Analysis of long non-coding RNAs and mRNAs in harvested kiwifruit in response to the yeast antagonist, *Wickerhamomyces anomalus*

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ABSTRACT

Biological control utilizing antagonistic yeasts is an effective method for controlling postharvest diseases. Long non-coding RNAs (lncRNAs) have been found to be involved in a variety of plant growth and development processes, including those associated with plant disease resistance. In the present study, the yeast antagonist, *Wickerhamomyces anomalus*, was found to strongly inhibit postharvest blue mold (*Penicillium expansum*) and gray mold (*Botrytis cinerea*) decay of kiwifruit. Additionally, lncRNA high-throughput sequencing and bioinformatic analysis was used to identify lncRNAs in *W. anomalus*-treated wounds in kiwifruit and predict their function based on putative target genes. Our results indicate that lncRNAs may be involved in increasing ethylene (ET), jasmonic acid (JA), abscisic acid (ABA), and auxin (IAA) levels, as well as activating signal transduction pathways that regulate the expression of several transcription factors (*WRKY72*, *WRKY53*, *JUB1AP2*). These transcription factors (TFs) then mediate the expression of downstream, defense-related genes (*ZAR1*, *PAD4*, *CCR4*, *NPR4*) and the synthesis of secondary metabolites, thus, potentially enhancing disease resistance. Notably, by stimulating the accumulation of antifungal compounds, such as phenols and lignin, disease resistance in kiwifruit was enhanced. Our study provides new information on the mechanism underlying the induction of disease resistance in kiwifruit by *W. anomalus*, as well as a new disease resistance strategy that can be used to enhance the defense response of fruit to pathogenic fungi.

1. Introduction

Kiwifruit is an economically-important fruit crop and is cultivated worldwide [1]. Recent FAO data from 2019 indicated global production of kiwifruit was over 6.5 million tons (http://www.fao.org/faostat/en/#data/QC). Postharvest diseases during transportation and storage result in serious economic losses. Blue mold, caused by *Penicillium expansum*, and gray mold, caused by *Botrytis cinerea*, represent two major postharvest diseases of kiwifruit [2,3]. Although the use of chemical fungicides remains the principal method used to control postharvest decay, the development of alternative approaches has been pursued to address environmental, health, and fungicide resistance concerns. Among alternative approaches, the use of antagonistic yeasts has been considered a promising substitute for synthetic chemical

fungicides and their use has been explored on a wide range of fruits and vegetables [4,5]. For example, Cheng et al. utilized *Hanseniaspora uvarum* to control *B. cinerea* and *Alternaria alternata* on kiwifruit [3], and *Candida tropicalis* YZ27 was reported to be an effect alternative to SO_2 fumigation for the control of postharvest decay and quality in litchi fruit [6,7].

Antagonistic yeasts act through a variety of mechanisms, including competition for nutrients and space, fungal parasitism, induction of host resistance, and the production of volatile organic compounds (VOCs) and other inhibitory compounds [7]. Among these various mechanisms, the induction of host resistance is especially intriguing. Studies have shown that antagonistic yeasts can increase resistance-related enzyme activity [such as β-1,3-glucanase, phenylalanine ammonia-lyase, peroxidase, (POD), and polyphenol oxidase (PPO)] in fruit, and the expression of stress resistance genes that have been associated with enhanced resistance to biotic and abiotic stress [8,9]. Therefore, a comprehensive understanding of the mechanisms underlying the ability of an antagonistic yeast to prevent postharvest infections and maintain

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quality can provide information that can be used to apply this technology in an efficient and effective manner.

Long non-coding RNAs (lncRNAs) are a general term for a class of RNA having a length of more than 200 nt but does not encode a protein. LncRNAs were initially considered as the transcriptional "noise" of protein-coding genes, and often ignored in transcriptome analyses [10,11]. As sequencing technology and transcriptome analysis continued to evolve, however, lncRNAs have been demonstrated to play an important role in most biological processes in plants and mammals [12]. The potential regulatory impact of lncRNA can be inferred by identifying their target mRNA and the function of its target. Studies on lncRNAs in crops have been increasing. Zhang et al. identified the presence of lncRNA in mature red and yellow sea buckthorn fruit (*Hippophae rhamnoides* Linn) and their putative target genes [12]. They identified 61 differentially expressed lncRNAs and identified their putative target genes. Different lncRNAs have been found to exert cis and trans effects on genes involved in carotenoid biosynthesis, as well as ascorbic acid and fatty acid metabolism. Deep sequencing and bioinformatic analysis were used to characterize the regulatory role of lncRNAs in chilling injury in tomato fruit. A total of 1,411 lncRNAs were identified along with a large number of putative target genes, many of which encoded stress-related proteins and genes involved in SA and abscisic acid (ABA) metabolism [13]. LncRNAs also appear to play a role in the regulation of resistance to anthracnose (*Colletotrichum gloeosporioides*) disease in walnut (*Juglans nigra*) trees [10]. Although lncRNAs have been linked to plant disease resistance, little is known about their role in disease resistance in kiwifruit.

In the present study, Illumina HiSeqTM 2500 sequencing was used to conduct deep sequencing of pulp samples of kiwifruit treated with *Wickerhamomyces anomalus* (W) and untreated, control samples (Control) treated with sterile water. In both cases pulp tissues were obtained from tissue surrounding an administered wound site. The study: (1) determined if *W. anomalus* can control blue mold decay and gray mold decay in kiwifruit (2) identified the number and characteristics of lncRNAs in kiwifruit wounds inoculated with *W. anomalus*, compared to untreated wound tissues; (3) provided a functional prediction of the target genes of the lncRNAs in relation to disease resistance.

2. Materials and methods

2.1. Microorganisms

W. anomalus isolated from orchard soil in Zhejiang, Jiangsu province, was identified based on 5.8S rDNA sequence analysis. The yeast was cultured in nutrient yeast dextrose broth (NYDB, nutrient broth 8 g/L, yeast extract 5 g/L and dextrose 10 g/L) medium for 24 h. Cells were harvested after culture by centrifugation (4000 *g*, 15 min) and washed twice with sterile distilled water to remove any medium. The cells were then re-suspended in sterile distilled water and adjusted to a concentration of 1×10^8 CFU/mL.

P. expansum and *B. cinerea* were isolated from diseased kiwifruit and cultured on potato dextrose agar (PDA, extracts of 200 g potatoes, glucose 20 g/L; agar 20 g/L) at 25 ℃ for 7 d. Spores were then washed from the plates and collected in sterilized, distilled water. The spore concentration was then adjusted to 1×10^5 CFU/mL with the aid of a hemocytometer [14].

2.2. Assessment of the biocontrol efficacy of W. anomalus against postharvest blue mold and gray mold of kiwifruit

Similar-sized kiwifruit (*Actinidia chinensis* cv. Hongyang with an average weight of 84 g per fruit) at commercial maturity and with no evidence of mechanical injury, fungal infections, or insect pest damage, were selected for use in the study. Prior to use in the biocontrol assay, fruit were soaked in 2% sodium hypochlorite for 5 min to disinfect the fruit and then washed three times in distilled water to remove the residual sodium hypochlorite solution or other impurities. After air-drying, the kiwifruits were divided into four treatment groups of 12 fruit each. Each treatment had three replicates and the experiment was repeated twice. Three (4 mm diameter and 3 mm deep) wounds were made around the equatorial area of each kiwifruit with a sterilized punch, which ensured that the wounds were significantly separated from each other. The wounds were allowed to air dry, after which 20 μL of *W. anomalus* (1×10^8 CFU/mL) were pipetted into each of the wounds of kiwifruit in two of the treatment groups, while the other two groups of kiwifruits were administered 20 μL of sterile water as a control. After two hours, each wound was inoculated with 20 μL of a spore suspension of *P. expansum* or *B. cinerea* (1×10^5 CFU/mL) [14]. The two treatment groups were yeast followed by either *P. expansum* or *B. cinerea* and the two control groups were sterile water followed by either *P. expansum* or *B. cinerea*.

