

Long non-coding RNAs (lncRNAs) are a class of poorly conserved RNAs longer than 200 nucleotides and short of protein encoding capability (Mercer et al. 2009). Most lncRNAs are transcribed by RNA polymerase II. However, some lncRNAs are transcribed by RNA polymerase III, and a small number of lncRNAs in plants are produced by plant-specific RNA polymerases IV and V (Wu et al. 2020). According to the localization of lncRNAs with reference to adjacent protein-coding genes in the genome, lncRNAs are divided into five types: sense lncRNA, antisense lncRNA, bidirectional lncRNA, intronic lncRNA (incRNA), and large intergenic lncRNA (lincRNA) (Ponting et al. 2009). LncRNAs may interact with many large molecules, such as DNA, RNA, and proteins, and regulate protein modification, chromatin remodeling, protein functional activity, and RNA metabolism in vivo at the transcriptional, post-transcriptional and epigenetic levels (Ma and Yan 2019). The lncRNA action mechanisms have cis- and trans-associated functions. Cis-acting lncRNAs are located in upstream or downstream of the encoded protein and interplays with cis-acting elements of the promoter or co-expressed genes, thereby modulating gene expression at the transcriptional or post-transcriptional level. Trans-acting lncRNAs may

act as signals, guides or scaffolds for the chromatin to mediate the expression of target genes located in distant chromosomal domains or even in different chromosomes (Zhang et al. 2019). Up to now, the regulatory roles of lncRNAs have been broadly studied in yeast and mammals, but much less is known about their roles in plants (Li et al. 2021).

With the rapid development of sequencing technology, the functions and mechanisms of plant lncRNAs have attracted more and more attention. Some studies have revealed that lncRNAs are broadly involved in plant growth and development. For example, the lncRNA *APOLO* (*AUXIN-REGULATED PROMOTER LOOP*) is transcriptionally regulated by auxin and modulates later root development through mediating the formation of a chromatin loop (R-loop) encompassing the promoter of its neighboring gene *PINOID* (*PID*) and suppressing the *PID* transcript (Amor et al. 2009; Ariel et al. 2014). A lncRNA [termed COLD ASSISTED INTRONIC NONCODING RNA (COLDAIR)] recruits the protein complex, polycomb repressive complex 2 (PRC2) to activate tri-methylated histone H3 Lys^{27} to maintain the low-level expression of the flowering repressor, *FLOWERING LOCUS C* (*FLC*) in *Arabidopsis thaliana*, while lncRNA COOLAIR involves alternative splicing (AS) and can indirectly inhibit *FLC* expression through transcription interference (Heo and Sung 2011; Swiezewski et al. 2009). More importantly, overexpressing lncRNA *LAIR* [*LRK* (leucine-rich repeat receptor kinase) *Antisense Intergenic RNA*] transcribed from the antisense strand of the neighbouring gene *LRK* cluster increases grain yield and regulates neighbouring gene cluster expression in rice and it may be useful targets for crop breeding (Wang et al. 2018). Secondly, Some studies have also proved that lncRNA is involved in plant response to all kinds of abiotic stress. For instance, The lncRNA *APOLO* interacts with the transcription factor WRKY42 to trigger root hair cell expansion in response to cold (Moison et al. 2021). Potato central clock output transcription factor CYCLING DOF FACTOR 1 (*StCDF1*) and a long non-coding RNA (lncRNA) counterpart, named *StFLORE* also link tuber development and drought response. Both natural and CRISPR-Cas9 mutations in the *StFLORE* transcript produce plants with increased sensitivity to water-limiting conditions. Conversely, regulating expression of *StFLORE*, both by the overexpression of *StFLORE* or by the downregulation of *StCDF1*, results in an increased tolerance to drought through reducing water loss (Ramírez et al. 2021). Long noncoding RNA lncRNA354 that was localized at the nucleus and cytoplasm in cotton functions as a competing endogenous RNA of miR160b to regulate the auxin response factor gene *ARF* in response to salt stress in upland cotton (Zhang et al. 2021). LncRNA973 from cotton was localized in the nucleus and its expression was increased by salt treatment, the lncRNA973-overexpression lines had increased salt tolerance compared with the wild type, while the lncRNA973 knockdown plants had reduced salt tolerance (Zhang et al. 2019). MSTRG.85814.11, a long non-coding RNA from *Malus domestica* (apple), positively promotes the small auxin upregulated gene *SAUR32* expression as a transcriptional enhancer of *SAUR32* which then activate proton extrusion involved in the Fe-deficiency response and contributes to the Fe-deficiency response (Sun et al. 2020). Additionally, plant lncRNAs also important in responding to all sorts of biological stress. For example, Sl-lncRNA15492 from tomato (*Solanum lycopersicum* Zaofen No. 2) plants, as a positive regulator, interacts with Sl-miR482a (miRNAs) and affects *Solanum lycopersicum* immunity against *Phytophthora infestans* (Jiang et al. 2020). LncRNA39026 from tomato Zaofen No. 2 might function to decoy miR168a (miRNAs) and affect the expression of pathogenesis-

related (*PR*) genes in tomato plants, increasing resistance to disease (Hou et al. 2020). However, in contrast to the studies of lncRNA functions in plants responding to pathgens, herbivore-responsive lncRNAs in plants are poorly investigated (Wang et al. 2021).

As an economically important crop, cotton (*Gossypium* spp.) produces a natural renewable fiber for the textile industry, provides edible protein for livestock feed and is a source of oil and biofuel. However, the damage from *Aphis gossypii* are often challenged to cotton production. It is an environmentally friendly way using plant resistance to control cotton aphids. Therefore, it is imperative to study the functions and mechanisms of aphid resistance genes in cotton and cultivate aphid resistant varieties. In our previous study, we identified differentially expressed lncRNAs in cotton leaves damaged by *Aphis gossypii* (Zhang et al. 2019). And it was found that the *lncRNA149.1* is 740 bp long and that its expression level changes in cotton plants after the damage of *Aphis gossypii*. Therefore, we speculate that *GhlncRNA149.1* may affect cotton immunity against *Aphis gossypii*. On that basis, we analyze the functions and mechanism of *GhlncRNA149.1* in cotton response to the damage of *Aphis gossypii*. Our results will not only elucidate *the GhlncRNA149.1* function and molecular mechanisms in cotton response to aphid damage but also will also provide candidates for plant aphid-resistance breeding.

