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Research

Apple ZAT11, a C2H2-type zinc finger protein, enhances resistance to *Penicillium expansum* by affecting jasmonic acid biosynthesis in apple

Yuchun Lu[1], Kaili Wang[1], Yingying Su[1], Solairaj Dhanasekaran[1,2], Qiya Yang[1] and Hongyin Zhang[1]

[1]School of Food Science and Engineering, Jiangsu University, Zhenjiang, 212013, Jiangsu, China; [2]Department of Biotechnology, PSGR Krishnammal College for Women, Coimbatore, 641004, India

Author for correspondence:
Hongyin Zhang
Email: zhanghongyin126@126.com

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Summary

- Blue mold, caused by *Penicillium expansum*, is a prevalent postharvest disease of apple and significantly limits apple quality. Apple have key genes underlying their biological stress responses and corresponding functions that remain largely unexplored. Here, we identified MdZAT11, a Cys2/His2 (C2H2)-type zinc finger protein, as a crucial positive regulator of apple resistance to *P. expansum* infection.
- Utilizing transcription factor-centered yeast one-hybrid (TF-centered Y1H) and RNA sequencing analyses, we discovered 1059 potential target genes of MdZAT11, with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment indicating significant enrichment in stress-responsive pathways, particularly jasmonic acid (JA) biosynthesis.
- Further investigation revealed that MdZAT11 enhances the expression of JA biosynthesis genes (*MdAOS*, *MdAOC*, *MdLOX6*, and *MdLOX3.1*) by directly binding to their promoters, as demonstrated by electrophoretic mobility shift assays (EMSA), dual-luciferase reporter (DLR) assays, and RT-qPCR analysis, thereby augmenting JA biosynthesis.
- Taken together, our findings advance understanding of the transcriptional regulation of defense against *P. expansum* in apple and identify *MdZAT11* as a candidate gene worth further investigation for disease resistance in this species.

Introduction

Apple (*Malus domestica*) is among the most popular fruits worldwide. However, apples are prone to damage during transportation and storage, leading to pathogen infestation and apple rot. Blue mold, caused by *Penicillium expansum*, is the most prevalent postharvest fungal disease of apple, resulting in a 15–20% yield loss during storage (Jijakli & Lepoivre, 2004; Wang *et al.*, 2023a ; Al Riachy *et al.*, 2024). A major approach to improving disease resistance in apples involves locating resistance-associated genes and analyzing their functional mechanisms.

Plant hormones are endogenous trace compounds within plants that can be induced by external stimuli and play critical roles in growth, development, reproduction, and stress responses (Waadt *et al.*, 2022). Jasmonic acid (JA), a fatty acid derivative, is a critical hormone that serves a central function in regulating defense reactions against pathogens (Chen *et al.*, 2024). JA is produced through the octadecanoid pathway using linolenic acid and through the hexadecanoid pathway using hexadecatrienoic acid, catalyzed sequentially by lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC), and completed via β-oxidation (Wasternack & Hause, 2013; Monte, 2023). Clarifying these biosynthetic and regulatory pathways is vital for understanding JA-mediated disease resistance. In tobacco, NaWRKY70 plays a crucial role in augmenting JA-isoleucine (JA-Ile) levels by directly upregulating the expression of pivotal genes involved in the JA pathway, including *NaAOS* and *NaJAR4*. This upregulation consequently enhances plant resistance to the pathogen *Alternaria alternata* (Song & Wu, 2024). Under typical physiological conditions, plant cells maintain low concentrations of JA, while Jasmonate ZIM-domain (JAZ) proteins are present and bind to MYC2, a basic helix–loop–helix (bHLH) transcription factor, thereby repressing the expression of JA-responsive genes. In response to stress, JA levels increase within plant cells, alleviating the inhibitory effect of JAZ proteins on MYC2 and activating JA-responsive gene expression (Chini *et al.*, 2007; Kazan & Manners, 2013). Transcription factors are recognized as key regulators of the JA signaling pathway. Specifically, CpWRKY50 directly binds to the promoters of *CpMYC2* and *CpPR4*, thereby enhancing JA-mediated defense responses and improving papaya resistance to *Colletotrichum brevisporum* (Yang *et al.*, 2024). Recent studies have begun to elucidate the transcriptional mechanisms underlying JA-mediated fruit defense. For instance, agaro-oligosaccharides (AO) activate JA

biosynthesis and signal transduction in peach fruit, leading to the upregulation of *PpLOX5*, *PpAOC*, *PpAOS*, and *PpOPR2*, increased JA content, and subsequent PpMYC2-mediated activation of defense genes such as *PpPGIP1* (Li *et al.*, 2025). These findings highlight TF-mediated regulation of JA-mediated defense by targeting downstream defense genes or signaling regulators in postharvest fruit. However, whether TFs directly target JA biosynthetic genes to modulate resistance against *P. expansum* in apples remains poorly understood.