The four groups of fruit were placed in separate clean plastic baskets in a manner that ensured that the fruit did not touch each other. Polyethylene film was placed over each basket to prevent the fruit from drying out and ensuring that a high relative humidity (85–90%) was maintained. Each basket was sprayed with 75% alcohol before use and sterilized by UV light on an ultraclean laboratory bench for two hours. The treated fruit were stored in an incubator at a constant temperature of 20 °C. Disease incidence and lesion diameter of wounded kiwifruit were determined after 4 d. Disease incidence was assessed by the presence of hyphae and wounds that were discolored and soft.

2.3. Preparation of samples for deep sequencing

Pretreatment of kiwifruit was the same as described in section 2.2, after which wounds were treated with either *W. anomalus* (1×10^8 CFU/mL) or sterilized water as described above. All kiwifruits were placed in a constant temperature incubator for 24 h, after which approximately 2 g of tissue was removed with a sterilized scalpel from around and inclusive of the wound and placed in a sterilized centrifuge tube. The tubes were placed in liquid nitrogen to ensure rapid freezing of the tissue and then samples were placed at −80 °C until further processing. Three biological replicates were obtained from each to the two groups (treated and control) for a total of six samples [15].

2.4. RNA extraction, library construction, and sequencing analysis

The treated and untreated tissue samples were sent to Shanghai OE Biomedical Science and Technology Company (Shanghai, China) for transcriptome sequencing and bioinformatic analyses. Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion) following the manufacturer's instructions. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples having an RNA Integrity Number (RIN) ≥ 7 were used for further processing. The libraries were constructed using TruSeq Stranded Total RNA with Ribo-Zero Gold according to the manufacturer's instructions. The prepared libraries were sequenced on an Illumina sequencing platform (HiSeqTM 2500) and generated 150 bp paired-end reads. Raw reads were filtered by Trimmomatic software to remove Illumina sequencing adapters and primer sequences, as well as low-quality reads. Read quality was assessed using FASTQC software to ensure the quality and reliability of the reads. The filtered reads were then referred to as clean reads and used in the subsequent bioinformatic analyses. Raw sequence data was uploaded

to the SRA NCBI database (https://www.ncbi.nlm.nih.gov/SRA), under the accession number: PRJNA689627. Clean reads were matched to the kiwifruit reference genome (http://kiwifruitgenome.org/) using Hisat2, and the number and proportion of the various type's reads were determined and evaluated using an RSeQC software. Based on the results of transcriptome assembly, candidate lncRNA were obtained for subsequent analysis through a series of rigorous screening steps as follows. (1) The assembled transcripts were annotated using the Cuffcompare software. According to Cuffcompare, only transcripts class-coded as "i" (a transfrag falling entirely within a reference intron), "u" (unknown intergenic-transcript), and "x" (exonic overlapwith reference on the opposite strand), "o" (Generic exonic overlap with a reference transcript) were selected as candidate lncRNAs. (2) Transcripts with lengths greater than 200 bp and at least two exons were retained. (3) CPC, CNCI, PLEK and Pfamtools were utilized to predict the coding ability of transcripts. The intersection of CPC score < 0, CNCI score < 0, PLEK score < 0 and Pfam result"noncoding" were the final newly predicted lncRNAs. Bowtie2 and Express software were used to calculate expression abundance of each transcript in each sample, calculate the fragments per kilobase of exon per million fragments mapped (FPKM), and calculate the fold change (FC) and P value. DESeq can detect and correct the dispersion of high-throughput data in counts between different samples. Log$_2$|FC| > 1 and P < 0.05 of lncRNAs and mRNAs were considered to be differentially expressed transcripts [13].

2.5. Target gene prediction and functional analysis

The identified lncRNAs were placed in three different categories based on their regulatory relationship to putative target genes. (1) cis regulation, lncRNAs that were transcribed within 100 kb before or after a coding gene were designated cis-lncRNAs, these lncRNAs aligned with genes that had significant co-expression based on the Pearson correlation coefficient (pearsonr) value of not <0.8, and P value less than or equal to 0.05. (2) trans regulation, RNA interaction software research-2.0 was used to predict the binding of candidate lncRNA and gene at nucleic acid level, the base number of direct interactions between two nucleic acid molecules was no <10, and the base binding free energy was no more than – 50. According to the free energy, from small to big, take the top 200 items to further analysis. (3) ceRNA regulation. The regulatory relationship of miRNA was predicted using miRanda. The score was calculated by combining the results of ceRNA-MuTATE analysis with the probability of sharing some miRNAs with ceRNA. The correlation between mRNA and lncRNA in the 6 samples was calculated by pearsonr. The threshold for a significant correlation was set at a correlation coefficient $\geq$ 0.90 and a P value $\leq$ 0.05. Gene function was annotated based on the following databases: Nr (NCBI non-redundant protein sequences); Swiss-Prot (Amanually annotated and reviewed protein sequence database); GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes Genomes); A hypergeometric distribution test was used to calculate the enrichment significance of lncRNA-regulated mRNAs in GO or KEGG entries, as functional annotations of lncRNAs. A significance level of P < 0.05 was used to indicate a significant enrichment of differentially expressed genes (DEGs).

2.6. Reverse transcription - quantitative polymerase chain reaction (RT-qPCR) validation

Verification of transcriptome data was conducted as described by Xu et al, with some modifications [16]. Total RNA was converted to cDNA using HiFiScript gDNA Removal RT Master Mix (CoWin Biosciences, Jiangsu, China) according to the manufacturer's instructions. RT-qPCR was performed using TB Green Real-Time Polymerase Chain Reaction Kit (Takara Bio). Primer sequences were designed using Primer 5.0 software (Biosoft Premier, Palo Alto, California, USA), Gene-specific primers were designed using Primer 5.0 software and are listed in Table S5. The $2^{-\Delta \Delta Ct}$ method was used to determine relative expression. Presented data represent the mean ± standard error of three biological replicates.

2.7. Statistical analysis

For the samples with biological replicates, data were analyzed using SPSS Statistics (SPSS version 17.0 for Windows, 2009). An independent sample t-test was used for comparison between the two treatment groups, and differences at P < 0.05 were considered to be statistically significant. Genes, and lncRNAs with an adjusted P < 0.05 and an absolute value of log$_2$ |FC| >1, were designated as being differentially expressed.

3. Results

3.1. Efficacy of W. anomalus against blue mold decay and gray mold on kiwifruit

All the wounds in the 36 kiwifruits of the control group were infected at 4 days post-inoculation (Fig. 1A). In contrast, *P. expansum* and *B. cinerea* disease incidence in kiwifruit treated with *W. anomalus* was reduced to 45.75 % and 31.3 %, respectively. Lesion diameter in *W. anomalus*-treated kiwifruit was also significantly reduced compared to lesion size in the control group (Fig. 1B). Average lesion diameter in *P. expansum* inoculated fruit was 18.89 mm in the control group and 10.26 mm in *W. anomalus*-treated kiwifruit. Average lesion diameter of *B. cinerea* inoculated kiwifruit was 22.55 mm in the control group was, and 13.89 mm in the *W. anomalus* treatment group. Representative photos of the phenotype of kiwifruit treated with sterile water (control) or *W. anomalus* (W) followed by inoculation with either *P. expansum* or *B. cinerea* on the fourth day after inoculation are presented in Fig. 1C and 1D, respectively. These results demonstrate that *W. anomalus* exhibits significant biocontrol efficacy against *P. expansum* and *B. cinerea* decay in kiwifruit for at least four days.