Materials And Methods

Materials and growth conditions

Cotton variety Zhongjixin No.7 (*Gosisyphim hirsutum*) was provided by Mr. Dingguo Li from Yangtze University. The seeds were soaked in sterile water for 24 h till the radicle broken through the seed coat, and they were sown directly in the 25-cm-diameter plastic pots filled with the nutrient soil (a mixture of vermiculite, plant ash and soil in a ratio of 1 : 1 : 2). The pots were put into in a culture chamber (25 °C, 16 h/8 h light/dark cycle and 70% relative humidity). The seedlings with 2 cotyledons or 4 true leaves were used in this experiment. *Aphis gossypii* used in this experiment was collected from the laboratory cotton fields in Yangtze University. They were inoculated on Zhongjixin No.7 and reared in the culture room (25 °C, 16 h light / 8 h dark cycles and 70% relative humidity).

Cotton aphid and hormone treatment

Cotton plants at the 14 th d after sowing were used in the experiment. 50 cotton aphid adults were inoculated on the true leaves of cotton plants. Cotton plants without cotton aphid damage were used as CK (contrast). At 24 h, 48 h and 72 h after aphid feeding, the true leaves, stems and roots from cotton plants with cotton aphid damage and without cotton aphid damage were collected to extract their total RNAs using Spectrum^{TM} plant total RNA kit (Sigma-Aldrich, USA). Moreover, salicylic acid (5 Mm)、Methyl jasmonate (0.5 Mm) and sterile water (CK) were respectively sprayed onto cotton plants until their liquid drops dropped. Each plants was covered with transparent plastic bags and removed after 6 hours. At 24 h, 48 h and 72 h after spraying, cotton leaves at each stage were collected for the extraction of cotton total RNA using the Spectrum^{TM} Plant Total RNA Kit.

Vector construction and transformation

Cotton gene *GhlncRNA149.1* was ligated into the EcoR I and Sma I sites of the overexpression vector pBI121 with 35S promoter of Cauliflower Mosaic virus and the vector pTRV2 used for virus induced gene silence (VIGS) respectively. The vectors including pBI121-*GhlncRNA149.1,* pBI121, pTRV2-*GhlncRNA149.1,* pTRV2 and pTRV1 was transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. *Agrobacterium* liquids containing pBI121-*GhlncRNA149.1* and pBI121 were injected into cotton seedlings at 7th d after sowing by the micro-injection method respectively to generate pBI121-*GhlncRNA149.1* transgenic cotton plants (pBI121-*GhlncRNA149.1*) and pBI121 transgenic cotton plants (pBI121). Total RNA was extracted from the cotton cotyledons at 24 h, 48 h and 72 h after injection of *Agrobacterium* liquids and then reverse transcribed into cDNA to be used as a template for qPCR to detect the transformated effciency. Similarly, the *Agrobacterium* liquids containing pTRV2-*GhlncRNA149.1* and the *Agrobacterium* liquids containing pTRV1, the *Agrobacterium* liquids containing pTRV2 and the *Agrobacterium* liquids containing pTRV1 were mixed at the same ratio respectively and injected into cotton seedlings at 7th d after sowing by the micro-injection method respectively to format the TRV:*GhlncRNA149.1* transgenic cotton plants (TRV:*GhlncRNA149.1*) and TRV:00 transgenic cotton plants (TRV:00). Total RNA was extracted from cotton cotyledons（WT, TRV:00, TRV:*GhlncRNA149.1*）at 8 d, 15 d and 22 d after injection and then reverse transcribed into cDNA to be used as a template for qPCR.

Analysis of *GhlncRNA149.1* overexpressed cotton plant resistance to cotton aphids

The choice experiment was carried out in a 15-cm-diameter petri dish. The cotton leaves (WT, pBI121 and pBI121-*GhlncRNA149.1*) were selected at 24 h after injection and placed symmetrically on the inner edge of the culture dish. Ten adult aphids were selected and placed in the middle of the culture dish. The number of aphids on each leaf was investigated at 24 h, 48 h and 72 h after the feeding of cotton aphids. The no-choice was carried out in the same petri dish. The cotton leaves (WT, pBI121 or pBI121-*GhlncRNA149.1*) were selected at 24 h after injection and placed in the middle of the culture dish. Ten adult aphids were selected and placed on the middle of cotton leaf. The number of aphids on each leaf was counted at 24 h, 48 h and 72 h after the feeding of cotton aphids. The petioles of cotton leaves in all the experiments were wrapped with cotton balls soaked with MS nutrient solution, and pBI121 and WT plants were used as control, Each treatment repeated 4 times.

Analysis of *GhlncRNA149.1* silenced cotton plant resistance to cotton aphids

The choice experiment was performed in a 15-cm-diameter petri dish. The cotton leaves (WT, TRV:00 and TRV:*GhlncRNA149.1*) were selected at 15 d after injection and placed symmetrically on the inner edge of the culture dish. Ten adult aphids were selected and placed in the middle of the culture dish. The total number of cotton aphids on the cotton leaves was investigated at 24 h, 48 h and 72 h, respectively. The no-choice assay was carried out in the same petri dish. The cotton leaves (WT, TRV:00 or TRV:*GhlncRNA149.1*) were selected at 15 days after injection and placed in the middle of the culture dish. Ten adult aphids were selected and placed on the middle of cotton leaf. The number of aphids on each leaf was investigated at 24 h, 48 h and 72 h after the feeding of cotton aphids. The petioles of cotton

leaves were wrapped with cotton balls were soaked with MS nutrient solution in all the experments, and TRV:00 and WT plants were used as control. Each treatment was repeated 4 times.

Quantification of Cotton Aphid Excreted Honeydew

The content of aphid honeydew was measured by ninhydrin staining. Cotton plants (WT, pBI121, pBI121-*GhlncRNA149.1* , TRV:00, TRV:*GhlncRNA149.1*) were used in the experiment. At 24 h (WT, pBI121, pBI121-*GhlncRNA149.1*) or 15 d (WT, TRV:00, TRV:*GhlncRNA149.1*) after injecting the bacterial solution, the front of the isolated leaves were pasted on the prepared Agar medium plate, and 10 cotton aphid adults were inoculated on the leaves. The plates were placed upside down on Whatman filter paper and cultured in an incubator. At 1 d, 2 d and 3 d after feeding, the filter paper was collected under the isolated leaves. Each treatment was repeated for 3 times. The collected filter paper was immersed in 0.1 % (W/V) acetone solution of ninhydrin, and dried in the oven at 65 ℃ for 30 min until the honeydew on the filter paper showed purple spots, and the amount of honeydew of cotton aphids was measured (Kim et al. 2007). Moreover, the filter paper was torn into pieces, and stained in 1 mL 90% (V/V) methanol solution at 4℃ for 1 hour, during which the filter paper was continuously stirred. After centrifugation at 5000 g for 60 s, the supernatant absorbance at 500 nm was determined and 90% methanol was used as blank control (Nisbet et al. 1994).