As one of the most extensive TF families, C2H2 (Cysteine-2/Histidine-2) zinc finger proteins (C2H2-ZFPs) play a crucial role in regulating plant responses to various stresses (Han *et al.*, 2020). So far, C2H2-ZFPs have been identified in various plants (Lu *et al.*, 2024). C2H2-ZFPs differ in the number of zinc finger motifs, each stabilized by zinc ions binding to two Cys and two His residues (Laity *et al.*, 2001; Gupta *et al.*, 2012; Kiełbowicz Matuk, 2012). Plant C2H2-ZFPs contain conserved QALGGH motifs that contribute to DNA binding and gene regulation (Laity *et al.*, 2001; Han *et al.*, 2020). In recent studies, functional analyses have demonstrated that CgZAT11 acts as a transcriptional activator in lignin biosynthesis by directly binding to the promoters of *CgCAD8* and *CgPOD16*, thereby promoting lignin accumulation and the thickening of secondary cell walls (Huang *et al.*, 2026). These findings indicate that ZAT11 can act as a transcriptional regulator of secondary metabolism in fruit tissues, although its role in defense responses against necrotrophic pathogens remains less well understood. Increasing evidence indicates that C2H2-ZFPs are crucial in plant immunity. For example, VvZFP11 increases plant resistance to powdery mildew via the salicylic acid (SA) pathway (Li *et al.*, 2022). GmZFP03 binds to and activates *SOD1* transcription, enhancing soybean tolerance to *Phytophthora sojae* (Li *et al.*, 2023). PtrC2H2.2–6 reduces drought resistance in poplar leaves by inhibiting key genes involved in cutin and wax synthesis, specifically *PtrCYP86A7/A8*. However, under drought stress, PtrPPK1 mediates its degradation, thereby lifting the inhibition on *PtrCYP86A7/A8*, which promotes wax accumulation and enhances stress adaptability (Zhao *et al.*, 2026). In another study, PtrCBF1 binds to the *PtrZAT12* promoter to activate its transcription, which may then upregulate various Cold-responsive (*COR*) genes to alleviate the effects of cold stress (Zhang *et al.*, 2024). Collectively, these findings indicate the importance of C2H2-ZFPs in plant responses to adverse environmental conditions. While the functions of numerous C2H2-ZFPs in stress tolerance have been identified, their specific roles and mechanisms, particularly in the defense against pathogen infection in woody plants, remain largely unexplored.

Earlier research showed that the gene *MdZAT11*, which encodes a C2H2-ZFP in apple, was upregulated during *P. expansum* infection. This study investigated whether MdZAT11 is important for enhancing apple resistance against *P. expansum* by analyzing its functional role and underlying mechanisms. Using transient expression assays, transcriptome analysis, electrophoretic mobility shift assays (EMSA) and dual-luciferase reporter (DLR) assays, we found that *MdZAT11* enhances apple resistance to *P. expansum*. Mechanistically, MdZAT11 directly binds to the promoters of four key JA biosynthetic genes, namely *MdAOS*, *MdAOC*, *MdLOX6*, and *MdLOX3.1*, thereby activating their transcription, increasing endogenous JA levels, and ultimately augmenting defense responses. Our findings suggest that the MdZAT11-mediated JA biosynthesis module may be a pivotal component of hormone-mediated disease resistance in postharvest apple fruit.

Materials and Methods

Plant materials and pathogen inoculation

Apple fruits (*Malus domestica* Borkh.) were harvested at commercial maturity in Yantai, Shandong Province, China. Fruits were transported to the laboratory and stored at 4°C before use. Apple fruits of uniform maturity and free of mechanical damage were selected for the experiment. The apples underwent a rinsing process and were subsequently randomly distributed into sterilized fruit baskets, allowing the surface moisture to air-dry at room temperature.

Tobacco (*Nicotiana benthamiana* Domin) plants used for experiments were grown in an air-circulating incubator. Leaves from 6-week-old tobacco were selected for transient expression assays (Si *et al.*, 2024).

Our research team successfully isolated, identified, and preserved *Penicillium expansum* from the surface of a decayed apple. The spore concentrations of *P. expansum* were standardized to 5×10^6 and 1×10^7 spores ml^{-1} (adapted from Wang *et al.*, 2023b).

Three uniformly distributed perforations, each measuring 5 mm in depth and 5 mm in diameter, were creatd in the equatorial region of each washed apple using a sterile hole punch. The juice from these perforations was allowed to air-dry for subsequent use. Subsequently, 30 µl of a *P. expansum* spore suspension was inoculated into the holes. Inoculated fruits were tightly sealed with plastic wrap and stored in an incubator at a constant temperature of 25°C and 80–95% relative humidity. Each treatment group consisted of 12 fruits. Apple tissue surrounding the 1 mm wound in contact with the spore suspension was excised using a sterile scalpel. Tissue located 3 mm from the wound (excluding the peel) was collected, powdered, and stored at −80°C for future analysis.

Isolation and bioinformatics analysis of MdZAT11

The MdZAT11 coding sequence (CDS) was amplified from apple cDNA using specific primers (see Supporting Information Table S1). The amino acid sequence was analyzed using DNAMAN, and a phylogenetic tree was created using MEGA software. NovoPro (https://www.novopro.cn/tools/) was used to analyze the molecular weight, isoelectric point and nuclear localization signal. Furthermore, the conserved domain of the MdZAT11 protein was examined through the SMART database (https://smart.embl.de/). The structure of MdZAT11 was predicted and molecular docking was performed using NeuroSnap (Neurosnap Inc., 2022), which utilizes the Chai-1 model

(Boitreaud *et al.*, 2024). The predicted structure was then visualized, annotated, and analyzed using PyMOL (The PyMOL Molecular Graphics System, v.3.1.6.1, Schrödinger, LLC). PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was utilized to analyze *cis*-regulatory elements in the *MdZAT11* promoter sequence, and the results were visualized using TBtools.

Subcellular localization

The CDS of *MdZAT11*, excluding the stop codon, was inserted into the pCAMBIA1300 vector to generate the MdZAT11-GFP fusion expression construct. The empty vector pCAMBIA1300, along with the MdZAT11-GFP construct, was individually introduced into the *Agrobacterium tumefaciens* strain GV3101 (P19) and transiently expressed in *N. benthamiana* leaves via agroinfiltration (Zeng *et al.*, 2024). The agroinfiltrated tobacco samples were incubated in darkness for a duration of 12 h, followed by their transfer to an incubator set at 25°C with a photoperiod of 16 h : 8 h, light : dark. After 48 h, fluorescence signals were detected using laser confocal microscopy (TCS SP5; Leica, Wetzlar, Germany).

Transcriptional self-activation assays

To assess the transcriptional self-activation activity of MdZAT11 in yeast, the *MdZAT11* CDS was cloned into the pGBKT7 vector to generate the MdZAT11-pGBKT7 construct. Subsequently, both the pGBKT7-Empty and MdZAT11-pGBKT7 vectors, together with the empty pGADT7 vector, were introduced into the yeast strain Y2HGold. The transcriptional self-activation activity was evaluated by observing the growth of the transformants on a synthetic dropout (SD) medium deficient in tryptophan, histidine, adenine and leucine (SD/-Trp-His-Ade-Leu) (adapted from Bao *et al.*, 2022).