3.2. Identification of lncRNAs in wounded and wounded/yeast-inoculated tissues of kiwifruit

The complete results of the sequencing of the transcripts of six samples (three wounded and three wounded/yeast inoculated) are provided in Table S1. A total of 66.72 Gb of clean data were obtained. The effective data volume of each sample ranged from 10.57 to 11.84 Gb, the Q30 base distribution ranged from 94.66 to 94.96 %, and the average GC content was 44.67 %. The obtained reads were aligned to the reference genome, achieving an alignment rate of 83.65–86.15 %. Sequences with multiple alignment positions on the reference genome accounted for 3.93–4.01 %, and the number of sequences with unique alignment positions on the reference sequence accounted for 79.72–82.18 %. A total of 4482 lncRNAs were identified, with a total length of 2,733,788 nt and an average length of 609.95 nt, among them, the number of lncRNAs was the largest between 201 and 300 nt (Fig. 2A). Analysis of exon number distribution found that the number of lncRNAs with exon 2 was the largest (3005). The second was lncRNAs with exon number of 3 (1054) (Fig. 2B). The identified lncRNAs were categorized into two main groups, sense and antisense types (Fig. 2C) with four sub-types in each group; genic_ exonic; genic_ intronic; intergenic_ downstream; and, intergenic_ upstream. The groups with the most lncRNAs were sense_ intergenic_ upstream (776) and sense_ genic_ exonic (736).
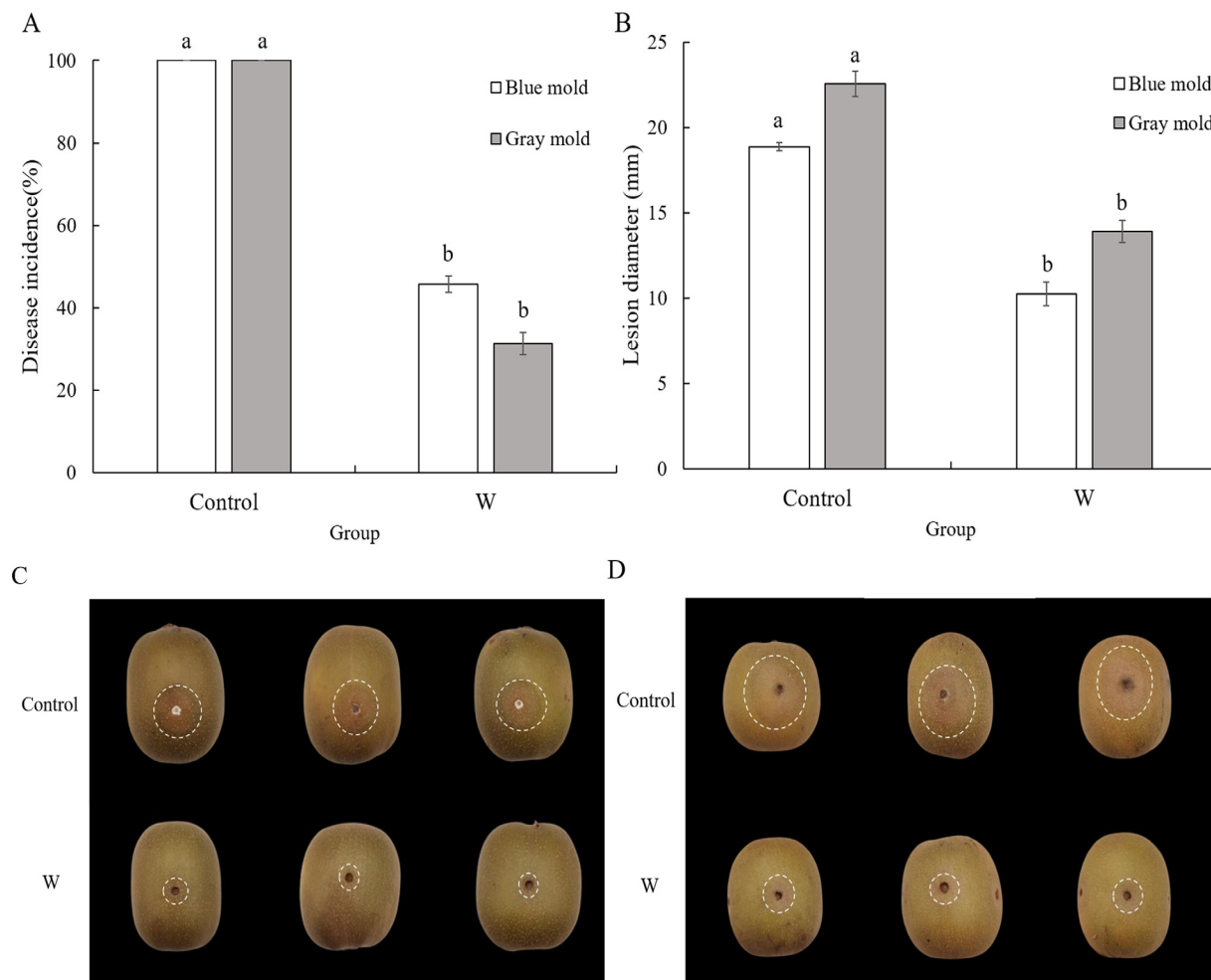
Fig. 1. Effect of *W. anomalus* on disease incidence (A) and lesion diameter (B) of blue mold (*P. expansum*) and gray mold (*B. cinerea*) in kiwifruit stored at 20 °C. Representative photographs of the phenotype of kiwifruit samples infected with *P. expansum* (C) and *B. cinerea* (D) in control and *W. anomalus* treatment groups of kiwifruit stored at 20 °C for four days post-inoculation. The dashed white lines represent the perimeter of the lesion area in infected kiwifruit. Control represents wounded fruit inoculated with sterile water, and W represents wounded fruit treated with a suspension (1×10^8 CFU/mL) of yeast (*W. anomalus*).

3.3. Differential expression of lncRNAs and mRNAs in wounded and wounded/yeast-inoculated tissues of kiwifruit

The analysis identified 149 differentially-expressed lncRNAs between the two sample groups. Further analysis indicated that 91 lncRNAs were up-regulated and 58 lncRNAs were down-regulated in the yeast-treated group compared to the untreated control group. At the same time, 745 differential mRNAs were screened, among which 487 were up-regulated and 258 were down-regulated compared with the control group. A cluster diagram of the differentially-expressed mRNAs and lncRNAs is presented in Supplementary Figs. S1A, B. The three samples of the yeast-treated group and the untreated sample group clustered separately within their respective treatment groups. These results demonstrated that the expression levels of lncRNAs and mRNAs in the *W. anomalus*-treated samples differed from the water-treated (control) samples.

3.4. GO and KEGG enrichment analysis of differentially expressed mRNA

GO enrichment can be used to analyze the function of differentially expressed mRNAs. A total of 495 DEGs (329 up-regulated and 166 down-regulated) were annotated into GO categories. The number of differentially enriched genes in Biological Process (BP) was

greater than the number in Cell Component (CC) and Molecular Function (MF). The DEGs were enriched in BP were associated with the terms biological regulation, cellular process, metabolic process, regulation of biological process, and response to stimulus, The DEGs were enriched in CC were associated with the terms cell, cell part, membrane, membrane part, and organelle. The DEGs were enriched in MF were associated with the terms binding, and catalytic activity (Supplementary Fig. S2A). The GO terms that were enriched in the upregulated DEGs were mainly related to resistance, including response to stimulus, catalytic activity, antioxidant activity, signaling, and immune system process.