Determination of *POD*, *CAT* and *SOD* activity

Cotton leaves (WT, pBI121 and pBI121-*GhlncRNA149.1*) at 48 h after the damage of *Aphis gossypii* were ground to a fine powder in the mortar placed on ice with pestle and were used to detect the activities of ROS-related enzyme including *SOD* , *POD* and *CAT* . The *POD*, *CAT* and *SOD* activity were determined according to kit instructions (Suzhou Comin Biotechnology Co. Ltd., Jiangsu, China).

Expression analysis of defense pathway related genes in transient overexpressing lncRNA149.1 cotton plants

At 24 h after cotton leaves were injected by injection, total RNA was extracted from cotton leaves(WT, pBI121, pBI121-*GhlncRNA149.1*), and then reverse transcripted into cDNA, which was used as a template for qPCR in subsequent experiments. qPCR was used to detect the expression levels of defense pathway related genes including *POD, CAT, SOD, PAL,PPO, EDS1* and *NPR1* in cotton plants.

RNA isolation and quantitative real-time PCR (qPCR)

Total RNA was extracted from cotton roots, leaves, and stems using a Fast pure plant total RNA isolation kit (Vazyme, Nanjing, China) according to the manufacturer's instructions. Total RNA from each sample was treated with RNase free DNaseI (Promega, USA) and was reverse transcribed into cDNA with transcriptase (Promega, USA), which was used as a template for quantitative real-time PCR (qPCR). The cotton gene *GhUBI1* (GenBank accession number: EU604080) served as an internal control. The *GhUBI1* primer sequences is shown in Table 1**.** The relative value of each gene was showed as mean values of four independent tests, and three replicates were performed for each test.

Statistical analysis

The difference between each treatment and control was statistically analyzed with a *t*-test for independent samples. Two significance levels were used (*, $P<0.05$ and **, $P<0.01$), three significance levels were used (different letters, $P<0.05$), The statistical significance of the difference among treatments was analyzed using one-way ANOVA. The data was analyzed using statistical software SPSS 26.0 (SPSS Inc., Chicago, IL, United States of America). Statistical significance was considered when the p-value was less than 0.05.

Result

Identification of *GhlncRNA149.1* and its target *GhA01G0129* in cotton

In our previous study, the whole transcriptome sequencing was performed on cotton leaves induced by cotton aphids, and the lncRNAs that were induced significantly by the cotton aphid feeding were identified (Zhang et al. 2019). Among these lncRNAs, *GhlncRNA149.1* was chosen for further study because its target genes were specific. *GhlncRNA149.1* is 740 bp in length and was located on the sense chain of chromosome 1 in the A genome of *Gossypium hirsutum*. A bioinformatic analysis (http://cpc2.gao-lab.org/) revealed that *GhlncRNA149.1* got Fickett score 0.35472 with a pI 9.69085693359. Therefore, it was classified as a noncoding sequence with coding probability 0.0117256 (Fig. 1). The ORF Finder online tool was used to predict the *GhlncRNA149.1* ORFs that ranged from 26 to 31 amino acids. Furthermore, according to homology searches of large protein families and domain databases (Pfam and SMART, respectively), no functional domains matching any of the peptides were found. According to the positional relationship between lncRNA and mRNA, that is, the neighboring genes within 100 kb of lncRNA were its target genes, it was found that *GhlncRNA149.1* had 19 cis- target genes (Table 2). *GhA01G0129* attracts our insights because it belongs to the CC-NBs-LRR family gene, which is involved in insect resistance in plants. Therefore, it is speculated that *GhlncRNA149.1* may improve cotton resistance to cotton aphids by regulating the expression level of *GhA01G0129.*

The expression levels of *GhlncRNA149.1* and its target gene *GhA01G0129* were induced in cotton defense response to cotton aphids

In order to analyze the roles of *GhlncRNA149.1* and its target gene *GhA01G0129* in cotton defense response to cotton aphids (*Aphis Gossypii*), the expression levels of cotton *GhlncRNA149.1* and its target gene *GhA01G0129* in cotton cotyledons, stems and roots at 24 h, 48 h and 72 after the damage of cotton aphids were determined by qPCR (Fig. 2). The results showed that the expression levels of *GhlncRNA149.1* in the cotyledon were very significantly down-regulated at 24, 48 and 72 h under the attack of cotton aphids by comparison with CK. The transcripts of *GhlncRNA149.1* in the stem were significantly up-regulated at 24 h under the attack of cotton aphids by comparison with CK, but down-regulated at 48 and 72 h and reached very significantly difference at 72 h. The expression levels of *GhlncRNA149.1* in the roots showed an very significantly upward trend at 24 h and 48 h by comparison with CK , but a significantly downward trend at 72 h (Fig. 2A).Compared with CK, the expression levels of

GhA01G0129 in the cotyledon were very significantly down-regulated at 24, 48 and 72 h under the attack of cotton aphids. The expression levels of *GhA01G0129* in the stems were up-regulated very significantly at 24 h, 48 h and 72 h under the attack of cotton aphids. The expression levels of *GhA01G0129* in the roots showed an very significantly upward trend at 24 h and 48 h, but no significant downward trend at 72 h (Fig. 2B).

The transcripts of *GhlncRNA149.1* and its target *GhA01G0129* in cotton cotyledons were induced by SA and JA induction

It is known that the phytohormone signalling pathways mediated by jasmonates (JA) and salic acid (SA) play a crucial role in plant defence responses to herbivores. Therefore, the expression levels of *GhlncRNA149.1* and its target gene *GhA01G0129* in the cotton cotyledons under the induction of SA and JA was measured by qPCR (Fig. 3). The results showed that the expression levels of *GhlncRNA149.1* in cotton cotyledons were down-regulated at 24 h, 48 h and 72 h after SA treatment, and reached a very significant different level at 24 h and 48 h and a significantly different levels at 72 h after SA induction (Fig. 3A). The expression levels of *GhlncRNA149.1* in cotton cotyledons were down-regulated at 24 h, 48 h and 72 h after MeJA treatment, and reached a very significant different level at 24 h and a significantly different levels at 72 h (Fig. 3B). The expression levels of *GhA01G0129* in cotton cotyledons was up-regulated at 24 h, 48 h and 72 h after SA or MeJA induction, and reached a very significant level at 24 h and 72 h after SA induction. and a significant level at 48 h after MeJA induction (Fig. 3C, D).