Transient expression and phenotype analysis

The empty pCAMBIA1300 vector and the CaMV 35S promoter-driven constructs 35S::MdZAT11-pCAMBIA1300 were individually introduced into *A. tumefaciens* strain LBA4404, with the empty vector serving as the control. The transformed *A. tumefaciens* were subsequently grown to an OD_{600} of 0.6–0.8, collected by centrifugation, and resuspended in infiltration buffer. The method was slightly modified from Wang *et al.* (2023c).

The mature apple fruits were used for the transient expression assays. Apples, washed and well-drilled as described previously, were dried and inoculated with 30 µl of *A. tumefaciens* suspension carrying either the empty pCAMBIA1300 vector or the 35S::MdZAT11-pCAMBIA1300 construct. After 2 h, apples were tightly sealed with plastic wrap and incubated for 12 h in the dark. Next, 30 µl of a 5×10^6 spores ml^{-1} suspension of *P. expansum* was inoculated into the same wound sites. The fruits were incubated in the laboratory. Three days after inoculation, the lesion diameters were measured, and photographs of the apple fruit were taken. Samples of apple tissues transiently expressing empty vector or *MdZAT11* were used for gene expression analysis and JA content determination. Disease incidence and lesion diameter of apple from different groups were measured. Each set contained 12 apples, and the experiment was repeated three times.

Measurement of JA content

An enzyme-linked immunosorbent assay (ELISA) was conducted to quantify the JA content, utilizing an ELISA kit purchased from Shanghai Ruifan Biotechnology Co., Ltd. The JA content was expressed in units of nanomoles per kilogram of fresh weight (nmol/kg FW).

TF-centered Y1H and RNA-Seq

The TF-centered Y1H assay was performed by Nanjing Ruiyuan Biotechnology Co., Ltd (Nanjing, China), using a 7-bp random motif library from the yeast strain Y187. The TF-centered Y1H assay was performed as previously described (Ji *et al.*, 2014). pGADT7-MdZAT11 was introduced into competent yeast cells containing the random DNA library. The yeast was transferred to plates containing SD/-His/-Trp/-Leu medium supplemented with 75 mM 3-AT (to suppress background growth). Positive colonies were sequenced and analyzed using PLACE and PlantCARE to identify known *cis*-acting motifs.

The RNA-seq library was generated at 0, 6, 12, and 24 h after *P. expansum* inoculation (Table S2). Sequencing of the RNA was performed using the Illumina platform by OmicShare Biotechnology Co. (Guangzhou, China). Sequencing was performed on an Illumina NovaSeq 6000 platform with paired-end 150 bp reads (PE150), generating *c.* 6 Gb of data per sample. The resulting clean reads were aligned to the GDDH13_v1.1 apple genome assembly. Differentially expressed genes were identified based on FDR (false discovery rate) < 0.05 and $|\log_2$ fold change$| > 1$ (Love *et al.*, 2014).

Electrophoretic mobility shift assay

The CDS lacking stop codon for *MdZAT11* was cloned into the pET30a vector to create protein expression vector, which was then transformed into *Escherichia coli* Rosetta (DE3). Probes were designed based on the TF-centered Y1H assay and included both biotin-labeled and unlabeled probes. EMSA binding analysis was performed using a Chemiluminescent EMSA Kit from Beyotime Biotechnology (Shanghai, China).

Dual-luciferase reporter assay

Promoter fragments of *MdAOS*, *MdAOC*, *MdLOX6*, and *MdLOX3.1* were cloned into the pGreenII-0800-LUC reporter vector, whereas the full-length CDS of MdZAT11 were inserted into the pGreenII-62SK effector vector. An empty vector was used as a control. All vectors were transformed into *A. tumefaciens* strain GV3101 (Psoup) and co-infiltrated into 6-week-old tobacco leaves. At 3 d post-infiltration, dual-luciferase activity

was measured using the Dual-Luciferase Reporter Assay Kit (Beyotime Biotechnology, Shanghai, China), and the fluorescence signals from LUC were detected via a chemiluminescence imaging system. The experiment was repeated three times for biological replicates.

RNA extraction and quantitative real-time PCR analysis

Total RNA was isolated from apple samples using the Spin Column Plant Total RNA Purification Kit by Sangon Biotech Co., Ltd (Shanghai, China). The RNA concentration was determined, and cDNA was synthesized using a reverse transcriptase kit (Vazyme, Nanjing, China). Gene expression levels were then quantified using the LightCycler® 96 Instrument (Roche) and the ChamQ Blue Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). RT-qPCR was performed using the apple actin gene as the internal reference. All primers are listed in Tables S3 and S4. Results were normalized to the reference gene and analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Statistical analysis

Significant differences for pairwise comparisons were evaluated using Student's *t*-test, with significant differences indicated by asterisks as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data were analyzed by one-way ANOVA. Asterisks indicate significant differences. **, $P < 0.01$; ***, $P < 0.001$.