KEGG enrichment analysis of DEGs (Supplementary Fig. S2B) indicated that the up-regulated genes were associated with 15 pathways, and the down-regulated genes were associated with 20 pathways. The metabolic pathways enriched in the up-regulated genes were signal transduction, biosynthesis of other secondary metabolites, amino acid metabolism, and carbohydrate metabolism. The down-regulated genes were mainly associated with folding, sorting and degradation, carbohydrate metabolism, and energy metabolism.

3.5. Analysis of cis-interacting of lncRNAs and mRNAs

Interaction analysis indicated that there were 24 lncRNAs linked to 23 target gene mRNAs, which suggest that these lncRNAs
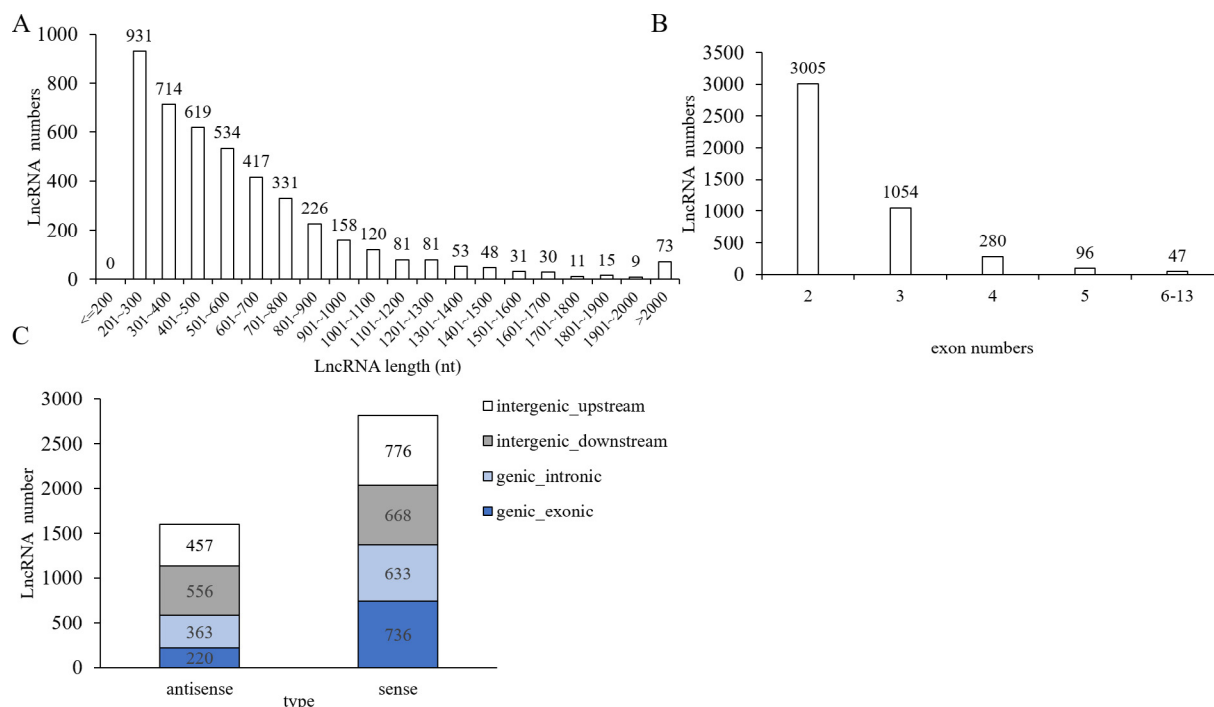
Fig. 2. Nucleotide length distribution (A), exon number distribution (B), and type distribution (C) of lncRNAs identified in control and yeast-treated fruit tissues of kiwifruit.

may be involved in regulating these mRNAs (Supplementary Fig. S3A). Fourteen of the target genes were enriched in 72 GO terms, and the enriched GO terms with more than two associated mRNAs were enriched in integral component of membrane, followed by cytoplasm, DNA binding transcription factor, and nucleus (Supplementary Fig. S3B), Additionally, eight KEGG pathways were enriched (Excel S1). We found that six lncRNAs and their five target genes were enriched in plant hormone signal transduction pathway. In alpha linolenic acid metabolism (Fig. 3A), the target gene of *TCONS 00048761*, *Actinidia19202* (*SAMT*), was up-regulated (log$_2$-FC = 2.76), promoting JA synthesis and the generation of Methyl Jasmonate (MeJA). In cysteine and methionine metabolism (Fig. 3B), the gene *ACS1*, it is also the cis target gene of *TCONS_00003444*, *TCONS_00003433*, *TCONS_00003434*, was up-regulated (log$_2$FC = 1.53), which promotes the transformation of S-adenosine-L-methionine into 1-aminocylopropane-1-carboxy late, a precursor of the plant hormone, ethylene (ET). *Actinidia17020* (encoding ornithine decarboxylase) (log$_2$FC = 1.76) (Fig. 4A), would promote the generation of L-ornithine generated putrescine. Putrescine can be converted into glutathione (GSH), which has been associated with increased resistance to environmental stress in plants. *ATHB-8* (encoding homeobox-leucine zipper protein like) (log$_2$FC = 1.35). Detailed information on a number of the DEGs is presented in Table S2.

3.6. Analysis of trans-interacting lncRNAs and mRNAs

The absolute value of binding free energy of *trans*-interacting target gene mRNAs and lncRNAs was arranged from large to small and the first 200 regulatory relationships were selected for functional enrichment analysis. A co-expression network diagram was also constructed (Supplementary Fig. S4A). DEGs with greater than two assigned GO terms were further assessed (Supplementary Fig. S4B). These DEGs were enriched in the nucleus, DNA-binding transcription factor activity, integral component of membrane and regulation of transcription, and DNA-template. Four of the target genes were associated with 10 metabolic pathways (Excel S2).

ABA plays an important role in carotenoid synthesis and plays a key role in regulating the level of adaptive response of plants under to stress conditions. *PYL8* (Fig. 3C), a receptor for ABA, was up-regulated (log$_2$FC = 1.43), which would increase the involvement of ABA in stress response. In the calcium signaling pathway (Fig. 4B), CaM is a highly-conserved regulatory protein, that has many physiological functions in both plants and animals. In this regard, *CML11* (encoding Calmodulin-like protein, log$_2$FC = 1.21) and *CML45* (encoding calcium-binding protein, log$_2$FC = 1.29) were found to be upregulated. We identified two transcription factors that may be trans regulated by lncRNA (Fig. 6), The WRKY transcription factor *WRKY72*, identified as the target of *TCONS_00034381* and *TCONS_00036668* (log$_2$FC = 1.42), was up-regulated. Additionally, the transcription factor JUNGBRUNNEN 1 (JUB1) (log$_2$FC = 1.43) may be *trans*-regulated by three lncRNAs (*TCONS_00015541*, *TCONS_00034381*, *TCONS_00036668*). Detailed information on some of the target genes regulated by *trans*-interacting lncRNAs is provided in Table S3.