Overexpression of *GhlncRNA149.1* enhanced cotton plant resistance to cotton aphids

After *GhlncRNA149.1* transiently overexpressed cotton plants were obtained successfully, the transcript of *GhlncRNA149.1* in cotton cotyledon in wild cotton plant (WT), pBI121cotton plants containing vector pBI121 and pBI121-*GhlncRNA149.1* cotton plants containing vector pBI121-*GhlncRNA149.1* was measured by qPCR. The result showed that the transcript of *GhlncRNA149.1* in pBI121-*GhlncRNA149.1* cotton plants were very significantly higher than that in WT plants and pBI121 cotton plants at 24 h, 48 h and 72 h after *GhlncRNA149.1* transiently over-expressed *Agrobacterium* solution was micro-injected (Fig. S1). Subsequently, the aphid-resistant experiments were performed. The no-choice assay showed that the number of cotton aphids in pBI121-*GhlncRNA149.1* cotton plants was lower than that in WT plants and pBI121cotton plants at 24 h, 48 h and 72 h after the damage of cotton aphids, and all reached a significant level at 24 h, 48 h and 72 h (Fig. 4A).The choice assay confirmed that the number of cotton aphids in pBI121-*GhlncRNA149.1* cotton plants was less than that in WT plants and pBI121 plants at 24 h, 48 h and 72 h, and all reached a significant level (Fig. 4B).The experimental results of honeydew secretion of cotton aphids demonstrated that the honeydew secretion of cotton aphids on the leaves in pBI121-*GhlncRNA149.1* cotton plants was very significantly less than that on WT cotyledons and pBI121 leaves at 24 h, 48 and 72 h after aphid feeding (Fig. 4C, D).These results indicated that *GhlncRNA149.1* transiently expression could enhance cotton plant resistance to cotton aphids.

Silencing of *GhlncRNA149.1* weakened the resistance of cotton plants to cotton aphids

GhlncRNA149.1 silenced cotton plants were obtained by VIGS (virus-induced gene silencing), and the transcript of *GhlncRNA149.1* in cotton cotyledons after gene silencing was determined by qPCR. The results showed that the transcription of *GhlncRNA149.1* gene in TRV:*GhlncRNA149.1* cotton plants was significantly lower than that in WT and TRV:00 cotton plants at 8 d, 15 d and 22 d after microinjection of *GhlncRNA149.1* gene silencing *Agrobacterium* solution (Fig. S2). The no choice test confirmed that after *GhlncRNA149.1* gene silencing for 15 d, the number of cotton aphis was significantly higher than that of WT plants and TRV:00 cotton plants at 24 h, 48 h and 72 h after aphid feeding (Fig. 5A). The choice test demonstrated that after *GhlncRNA149.1* gene silencing for 15 d, the number of cotton aphids was significantly higher than that of WT plants and TRV:00 cotton plants at 24 h, 48 h and 72 h after aphid feeding (Fig. 5B). In addition, after *GhlncRNA149.1* gene silencing for 15 d, the amount of cotton honeydew secretion was significantly higher than that of WT plants and TRV:00 cotton leaves at 24 h, 48 h and 72 h after aphid feeding(Fig. 5C, D).These results suggested that *GhlncRNA149.1* silence could weaken cotton plant resistance to cotton aphids.

The expression level of *GhlncRNA149.1* was positively correlated with the expression levels of *GhA01G0129*

In cotton with *GhlncRNA149.1* transient overexpression, the expression levels of *GhA01G0129* at 24 h, 48 h and 72 h were significantly higher than those in WT and pBI121 plants (Fig. 6A). Conversely, In *GhlncRNA149.1* silenced cotton plants, the expression of *GhA01G0129* at 8 d, 15 d and 22 d was significantly lower than that in WT and TRV:00 cotton plants (Fig. 6B). These result suggested that the expression level of *GhlncRNA149.1* was positively correlated with the expression levels of *GhA01G0129.*

Activities of Antioxidant enzyme was induced in *GhlncRNA149.1* transient overexpression cotton plants.

The activities of *SOD*, *POD* and *CAT* in pBI121-*GhlncRNA149.1* cotton plants were significantly higher than those in WT cotton and pBI121 cotton. (Fig. 7).These results indicated that the transiently over-expression of *GhlncRNA149.1* had the greatest effect on *SOD*, *POD* and *CAT* activities.

The expression levels of *GhlncRNA149.1* affected the expression level of antioxidase genes.

The expressions of *SOD, POD* and *CAT* of ROS scavenging genes were up-regulated in *GhlncRNA149.1* transient overexpression plants (Fig. 8A).On the other hand, the expressions of *SOD, POD* and *CAT* in cotton plants silenced by *GhlncRNA149.1* gene showed a downward trend (Fig. 8B).These result showed that *GhlncRNA149.1* may regulated the expression of *SOD, POD* and *CAT* of ROS scavenging genes in cotton response to cotton aphid damage.

Effects of transient overexpression of *GhlncRNA149.1* on the expression levels of genes involved in cotton defense pathways.

In order to clarify the defense mechanism of *GhlncRNA149.1* in cotton's defense response to cotton aphids, the expression levels of the genes involved in cotton defense response to cotton aphids including *PAL, PPO*, *NPR1* and *EDS1* were detected by qPCR. The results showed that the transcription levels of

PAL, PPO, *EDS1* and *NPR1* in pBI121-*GhlncRNA149.1* cotton plants were significantly higher than those in WT cotton plants and pBI121 cotton plants (Fig. 9).

Discussion

With the rapid development of sequencing technology, more and more plant lncRNAs have been identified and studied. However, there are few studies on the functions and mechanisms of lncRNAs in plant response to phytophagous insect damage. Therefore, in this study, on the basis of the whole transcriptome sequencing of cotton leaves induced by cotton aphid feeding, a cotton lncRNA *GhlncRNA149.1* was cloned and performed a preliminary study of its function and mechanism in cotton defense response to cotton aphid feeding. The results are of great significance for enriching the functions and mechanisms of lncRNAs.