Results

Isolation and expression analyses of MdZAT11

In a previous study, we used transcriptome sequencing to identify genes in the C2H2-ZFP family in apple and analyzed the expression of 15 of these genes at key points during *P. expansum* infection (Lu *et al.*, 2023). The expression of *MdZAT11* was significantly upregulated following *P. expansum* inoculation. Notably, at 3 and 6 h post-infection (hpi), the expression level of *MdZAT11* was increased by 2.54-fold and 12.89-fold, respectively, compared to the baseline level at 0 h (Fig. 1a), indicating a potential key role in apple defense against *P. expansum*. Subsequently, *MdZAT11* was isolated from apples using PCR. Protein analysis showed that the protein contained two C2H2 zinc finger domains with a QALGGH and an EAR motif (Fig. 1b). MdZAT11 is composed of 185 amino acids, with a molecular mass of 20.61 kDa and an isoelectric point of 8.26. Subsequently, a phylogenetic analysis of comparing MdZAT11 with 13 other plant C2H2-ZFPs revealed that MdZAT11 shares the closest evolutionary relationship with PcZAT11 (XP068319055.1) from *Pyrus communis* (Fig. 1c). To investigate the regulation of *MdZAT11* expression, the 2000 bp region upstream of its promoter was analyzed for *cis*-regulatory elements. The analysis revealed multiple light-responsive elements (e.g. G-Box, GATA motif) and MeJA-responsive elements (TGACG and CGTCA motifs) (Fig. 1d), suggesting that *MdZAT11* expression might be affected by MeJA. Visualization of MdZAT11 using the PyMOL molecular graphics system showed a tetrahedral configuration at the active site, with a zinc ion coordinated with two cys and two his residues (Fig. 1e). These results indicate that MdZAT11 may play a critical role in facilitating DNA binding.

MdZAT11 localizes to the nucleus and lacks self-activation activity in yeast

Bioinformatics analysis suggested that MdZAT11 is likely localized in the nucleus. To confirm this prediction, the subcellular localization of MdZAT11 was examined through a transient expression assay in tobacco. Confocal microscopy of the tobacco cells showed that the GFP signal from the control vector was distributed throughout the cell. By contrast, the *MdZAT11*-GFP fusion protein signal was confined to the nucleus, exhibiting complete colocalization with the DAPI (4′,6-diamidino-2-phenylindole) stain (Fig. 1f). These findings suggest that MdZAT11 functions as a nuclear protein. Subsequently, we performed a yeast self-activation assay to assess whether MdZAT11 possesses transcriptional activation activity. Yeast containing pGBKT7-MdZAT11 and pGADT7, as well as pGBKT7-empty and pGADT7, grew well on SD/−Trp-Leu but did not grow on SD/-Trp-His-Ade-Leu (Fig. 1g). The result indicate that MdZAT11 does not possess transcriptional self-activation activity in yeast.

Transient expression of *MdZAT11* enhances resistance to *P. expansum*

To validate the function of MdZAT11 in resistance against *P. expansum*, apple fruits were transiently expressed with *Agrobacterium tumefaciens* carrying either the empty pCAMBIA1300 (control) or the MdZAT11-pCAMBIA1300 construct (*MdZAT11*-OE). *MdZAT11*-OE apple exhibited slower disease progression than control, with significantly reduced lesion diameters (Fig. 2a–c). The expression of defense-related genes, including *PR4*, was analyzed. As illustrated in Fig. 2d, *MdZAT11* expression was elevated by 5.02-fold in the *MdZAT11*-OE group compared to the control group at 3 d after inoculation. As illustrated in Fig. 2e, the *PR4* expression was significantly higher in the MdZAT11-OE group than in the control group at 3 d after inoculation. Collectively, these results suggest that transient expression of *MdZAT11* in apple enhances disease resistance to *P. expansum*.

Identification of MdZAT11-binding motifs

To further investigate the influence of MdZAT11 on the regulatory network governing disease resistance in apple fruit, we performed the TF-centered Y1H technique to identify potential target sites directly regulated by MdZAT11, focusing on the screening of motifs within its binding sites. Following this methodology, a pGADT7-MdZAT11 yeast library incorporating 7-bp random fragments, as described by Ji *et al.* (2014), was constructed and screened. The constructs pGADT7-MdZAT11 + pHIS2-p53 and pGAD53m + pHIS2-p53 were introduced into