3.7. Regulatory mechanism of ceRNA

A total of 74,117 miRNA-mRNA relationship pairs and 6417 miRNA-lncRNA relationship pairs were identified, as well as a correlation between 12,542 pairs of mRNAs and lncRNA. A network diagram was constructed of the top 100 mRNA-lncRNA pairs with the highest MuTATE score ranking in the results of the ceRNA analysis (Supplementary Fig. S5). The mRNAs in the constructed ceRNA related pairs were subjected to GO and KEGG analyses. The GO enrichment analysis identified 545 enriched genes. A total of 299 genes were annotated within the BP category, 312 genes were annotated within the CC category, and 324 genes were annotated within the MF category. A bar chart was constructed illustrating the results of the GO enrichment analysis, and the top 10 annotations within the three different GO categories, are presented in the same graph (Supplementary Fig. S6A). The three most highly represented annotations in the three GO categories were DNA-binding transcription factor activity, nucleus, and response to chitin in
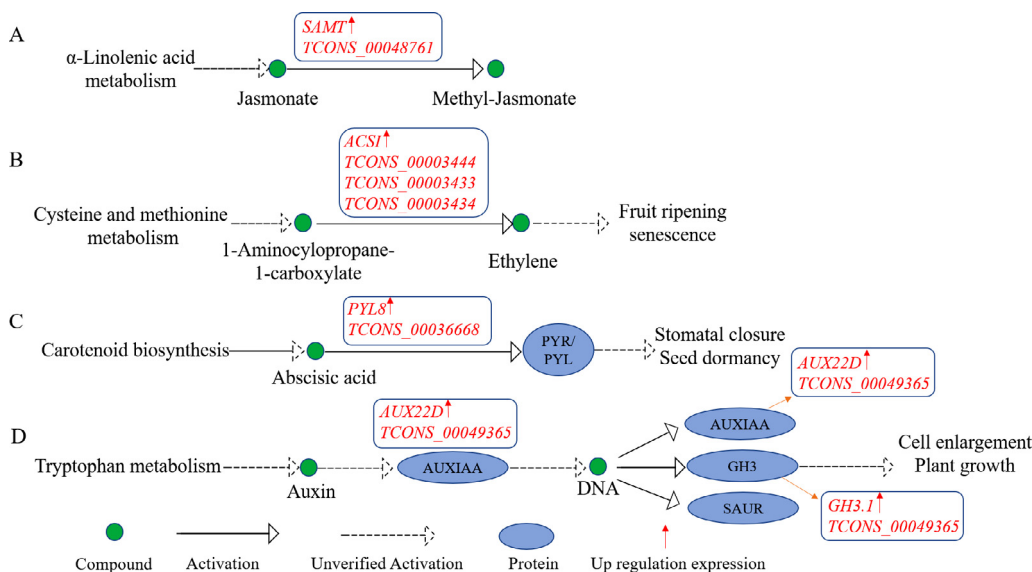
Fig. 3. Target genes involved in plant hormone signal transduction path ways. In the rectangular box are the lncRNAs involved in the pathway and the predicted target genes.
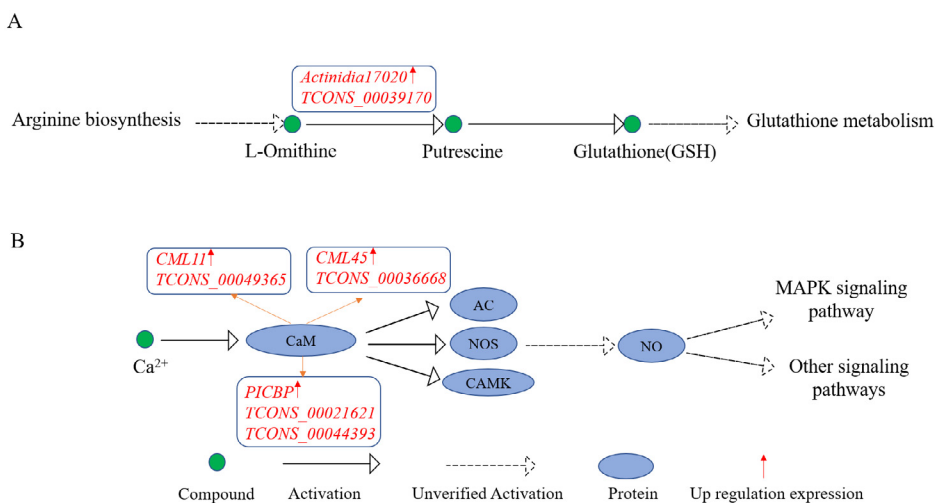

Fig. 4. Target genes involved in cysteine and methionine metabolism and glutathione metabolism (A), calcium signaling pathway (B). In the rectangular box are the lncRNAs involved in the pathway and the predicted target genes.

the MF, CC, and BP categories, respectively. KEGG enrichment analysis utilized the hypergeometric distribution to calculate the level of correlation between each pathway in KEGG and the DEGs identified in our study. Among a total of 545 DEGs, 79 were annotated in the KEGG pathway analysis. A bubble diagram of the results of the KEGG enrichment analysis of DEGs indicated that most of the DEGs were associated with plant hormone signal transduction, pentose and glucuronate interconversions, and phenylpropanoid biosynthesis (Supplementary Fig. S6B). The target genes *AUX22D* (log$_2$FC = 1.01) and *GH3.1* (log$_2$FC = 1.25) were up-regulated and associated with tryptamine metabolism, which is important for increasing the transport of IAA to specific cell types (Fig. 3D). The ce-regulated gene *Actinidia18707* (encoding 4-coumarateCoA ligase) (log$_2$FC = 1.17) was enriched in three pathways, including phenylpropanoid biosynthesis, ubiquinone and other terpenoid-quinone biosynthesis, and phenylalanine metabolism, and associated with *TCONS_00037962* (Fig. 5). The up-regulated expression of this gene would promote the production of 4-coumarate-Coa ligase (4CL) in three metabolic pathways. Additionally, the target genes, *GPAT5* (encoding Glycerol-3-phosphate acyltransferase,

log$_2$FC = 2.61), *ZAR1* (encoding Receptor protein kinase-like protein, log$_2$FC = 4.03), *NPR4*(encoding Ankyrin repeat-containing protein, log$_2$FC = 1.24), *PICBP* (encoding Calmodulin binding protein, log$_2$FC = 1.14), *CCR4* (encoding Serine/threonine-protein kinase like protein, log$_2$FC = 1.43), *PAD4* (encoding lipase like, log$_2$FC = 1.82), and two transcription factors *WRKY53* (log$_2$FC = 1.09) and *AP2* (encoding Ethylene-responsive transcription factor RAP2-7 like, (log$_2$FC = 1.02), were all up-regulated (Fig. 6). The mRNA information on the regulatory relationship to ceRNA is presented in the Table S4.