It is known that the most important characteristic of lncRNAs is that they lack encoding protein potentials. Therefore, it is very important to determine whether lncRNA has coding potential. CPC (Coding Potential Calculator), CNCI (Coding-Non-Coding Index), CPAT (Coding Potential Assessment Tool), Pfam microsoft are the most widely used methods for coding potential analysis (Kong et al. 2007; Sun et al. 2007; Wang et al. 2013; Finn et al. 2014). In the previous study, *GhlncRNA149.1* could be analyzed and predicted using these four methods. The result showed that *GhlncRNA149.1* is a lncRNA without protein-encoding function. In addition, according to the positional relationship between lncRNA and mRNA, we also found that *GhA01G0129* was a cis- target gene of *GhlncRNA149.1* (Zhang et al. 2019). Analysis of *GhA01G0129* sequence showed that *GhA01G0129* was a protein gene of plant NBS-LRR family. The relative study showed plant NBS-LRR proteins were associated with plant resistance to pathogenesis and insect pests. And what's more, Many NBS-LRR proteins could recognize effectors secreted by pathogens directly or indirectly that in turn activate downstream signaling pathways leading to activation of plant defense response against various classes of pathogens, including bacterial, fungal, viral, nematode and insect (Dubey and Singh 2018; Du et al. 2009). Therefore, it is possible that *GhlncRNA149.1* may improve the cotton defense response to cotton aphids.

The various gene expression levels in plants often changes greatly in the face of all sorts of environmental stress (Santamaria et al. 2016). Aphid, as a worldwide insect pest, often poses a serious threat to the safe production of crop (Jiao et al. 2021). Under the stress of aphids, the genes in plants must respond to the aphid stress (Züst and Agrawal 2016). Our study showed that the expression levels of *GhlncRNA149.1* and its target gene *GhA01G0129* in cotton root, stem and leaves were influenced to different extent under cotton aphid damage and under salicylic (SA) or Methyl jasmonate (MeJA) treatment. These preliminary results show that *GhlncRNA149.1* and its target gene *GhA01G0129* do respond to the induction of cotton aphids, salicylic acid and Methyl jasmonate. It is worth noting that their expression levels in cotyledons were inhibited by aphid feeding. These results are consistent with previous studies (Zhang et al. 2020; Zhong et al. 2021).

Previously thought to be dark matters of the genome, lncRNAs have been gradually recognized as crucial gene regulators in plant growth and response to all kinds of bio- and abio-stresses (Zhang et al. 2013; Zhang and Chen 2013). The functional studies on lncRANs involved the gene knockdown and overexpression of lncRNA. For example, overexpressing lncRNA *LAIR* increases grain yield and regulates neighbouring gene cluster expression in rice (Wang et al. 2018). In our studies, the function of *GhlncRNA149.1* were verified from positive and negative aspects using overexpression and virus-induced gene silencing methods. The results from choice, no-choice and honeydew secretion assay showed that overexpression of *GhlncRNA149.1* enhanced cotton plant resistance to cotton aphids. Conversely, silencing of *GhlncRNA149.1* weakened the resistance of cotton plants to cotton aphids. In short, the results indicated that that *GhlncRNA149.1* was positively regulating cotton defense response to cotton aphids.

LncRNAs can not only directly regulate the expression of DNA, protein and miRNA but also indirectly regulate the expression of other genes in plants. For instance, *ELENA1* has also been found to bind to MED19a to regulate *PRI* expression in response to pathogen stress and to increase plant immune response (Seo et al. 2019). In our study, we demonstrated that the expression level of *GhlncRNA149.1* was positively correlated with the expression levels of its target gene *GhA01G0129.* The activities and expressions of *SOD, POD* and *CAT* of ROS scavenging genes were up-regulated in *GhlncRNA149.1* transient overexpression plants. On the other hand, the expressions of *SOD, POD* and *CAT* in *GhlncRNA149.1* silenced cotton plants showed a downward trend. In addition, the transcription levels of *PAL PPO*, *EDS1* and *NPR1* were significantly up-regulated in the leaves with transient expression of *GhlncRNA149.1*. These result showed that *GhlncRNA149.1* may regulated the expression of many defense-related gene in cotton response to cotton aphid damage.

In short, our results indicated that *GhlncRNA149.1* transient overexpression could increase the cotton plant resistance to cotton aphids, while *GhlncRNA149.1* silence can reduce the tolerance of cotton plants to cotton aphids. *GhlncRNA149.1* and its target gene *GhA01G0129* positively regulated the cotton response to cotton aphid feeding together. In addition, some ROS-scavenging genes and *PAL, PPO, EDS1* and *NPR1* were regulated in *GhlncRNA149.1* transient overexpressed cotton plants and *GhlncRNA149.1* silenced cotton plants.

Abbreviations

LncRNA Long non-coding RNA

VIGS Virus-induced gene silencing

SA Salicylic acid

JA Jasmonic acid

qPCR Quantitative real-time polymerase chain reaction

SOD Superoxide dismutase

POD Peroxidase

CAT Catalase

PAL Phenylalanine ammonialyase

PPO Polyphenol oxidase

EDS1 Enhanced disease susceptibility 1

NPR1 Nonexpressor of pathogenesis-related genes 1

Declarations

Author contribution statement ZJM and YYZ conceived and designed the research. ZY, HZW, ZHR, ZM, CQ,WHN, LXC performed the experiments, ZJM , XD, WP, AHLand YYZ analyzed the data and wrote the manuscript.

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Ethics declarations **Conflict of interest** All the authors have no conflict of interest to declare.**Informed consent** Informed consent was not required as no human or animals were involved.**Human and animal rights** Our study has no research involved human participants or animals.

Data availability The data used in this study are available from the corresponding author on reasonable request.

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Tables 1-2

Tables 1-2 are available in the Supplementary files section.