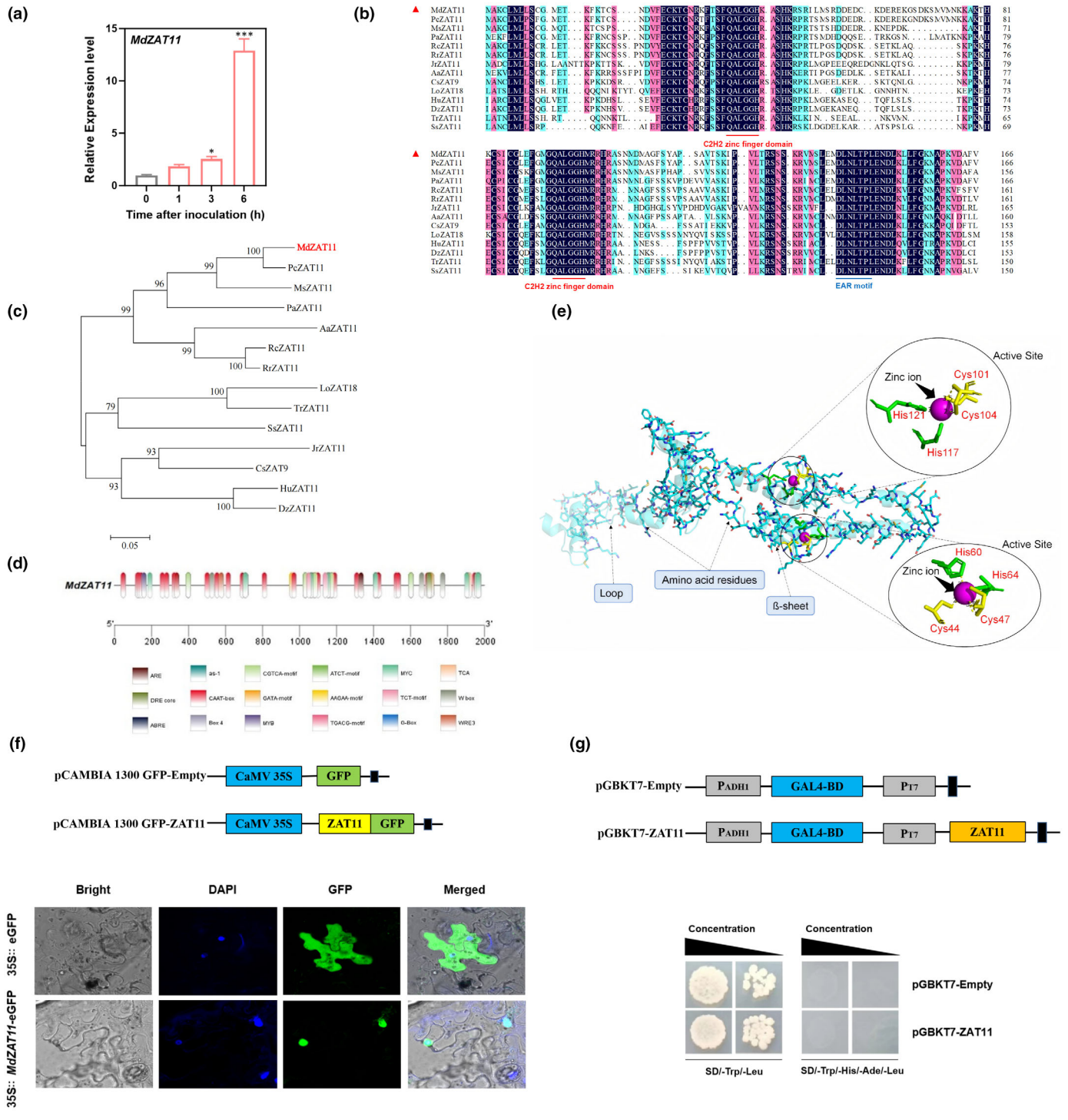

Fig. 1 Sequence analysis and characteristics of MdZAT11. (a) *MdZAT11* expression levels in apple at 0, 1, 3, and 6 h post-inoculation with *Penicillium expansum*. Data are presented as mean ± SD ($n = 3$) (Student's *t*-test, *, $P < 0.05$; ***, $P < 0.001$). (b) Multiple sequence alignment of MdZAT11 and 13 other C2H2 zinc finger proteins. The C2H2 zinc finger domain and an ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif are shown in red and blue lines, respectively. (c) Maximum likelihood phylogenetic tree of MdZAT11 and other C2H2 zinc finger proteins. The numbers of each node indicate the percentage bootstrap support. The scale bar indicates the genetic distance based on branch lengths. (d) *Cis*-elements in the promoter sequence of *MdZAT11*. (e) The structure of the MdZAT11 was predicted using Neurosnap (https://neurosnap.ai/) and visualized using PyMOL molecular graphics system. The zinc-binding sites are highlighted, with yellow and green colors, representing the cysteine (two Cys) and histidine (two His) amino acids, respectively. The coordination of the zinc ion is enlarged in the inset. (f) Schematic representation of the vector used for subcellular localization assays and the subcellular localization of MdZAT11 in tobacco (*Nicotiana benthamiana*) epidermis. Fusion constructs (35S:MdZAT11-GFP) or empty vector (35S:GFP) were transiently expressed in tobacco leaves. Microscopic observation of epidermal cells was performed under bright field and fluorescence (green for GFP and blue for DAPI). (g) Schematic representation of the vector used for transcriptional self-activation assays and validation of MdZAT11 in the yeast system.