3.8. RT-qPCR analysis of gene expression

The accuracy of the transcript sequence and expression data was verified by RT-qPCR. In total, 24 DEGs (12 mRNAs and 12 lncRNAs) were randomly selected for RT-qPCR analysis. The expression level of mRNA (*CML11*, *BHLH162*, *JUB1*, *PIK-1*, *ALA9*, *HSR4*, *GPAT5*, *UGT92A1* *CYP735A*2), and lncRNA (*TCONS_00014600*, *TCONS_00048761*, *TCONS_00024225*, *TCONS_00042508*, *TCONS_00000523*, *TCONS_00000525*, *TCONS_00004156*, *TCONS_00008032*, *TCONS_00014598*) increased in kiwifruit treated with *W. anomalus* (Fig. 7A), while the
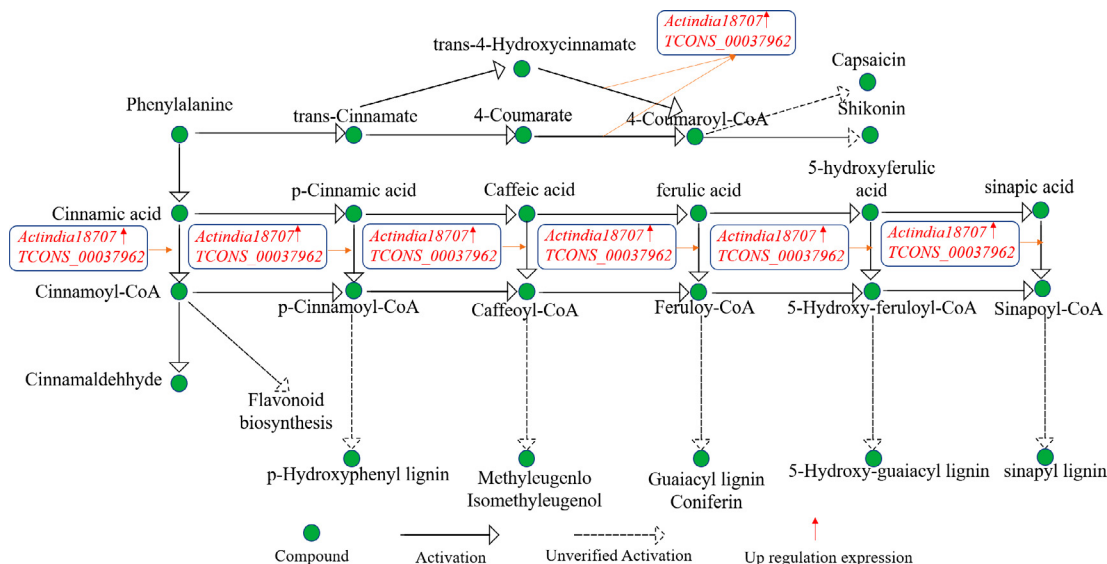
Fig. 5. Target genes involved in phenylpropanoid biosynthesis, ubiquinone and other terpenoid-quinone biosynthesis, phenylalanine metabolism. In the rectangular box are the lncRNAs involved in the pathway and the predicted target genes.
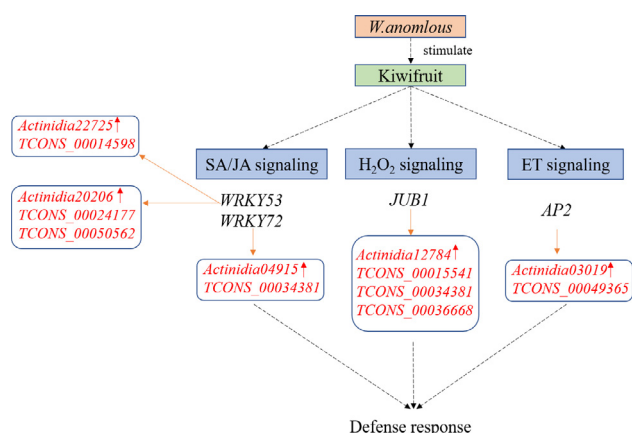

Fig. 6. Transcription factors induced by *W. anomalus* stimulated signal transduction in kiwifruit target genes. In the rectangular box are the lncRNAs involved in the pathway and the predicted target genes.

expression level of mRNA (*TBL38, TCP12, Actinidia31212*), and lncRNA (*TCONS_00003444, TCONS_00003434, TCONS_00051721*) decreased (Fig. 7B). The expression level of these DEGs and lncRNAs as determined by RT-qPCR was consistent with the transcriptome data, thus, verifying the reliability of the transcriptome data.

4. Discussion

Modern, high-throughput sequencing technologies provide an excellent tool to conduct research on molecular mechanisms. Comparative transcriptome analysis was performed on fruit bracts of 'F26′ (anthracnose-resistant) and 'F423′ (anthracnose-susceptible) walnut (*Juglans regia*) to identify differences in lncRNAs at five time points. In the early stages of the anthracnose infection, a significantly greater number of lncRNAs were up-regulated in 'F26′ (resistant), relative to 'F423′ (susceptible). Bioinformatic analysis indicated that the target genes of up-regulated lncRNAs were enriched in the activation of innate immune responses, defense responses to bacteria, immune system processes, plant hormone signal transduction, phenylpropanoid biosynthesis, and other

pathways [10]. Previous studies have also analyzed the transcriptome of infected kiwifruit [17,18]. Wang et al. conducted a transcriptome analysis of kiwifruit infected with *Pseudomonas syringae*, and identified a number of lncRNAs related to the regulation of various plant defense processes, including innate immune response, systemic-acquired resistance, and salicylic acid-mediated defense [19].

We previously reported that *W. anomalus* can significantly reduce naturally-occurring disease incidence in the fruit of pears and cherry tomatoes, without having a negative impact on fruit quality [20,21]. We also analyzed changes in the transcriptome and proteome of pear fruit that was induced in response to the yeast. Results indicated that *W. anomalus* induced the expression of several defense-related genes and their corresponding proteins [20]. Yeast can significantly enhance the activity of defense-related enzymes, such as polyphenol oxidase (PPO), peroxidase (POD), catalase (CAT), and phenylalanine ammonia-lyase (PAL) in cherry tomatoes [21]. Monika et al. reported that *W. anomalus* BS91 significantly reduced the incidence of apple brown rot, caused by *Monilinia fructicola*. *In vitro* experiments indicated that *W. anomalus* BS91 inhibited the mycelial growth of *M. fructicola*. Significant increases in POD and CAT activity were also observed in apple fruits inoculated with both *W. anomalus* BS91 and *Monilinia fructicola* [22].