Figures

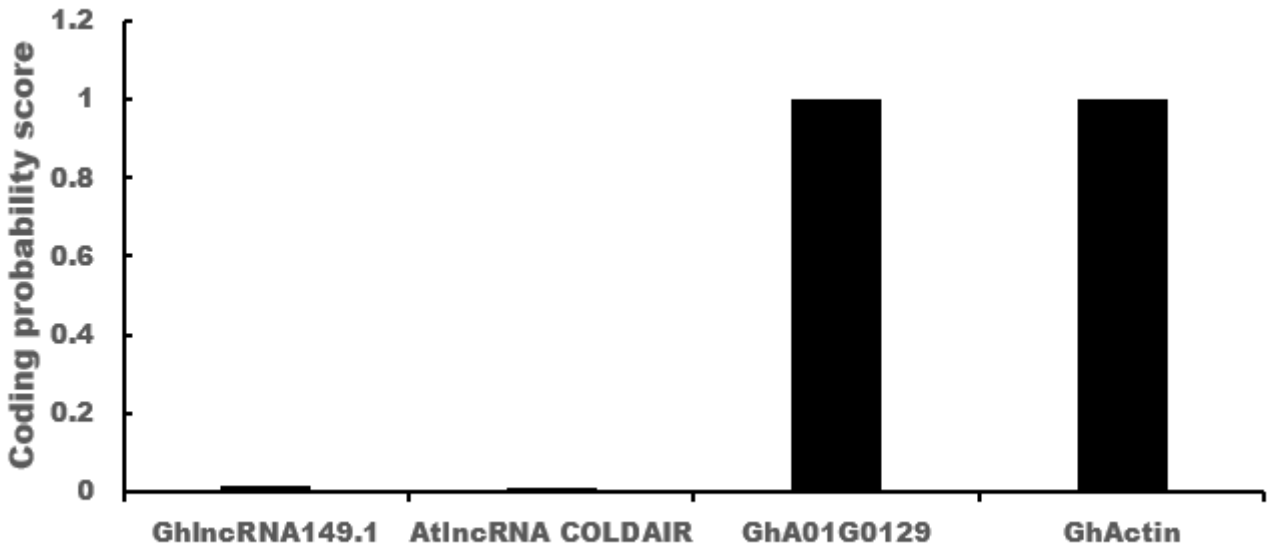

Figure 1

The coding potential of *GhlncRNA149.1* and its target gene *GhA01G0129*. Coding potential scores were generated using the CPC2 program. *GhAO1GO129* and *GhActin* are provided as coding examples, *AtlncRNA COLDAIR* represent non-coding examples

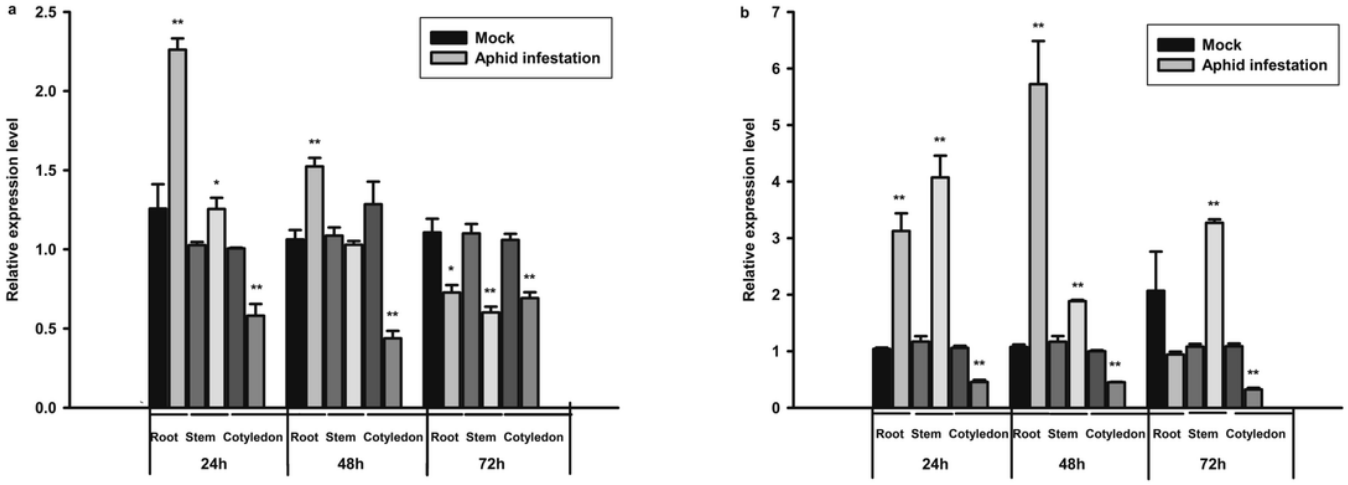

Figure 2

Expression levels of *GhlncRNA149.1* and *GhA01G0129* in cotton defense response to *Aphis gossypii* (**a, b**). Mock: treatment without the damage of *A. gossypii*, Aphid infestation: treatment with the damage of *A. gossypii*. Values are means ± SD (*n* = 3). Independent t tests were carried out to demonstrate whether there were significant（* P< 0.05）or very significant（** P< 0.01）differences between Aphid infestation and Mock

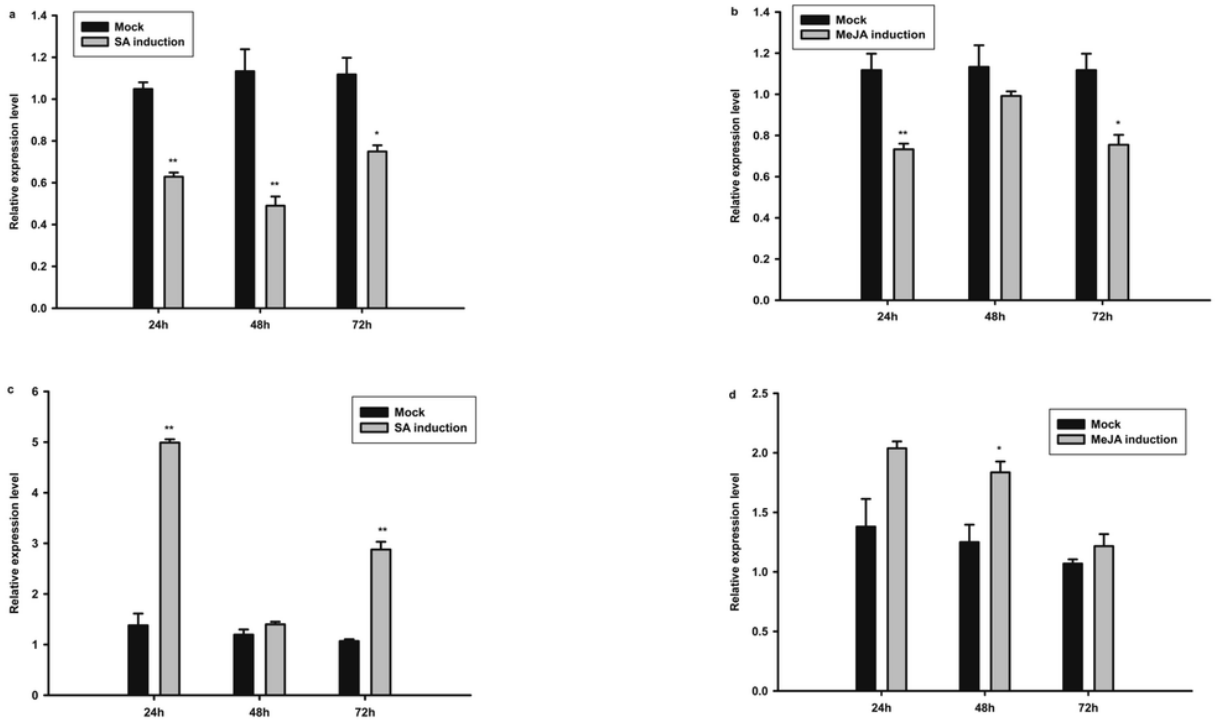

Figure 3

Expression levels of *GhlncRNA149.1* in cotton leaves under SA induction (**a**) and MeJA induction (**b**),and the expression levels of *GhA01G0129* in cotton leaves under SA induction (**c**) and MeJA induction (**d**). Mock: sterile water; SA induction：Salicylic acid; MeJA induction: Methyl jasmonate. Values are means ± SD (*n* = 3). Independent t tests were carried out to demonstrate whether there were significant (* P< 0.05) or very significant (** P< 0.01) differences between SA/JA induction and Mock