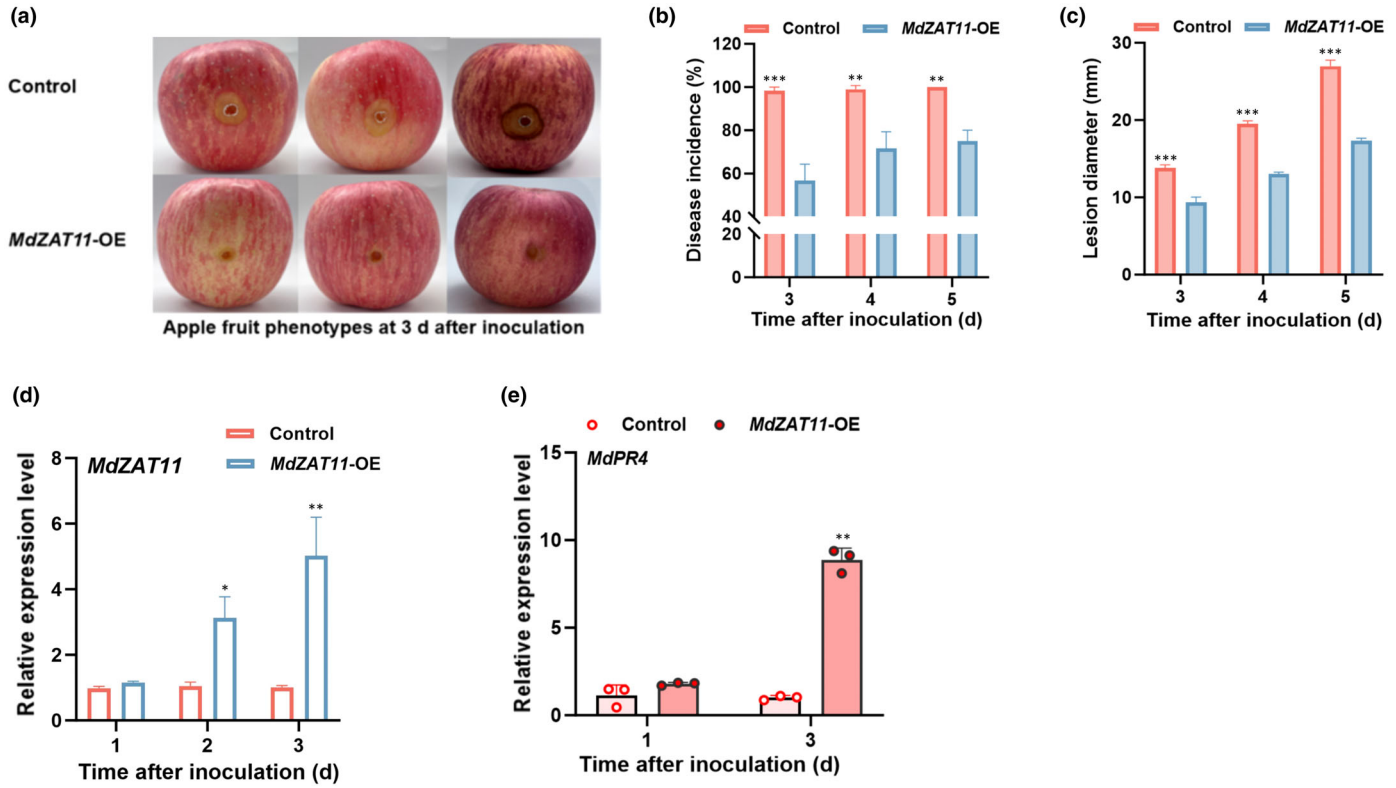

Fig. 2 Disease resistance to *Penicillium expansum* as affected by transient expression of *MdZAT11*. The empty pCAMBIA1300 vector was used as the control. (a) Apple fruit phenotypes at 3 d after inoculation. (b) Effect of *MdZAT11* transient expression on disease incidence. Data are presented as mean ± SD (n = 3). (c) Effect of transient expression of *MdZAT11* on the lesion diameter of disease spots. Data are presented as mean ± SD (n = 3). (d) Relative expression levels of *MdZAT11* at 1, 2, and 3 d post-inoculation. Values are mean ± SD (n = 3). (e) Expression analysis of resistant genes at 1 d and 3 d post-inoculation. Values are mean ± SD (n = 3) (Student's t-test, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Y187 yeast cells and cultured on SD/-Trp/-Leu /-His plates to isolate positive clones. The clones were then transferred to plates lacking 3-AT at different concentrations. Background screening showed minimal colony growth at 50 mM 3-AT, whereas no colonies appeared at 75 mM. Therefore, 75 mM 3-AT was used for the subsequent library screening. The validated pGADT7-MdZAT11 bait plasmid was subsequently screened against the yeast library with the motifs and placed on screening plates containing 75 mM 3AT. A total of 48 single colonies were obtained from this screening (Fig. 3a–c). Seqman and BLAST were used to analyze the 48 motifs, leading to the discovery of nine distinct motifs with possible binding affinity to MdZAT11 after removing duplicates. Subsequent one-to-one point plate verification on a yeast screening medium confirmed that MdZAT11 could recognize all nine motifs (Fig. 3d). These motifs predominantly contain the core sequence 'MYBPZM', functioning as *cis*-acting elements. Additionally, other *cis*-acting elements were identified, including CURECORECR, CGACGOSAMY3, CACTFTPPCA1, and PALBOXAPC (Fig. 4a).

Investigation and expression analysis of potential MdZAT11 target genes

An integrative analysis of TF-centered Y1H assays and RNA-seq data identified 1059 genes that both harbor MdZAT11-binding elements in their promoters and are differentially expressed upon infection, defining them as potential targets of MdZAT11 (Fig. 4b). Of these genes, 611 (57.7%) exhibited increased transcript levels and 448 (42.3%) showed decreased expression (Fig. 4c). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the 611 upregulated genes revealed significant enrichment in alpha-linolenic acid metabolism (ko00592), mitogen-activated protein kinase signaling pathway (ko04016), plant-pathogen interaction (ko04626), and biosynthesis of secondary metabolites (ko01110) in plants (Fig. 4d). These findings suggest that MdZAT11 may play a pivotal role in metabolic and signal transduction pathways, potentially regulating various biological processes by modulating critical downstream targets. Notably, alpha-linolenic acid metabolism was the most significantly enriched pathway, implicating MdZAT11 in the transcriptional regulation of JA biosynthesis upon *P. expansum* infection.

Alpha-linolenic acid metabolism serves as the entry point for JA, a vital phytohormone that regulates plant defenses against necrotrophic pathogens. This pathway is initiated by the oxygenation of α-linolenic acid through the action of LOXs, followed by a series of enzymatic reactions mediated by AOS, AOC, and 12-oxophytodienoate reductase (OPR) to produce JA, which is then conjugated to isoleucine by JAR1 to form the bioactive derivative JA-isoleucine (JA-Ile) (Wasternack & Hause, 2013). Considering the substantial enrichment of α-linolenic acid