While yeast antagonists have been demonstrated to induce host defense responses in fruit, no studies on the molecular mechanisms associated with the use of antagonistic yeast on kiwifruit have been reported. We found that *W. anomalus* exhibits good biocontrol activity against blue and gray mold disease of kiwifruit (Fig. 1). On the fourth day after inoculation with *W. anomalus* and either pathogen, both the percentage of disease incidence and lesion diameter were significantly lower in yeast-treated kiwifruit than they were in control fruit. We hypothesized that the mode of action by which *W. anomalus* reduces blue and gray mold infections and virulence on kiwifruit involved not only the direct inhibition of the growth of *P. expansum* and *B. cinerea*, but also the induction of host resistance by the upregulation of defense-related genes and increasing the abundance of defensive metabolites. Based on this hypothesis, we conducted deep sequencing of *W. anomalus*-treated kiwifruit tissues to explore potential biocontrol mechanisms at the molecular level.
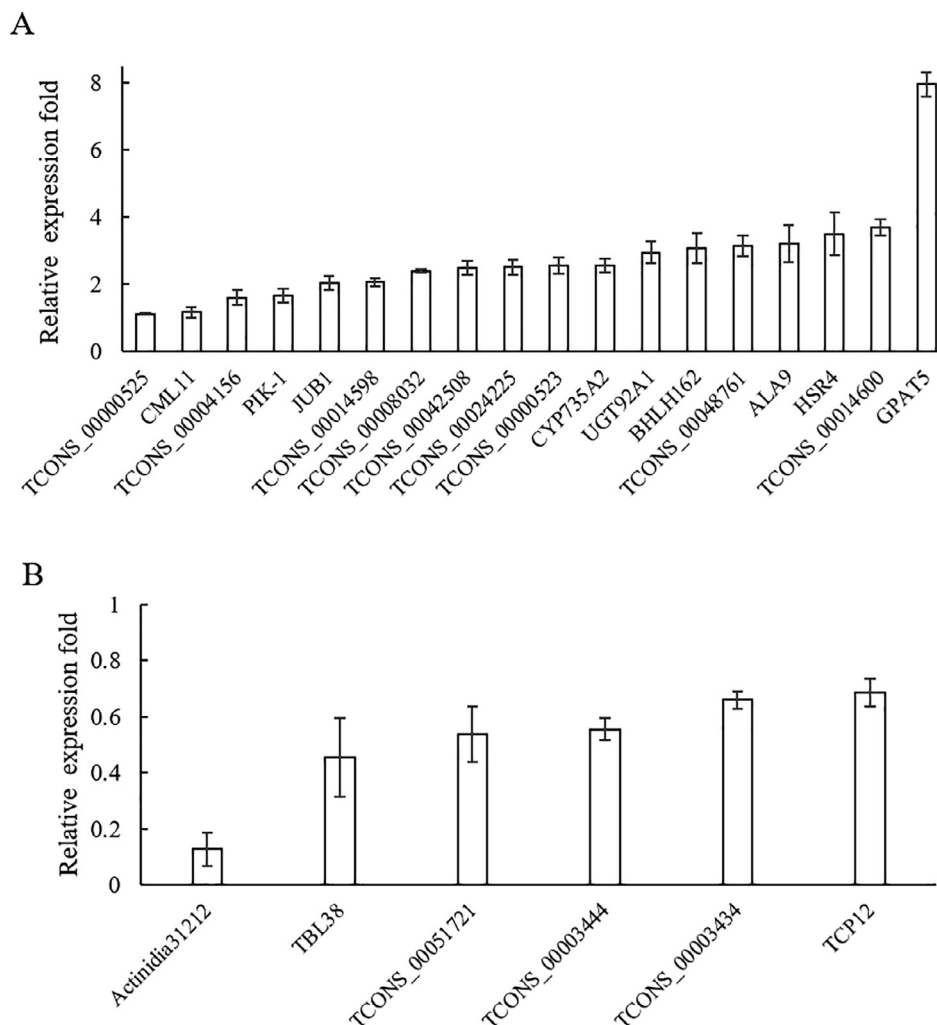
Fig. 7. Verification of differentially expressed genes (DEGs) by RT-qPCR. The analysis included a total of 24 randomly selected sequences (12 mRNAs and 12 lncRNAs). The expression level of 9 mRNAs and 9 lncRNAs were up-regulated in kiwifruit treated with *W. anomalus* (Fig. 7A), while 3 mRNAs and 3 lncRNAs were down-regulated (Fig. 7B).

In the present study, lncRNAs from wounded, yeast-treated (*W. anomalus*) and wounded, water-treated tissues of kiwifruit were identified and analyzed using RNA-seq data. A total of 4482 lncRNAs were identified, having an average length of 609.95 nt. The highest proportion of identified lncRNAs type was sense_intergenic_upstream, and the greatest number of exons was two (Fig. 2). We identified differentially expressed lncRNAs and predicted their target genes in three ways. The target genes were annotated by GO and KEGG databases for functional analysis. Results revealed that the lncRNAs were associated with plant hormone signaling transduction, calcium signaling pathways, and phenylpropanoid biosynthesis, as well as other pathways [23,24].

Plant hormones, including IAA, ABA, and ET play an indispensable role in the regulation of plant development and stress resistance [25]. The target gene, *SAMT,* identified in our study, promotes the production of MeJA (Fig. 3A), a common elicitor of disease resistance. Elicitors, such as MeJA, stimulate the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as antioxidant enzymes and defense-related genes [26].

Methionine is a starting material in the biosynthesis of ET, and 1-aminocyclopropane-1-carboxylic acid is a direct precursor. In our study, the up-regulated expression of the target gene, *ACS1*, would promote the generation of ET (Fig. 3B). ET plays a key role in the activation of defense-related responses in plants. It can

induce and activate the expression and accumulation of defense-related proteins and antimicrobial peptides, and increase the activity of enzymes related to phenolic metabolism. ET and JA can jointly promote the expression of plant defense-related genes in response to physical injury [27]. Watanabe et al. reported that the expression of three ACC synthase genes, *CM-ACS1*, *CM-ACS3* and, *CM-ACS4*, in the damaged pericarp tissues of winter pumpkin was regulated by ET, JA, and ROS, and that MeJA enhanced the accumulation of *CM-ACS1* transcripts [28]. JA and ET also coordinate and regulate plant stress responses through JAZS-MYC2 and EIN3/EIL1 TFs, especially in resistance to necrotrophic and semitrophic pathogens [29]. The ABA biosynthesis pathway involves both the terpenoid pathway and carotenoid pathway. ABA regulates stomatal closure, enhances water stress resistance, transmit signals, and induces the expression of stress-related genes [30]. ABA is also involved in other physiological processes, such as embryo morphogenesis, seed development, synthesis of storage proteins and oils, germination, leaf senescence, and pathogen defense [31]. In the present study, the target gene *PYL8* was up-regulated (Fig. 3C). *PYL8* has been reported to play an important role in drought stress response in *Arabidopsis* [30]. IAA can enhance the synthesis of ET by increasing the generation of ACC synthase. We found that the IAA - related gene, *ATHB-8* (which was cis regulated by *TCONS_00013800*, $\log_2$FC = 1.35), was upregulated. Baima et al. found that IAA induced the expression of *ATHB-8*, which in

turn positively regulated the activity of procambium and cambium cell differentiation [32]. We found that the expression of *GH3.1* was also up-regulated (Fig. 3D). Overexpression of *CsGH3.1* and *CsGH3.1L* in citrus reduces the susceptibility of plants to citrus canker by inhibiting auxin signal transduction and enhancing defense response, which indicates the important role of auxin homeostasis in citrus disease resistance [33].

We also identified target genes involved in other pathways. Increased expression of the target gene, *Actinidia17020* (encoding Ornithine decarboxylase), would promote the production of putrescine (Fig. 4A). Prabhavathi and Rajam reported that overexpression of a key polyamine biosynthesis gene, *arginine decarboxylase,* in eggplant improved its tolerance to abiotic stresses, such as salinity, drought, heavy metals, cold and heat, as well as its resistance to fungal wilt disease caused by *Fusarium oxysporum* [34]. Although Ca^{2+}-CaM has no intrinsic catalytic activity, it can activate the synthesis of many target proteins involved in a variety of cellular processes. Yu and Du reported that exogenous Ca^{2+} could improve the resistance of tomato seedlings to *B. cinerea* [35]. *Actinidia 35004*, the target gene of *TCONS_00050562*, was also up-regulated in our study. This gene promotes the up-regulation of *CML45* and also participates in calcium signaling pathways (Fig. 4B). Cheng et al. identified a defense related gene, *GhMYB108*, in cotton. Overexpression of *GhMYB108* enhances plant tolerance to pathogens. Further analysis revealed that *GhMYB108* interacts with *CML11* to form a positive feedback regulatory loop, which plays a positive regulatory role in defense against *Verticillium dahliae* infection [36]. *PICBP* was up regulated in yeast-treated kiwifruit in our study. Reddy et al. found that *PICBP* was induced by Ca^{2+}-CaM in response to inducers of plant pathogen response and defense-related genes [37].