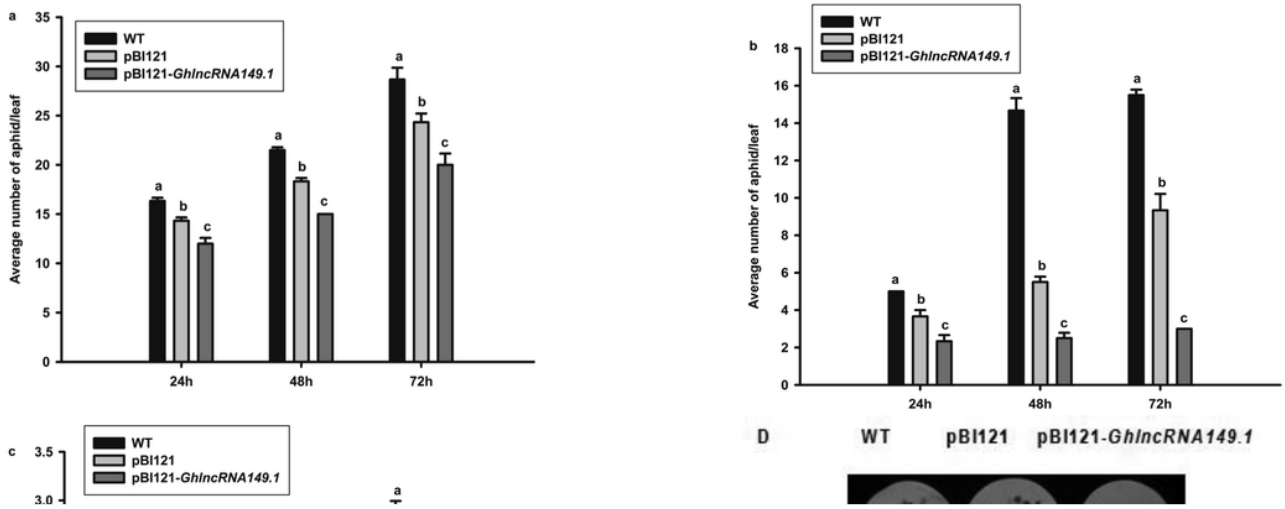

Figure 4

Transient overexpression of *GhlncRNA149.1* enhanced the resistance of cotton plants to cotton aphids. No-choice experiments (**a**), choice experiments (**b**), absorbance of aphid honeydew (**c**), and the color reaction of aphid honeydew (**d**). WT: wild cotton plants, pBI121: cotton plants containing vector pBI121, pBI121- *GhlncRNA149.1*: cotton plants containing vector pBI121-*GhlncRNA149.1*. One-way ANOVA analyzed data. Error bars represents the SD of three biological replicates. Tukey's HSD test showed that different letters indicated significant differences among WT, pBI121, pBI121-*GhlncRNA149.1* ($P < 0.05$)

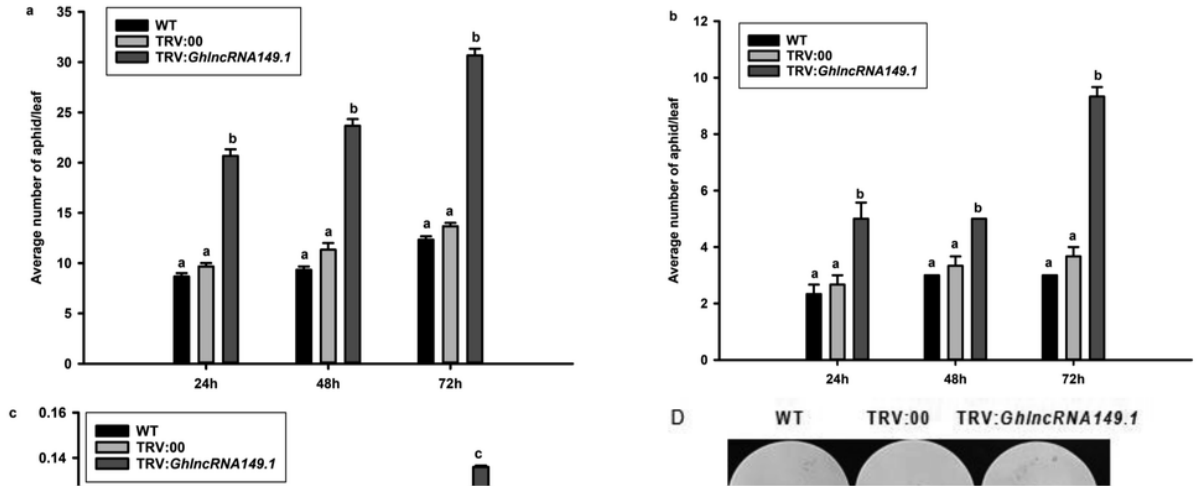

Figure 5

GhlncRNA149.1 silence weakened the resistance of cotton plants to cotton aphids. No-choice experiments (**a**), choice experiments (**b**), absorbance of aphid honeydew (**c**), and the color reaction of aphid honeydew (**d**). WT: wild cotton plants; TRV:00: cotton plants containing vector pTRV2 and pTRV1; TRV:*GhlncRNA149.1*: cotton plants containing vector pTRV2-*GhlncRNA149.1* and PTRV1. One-way ANOVA analyzed data. Error bars represents the SD of three biological replicates. Tukey's HSD test showed that different letters indicated significant differences among WT, TRV:00, TRV:*GhlncRNA149.1* ($P < 0.05$)