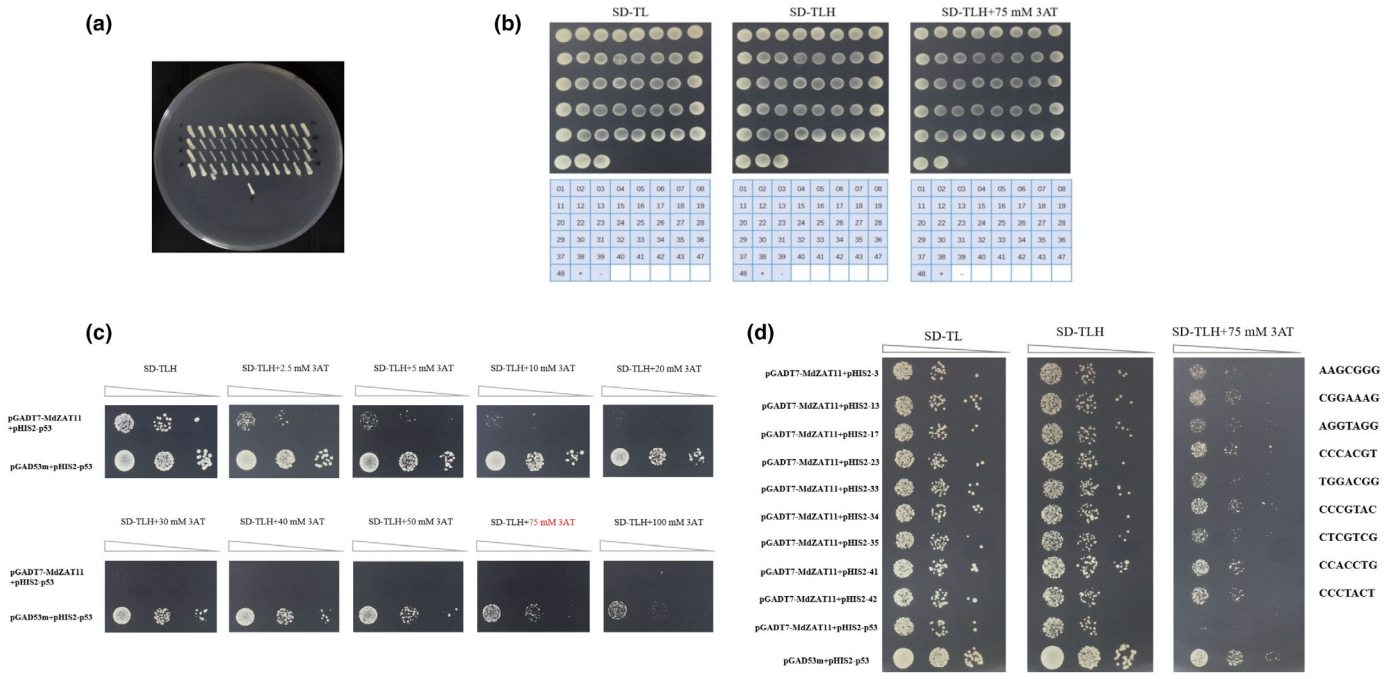

Fig. 3 Identification of MdZAT11 binding motifs by transcription factor-centered yeast one-hybrid (TF-centered Y1H). (a) Y1H screening on SD/-Trp/-Leu/-His + 75 mM 3-amino-1,2,4-triazole (3-AT) yielded 48 colonies. (b) Yeast co-transformation revalidation. Positive control (+): pGAD53m + pHIS2-p53; negative control (−): pGADT7-MdZAT11 + pHIS2-p53. Blue: growth; White: no growth. (c) The optimal 3-AT concentration for background suppression was determined by testing different concentrations on selective medium. At 75 mM 3-AT, no background colonies appeared, so this concentration was used for library screening. (d) One-to-one Y1H analysis was performed to validate the interaction between MdZAT11 and the nine candidate motifs. DNA sequences on the right indicate the corresponding motifs obtained from the apple genome scan.

metabolism among MdZAT11 target genes and the central role of this pathway in JA biosynthesis, we prioritized this pathway for further study.

To illustrate the transcriptional dynamics of JA biosynthesis during *P. expansum* infection, we constructed a comprehensive metabolic map showing the expression profiles of key pathway genes at 0, 6, 12, and 24 hpi (Fig. 4e). This analysis revealed a coordinated upregulation of core JA biosynthetic genes. By combining this expression landscape with our TF-centered Y1H screening data, we identified *MdAOS*, *MdAOC*, *MdLOX6*, and *MdLOX3.1* as potential MdZAT11-regulated genes. Our analysis revealed potential MdZAT11-binding sites in the promoters of these genes, suggesting that this C2H2 zinc finger protein may regulate their transcription to promote JA-mediated resistance against *P. expansum* in apple fruit.

MdZAT11 activates the genes in the JA biosynthesis pathway

As shown in Fig. 5a, RNA-seq analysis indicated a significant early upregulation of these four JA biosynthetic genes in *P. expansum*-infected apples, which was further confirmed by RT-qPCR (Fig. 5b).

We examined the promoter sequences of these genes for potential MdZAT11-binding motifs to determine if MdZAT11 directly regulates them. Bioinformatic analysis identified 1–3 potential binding sites within the promoter regions of *MdAOS*, *MdAOC*, *MdLOX6*, and *MdLOX3.1* (Fig. 5c). EMSA demonstrated that MdZAT11 specifically binds to these motifs. As anticipated, a shifted band was observed when the MdZAT11 protein was incubated with the biotin-labeled probe. Additionally, increasing the amount of the cold probe reduced the shift signal, while a mutant probe did not affect the binding between the MdZAT11 protein and the biotin-labeled probe (Fig. 5d).

To further elucidate whether MdZAT11 directly activates the transcription of these four genes, we performed DLR assays in tobacco. The promoter fragments of *MdAOS*, *MdAOC*, *MdLOX6*, and *MdLOX3.1* were cloned into the pGreenII-0800-LUC reporter vector, and MdZAT11 was cloned into the pGreenII-62SK effector vector. The luminescence signal was significantly enhanced, and LUC/REN activity was higher when MdZAT11 was co-expressed with each promoter construct compared to the empty vector control (Fig. 6a). These results indicate that MdZAT11 functions as a transcriptional activator of genes involved in JA biosynthesis.

Transient expression of MdZAT11 in apple fruits significantly upregulated *MdAOS*, *MdAOC*, *MdLOX6*, and *MdLOX3.1*, concomitantly increasing endogenous JA content (Fig. 6b,c). These findings suggest that MdZAT11 directly binds to the promoters of these JA biosynthetic genes and activates their transcription, which is associated with JA accumulation and enhanced resistance against *P. expansum*.