Plants can produce secondary metabolites, including terpenoids, phenols, and nitrogenous compounds, that inhibit the growth of pathogens [38]. We observed up-regulated expression of the target gene, *Actindia18707* (encoding 4-coumarateCoA ligase like – 4CL), a key enzyme in the phenylpropanoid metabolic pathway, plays a role in three branch pathways (phenylpropanoid biosynthesis, ubiquinone and other terpenoid-quinone biosynthesis, and phenylalanine metabolism) (Fig. 5). Flavonoids, coumarin, lignin and a variety of phenolic and disease-resistance-related compounds that are synthesized in the phenylpropanoid metabolic pathway, all increase host resistance to pathogen invasion [39–41]. *GPAT5* was up regulated in the yeast-treated group, and Beisson et al. indicated that polyester synthesis was affected in seed coats and roots in *GPAT5-1* and *GPAT5-2* mutants. This suggests that *GPAT5* plays a key role in cell wall biogenesis in seeds and roots, and is necessary for normal seed and root function [42].

TFs are important regulatory factors involved in signal transduction and the regulation of gene expression in response to environmental stress [43]. ROS and Ca^{2+} are involved in signal transduction and activate protein kinases, such as MPKs, to further transduce the signal and regulate the expression of TFs. TFs can bind to *cis*-acting elements in the promoters of target genes which inhibits or activates their transcription. Signal transduction processes and TFs provide plants with the ability to respond and adapt to biotic and abiotic stress [43]. Some transcription factor families (MYB, BZIP, AP2/EREBP, NAC) are specifically associated with stress response. Our results indicated that upstream signal transduction processes promote the expression of *WRKY53, WRKY72, JUB1*, and *AP2* (Fig. 6). Some WRKY TFs are rapidly induced in response to abiotic stress and regulate the expression of stress-related genes [44]. Zhang et al. reported that in peaches treated with *Pichia membranefaciens*, that WRKY-TFs, including *WKRY33, WRKY18* and *WRKY53,* were all up-regulated. This suggests that these TFs play an important role in induced resistance in peaches by stimulating SA-mediated defense response [14]. These results are consistent

with the report on the role of SA-mediated defense response in apples by Zhang et al. [45]. Bhattarai et al. reported that the transcription levels of *SLWRKY72a* and *SLWRKY72b*, homologs of *Arabidopsis thaliana* TFs, in tomato were up-regulated by R gene *Mi-1* mediated transcription during the activation of a disease resistance response [46]. Hou et al. reported that *WRKY72* regulates defense-related genes and JA biosynthesis genes [47]. AP2 is a class of TFs primarily found in plants. TFs in this family play important regulatory roles in many biological and physiological processes, such as plant morphogenesis, response to various stresses, hormone signal transduction, and regulation of metabolites [48]. The AP2/ERF transcription factor SMERF128 actively regulates the biosynthesis of the diterpenoid tanshinone by inducing the expression of *SMCPS1, SmKSL1,* and *SmCYP76AH1* in *Salvia miltiorrhiza* [49].

Plant epidermal waxes play a key role in protecting plants from both abiotic and biotic factors, especially drought. The AP2/EREBP transcription factor MDWRI4 mediates epidermal permeability and enhances plant resistance to abiotic stress by regulating the biosynthesis of apple waxes [50]. In our results, JUB1, an H$_2$O$_2$-induced NAC transcription factor in *Arabidopsis thaliana,* was one of three identified target genes of differentially expressed lncRNAs (Fig. 6). In *jub1-1* knockdown *Arabidopsis* plants, early fruit maturation and abiotic stress tolerance decreased, while the overexpression of *jub1* delayed senescence, inhibited the accumulation of intracellular H$_2$O$_2$, and enhanced tolerance to various abiotic stresses. Transcriptome analysis of *jub1* overexpression revealed that several reactive oxygen species (ROS) responsive genes, including heat shock protein (HSP) and glutathione S-transferase (GST), were up-regulated, which were further induced by treating plants with H$_2$O$_2$ [51].

Some target genes related to plant resistance were found to be associated with ceRNA, including *ZAR1, CCR4, PAD4, NPR4*. Lewis et al. identified a new resistance protein ZAR1, which recognizes *P. syringae* T3SE Hopz1A. HOPZ1A is a member of the YOPJ T3S superfamily. Their study indicated that ZAR1-mediated immunity is independent of known *Arabidopsis* resistant-related genes, suggesting that *ZAR1* has a novel signaling mechanism [52]. Liang et al. reported that CCR4 associated factor 1 (*CAF1*) mRNA plays a role in plant defense response. The tomato line, *DC3000*, is more susceptible to *P. syringae* than wild type plants, while high levels of expression of *AtCAF1a* in transgenic tomato plants, as well as *PR1* and *PR2,* increase disease resistance [53]. Citrus CaF1 (*CsCAF1*), a magnesium-dependent dehydrogenase, is associated with resistance to the citrus canker bacterium, *Xanthomonas* [54]. *PAD4* (log$_2$FC = 1.82) was regulated by DE- lncRNAs in our identified results. *Arabidopsis EDS1* and *PAD4* genes encode lipase-like proteins that play a role in (*R*) gene mediated disease resistance, as well as general plant disease resistance. *PAD4* is used to enhance the initial resistance response. Mutation of *PAD4* or the loss of SA affects the expression of *EDS1*. *PAD4* was demonstrated to be needed to provide salicylic acid in *EDS1*-dependent *R* gene induced resistance. Therefore, the main role of *PAD4* in this pathway may be to actively regulate the accumulation of salicylic acid [55]. *NPR4* was an up-regulated target gene for DE- lncRNA in our results (log$_2$FC = 1.24), NPR1 plays a key role in regulating salicylic acid (SA) -mediated gene expression and disease resistance. NPR3 and NPR4, the paragenetic homologues of NPR1, directly bind to SA, and this binding regulates their interaction with NPR1 [56]. Liu et al. found that a *npr4-1 Arabidopsis* mutant was more sensitive to the fungal pathogen, *Erysiphe cichoracearum*, and that the level of *NPR4* gene expressed in leaves of wild-type plants increased after pathogen attack or treatment with salicylic acid. The results suggested that *NPR4* is required for the fundamental defense against pathogens to be activated and may also be involved in crosstalk between salicylic and jasmonic acid-dependent signaling

pathways [57]. Therefore, we speculated that *PAD4* and *NPR4* may indirectly participate in plant resistance defense by affecting salicylic acid. The functional relationship between the transcription factors identified in our study and downstream defense genes, will be further examined in future studies.

5. Conclusion

The antagonistic yeast, *W. anomalus*, exhibits effective biocontrol activity against blue mold and grey mold on kiwifruit. Results of our present and previous studies, provide information on the potential involvement of lncRNAs in inducing disease resistance in kiwifruit in response to the yeast treatment. Our data indicate that when the antagonistic yeast is perceived by host cells, cell membrane receptors transduce the signal, which activates ET, JA, ABA, IAA, and other hormone signal transduction pathways in kiwifruit. Activation of these pathways modulates the production of defense-related TFs and the production of secondary metabolites. The TFs further mediate the expression of downstream defense-related genes that enhance disease resistance in kiwifruit. The present study provides new and novel information at the molecular level on the possible mechanism by which yeast enhance disease resistance in kiwifruit.

CRediT authorship contribution statement

Qianhua Zhao: Investigation, Data curation, Writing – original draft. **Qiya Yang:** Investigation, Methodology, Software. **Zhenshuo Wang:** Investigation, Methodology, Writing – review & editing. **Yuan Sui:** Data curation, Methodology. **Qi Wang:** Formal analysis. **Jia Liu:** Conceptualization, Supervision, Funding acquisition, Writing – original draft. **Hongyin Zhang:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2021.09.037.

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