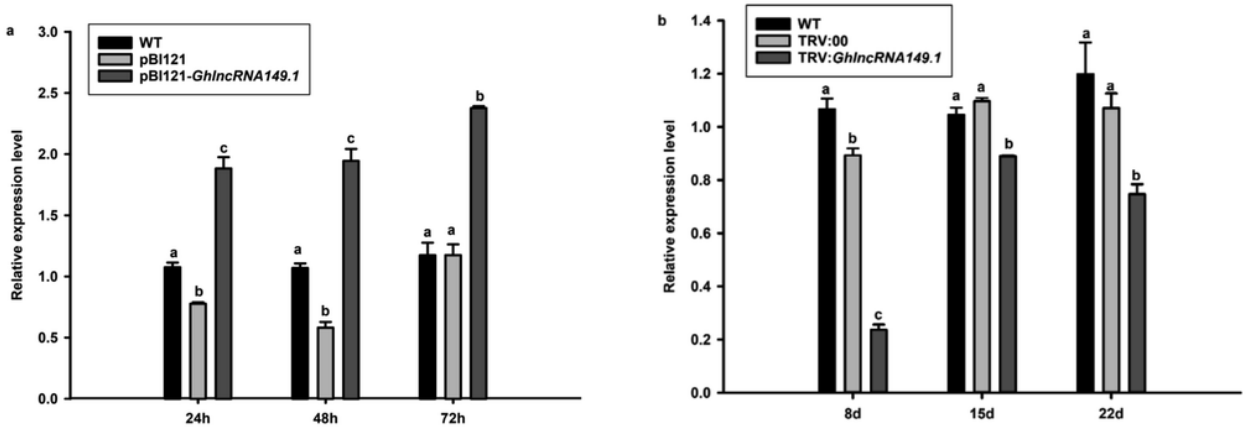

Figure 6

Expression levels of *GhA01G0129* in *GhlncRNA149.1* transiently overexpressed cotton plants (**a**) and silenced cotton plants (**b**). One-way ANOVA analyzed data. Error bars represents the SD of three biological replicates.Tukey's HSD test showed that different letters indicated significant differences among WT, pBI121, pBI121-*GhlncRNA149.1* and WT, TRV:00, TRV:*GhlncRNA149.1*($P < 0.05$)

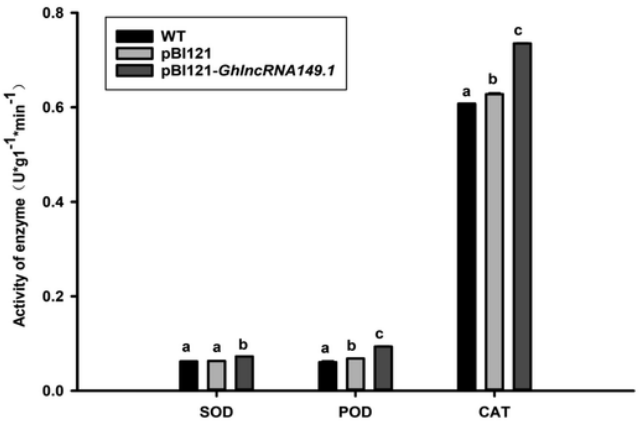

Figure 7

Activities of Antioxidant enzyme in *GhlncRNA149.1* transiently overexpressed cotton plants. One-way ANOVA analyzed data. Error bars represents the SD of three biological replicates. Tukey's HSD test showed that different letters indicated significant differences among WT, pBI121, pBI121-*GhlncRNA149.1*($P < 0.05$)

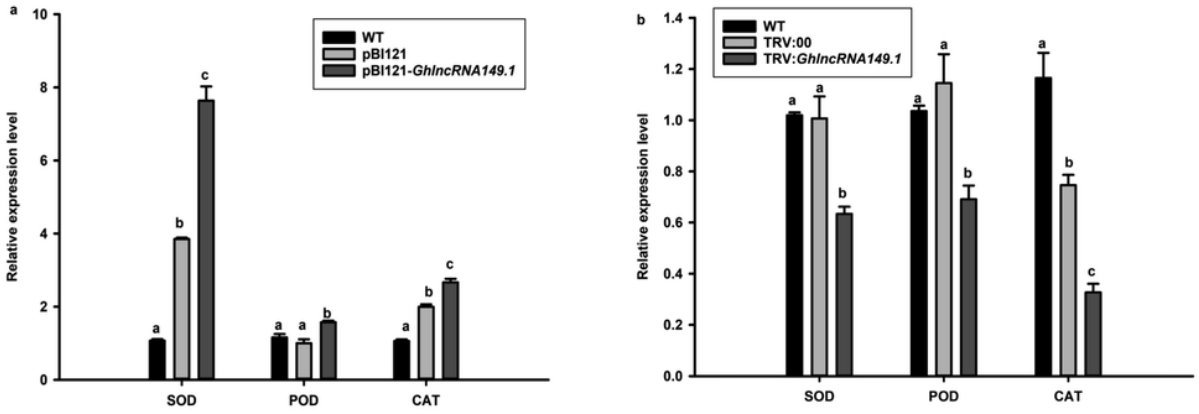

Figure 8

Expression levels of *SOD, POD* and *CAT* in *GhlncRNA149.1* transiently overexpressed cotton plants (**a**) and silenced cotton plants (**b**). One-way ANOVA analyzed data. Error bars represents the SD of three biological replicates.Tukey's HSD test showed that different letters indicated significant differences among WT, pBI121, pBI121-*GhlncRNA149.1* and WT, TRV:00, TRV:*GhlncRNA149.1*($P < 0.05$)

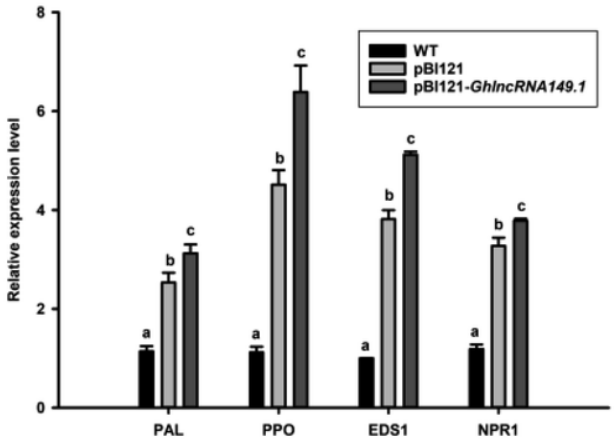

Figure 9

The expression levels of genes (*PAL, PPO*, *NPR1* and *EDS1*) involved in cotton defense pathways in transiently overexpressed cotton plants. One-way ANOVA analyzed data. Error bars represents the SD of three biological replicates.One-way ANOVA analyzed data. Error bars represents the SD of three biological replicates. Tukey's HSD test showed that different letters indicated significant differences among WT, pBI121, pBI121-*GhlncRNA149.1*($P < 0.05$)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1and2legends.doc
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- FigS1.png
- FigS2.png