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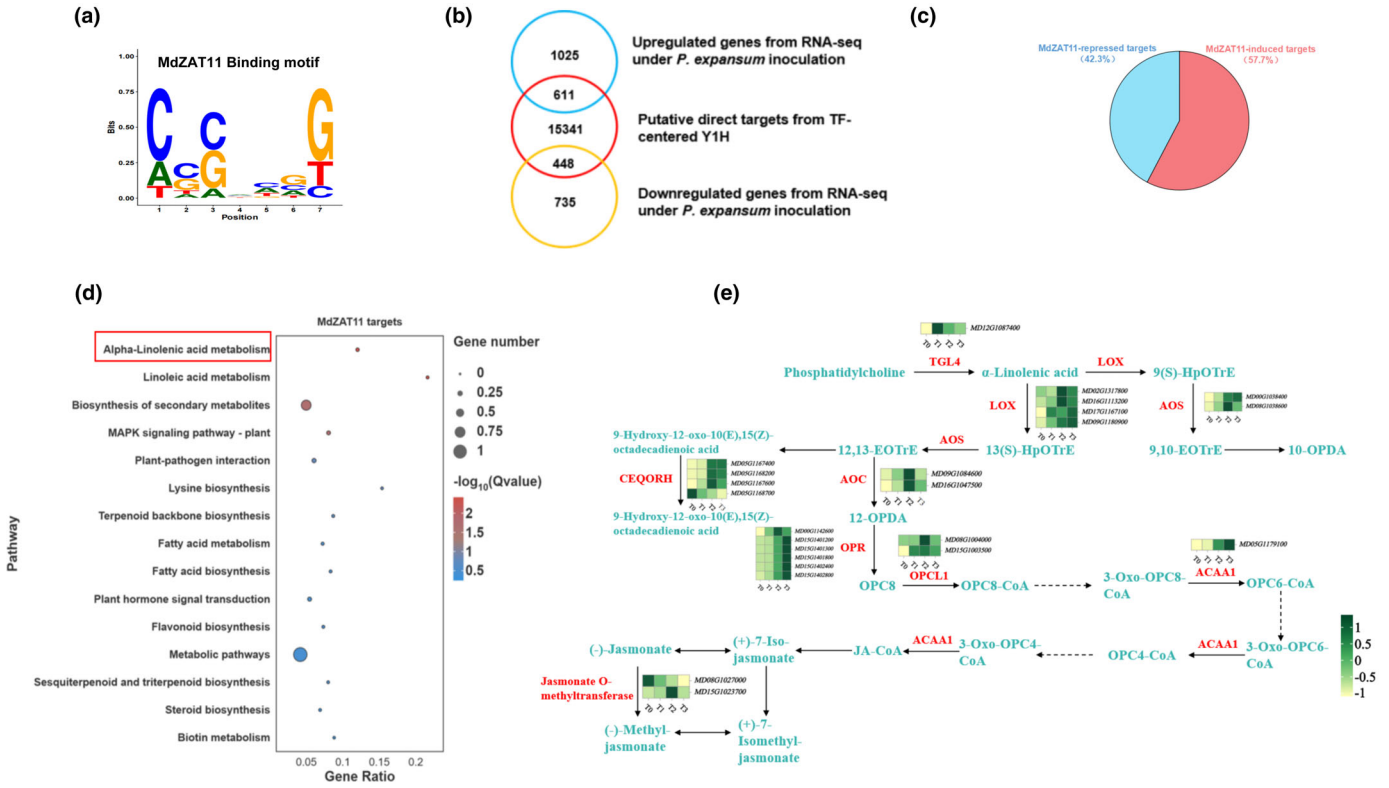

Fig. 4 Analysis of potential target genes of MdZAT11. (a) Diagram of MdZAT11 binding site. (b) The Venn diagram showing the overlap between genes containing MdZAT11-binding motifs in their promoters and differentially expressed genes upon *Penicillium expansum* infection. (c) The proportion of upregulated and downregulated genes among the 1059 potential targets of MdZAT11. (d) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the upregulated genes among these 1059 potential targets. The gene ratio represents the number of peak-associated genes in each pathway as a percentage of the number of all target genes. The dot size reflects the normalized gene number, and the color intensity corresponds to the $-\log_{10}$(Q value), with red indicating higher enrichment significance. (e) Heatmap visualization of transcript levels for these upregulated genes involved in α-linolenic acid metabolism. Gene expression values, represented as FPKM, were normalized and scaled to a range of −1 to 1 for heatmap generation. Time points T0, T1, T2, and T3 correspond to 0, 6, 12, and 24 h post-inoculation (hpi) with *P. expansum*, respectively.

Discussion

Apples are a major fruit crop, but their yield and quality are threatened by environmental stresses, particularly postharvest blue mold disease (Wang *et al.*, 2023a). Plants have evolved resistance mechanisms involving transcription factors that regulate gene expression (Han *et al.*, 2020; Bao *et al.*, 2022; Li *et al.*, 2023). Advances in genomics have helped identify genes and TFs linked to apple resistance to blue mold. However, the role of C2H2-ZFPs in apple's fungal immunity is still not well understood. During various stages of *P. expansum* infection, bioinformatics and transcriptome analyses have identified differential expression of C2H2-ZFP genes (Lu *et al.*, 2023). In this study, our current study identified *MdZAT11* upregulation in response to *P. expansum* infection (Fig. 1a). Our observations suggest that MdZAT11 may contribute to apple resistance against *P. expansum*. Further research is required to elucidate the underlying molecular mechanisms of this resistance.

C2H2-ZFPs are well-studied TFs that respond to environmental stresses by interacting with proteins or modulating gene expression. The EAR motif is the most common transcriptional repression motif in plants and is found in many C2H2-ZFPs, including ZAT5, ZAT10, and ZAT18, which contain EAR domains that enable them to repress downstream gene expression (Sakamoto *et al.*, 2004; Bao *et al.*, 2022; Gao *et al.*, 2022). MdZAT5, a nuclear C2H2-ZFP, enhances apple drought tolerance by regulating genes associated with drought response and microRNA biosynthesis (Bao *et al.*, 2022). Based on the finding that MdZAT5 lacks transcriptional self-activation activity in yeast, it was proposed that MdZAT5 may repress its target genes by recruiting the co-repressor MdTPL2 through its EAR motif under normal conditions. Under drought stress, the expression level of MdTPL2 decreases, leading to the release of MdZAT5, which subsequently activates the expression of target genes, thereby augmenting drought resistance (Bao *et al.*, 2022). By contrast, ZAT18, induced by *Pseudomonas syringae*, reduces plant disease resistance by suppressing *EDS1* expression and decreasing SA levels (Gao *et al.*, 2022). Similarly, ZmC2H2–149 lowers drought tolerance in maize by inhibiting *Hydroxysteroid dehydrogenase 1* (*HSD1*) (Liu *et al.*, 2024). In this study, we identified MdZAT11, an apple C2H2-ZFP with an EAR domain at its C-terminus (Fig. 1b). Phylogenetic analysis revealed that

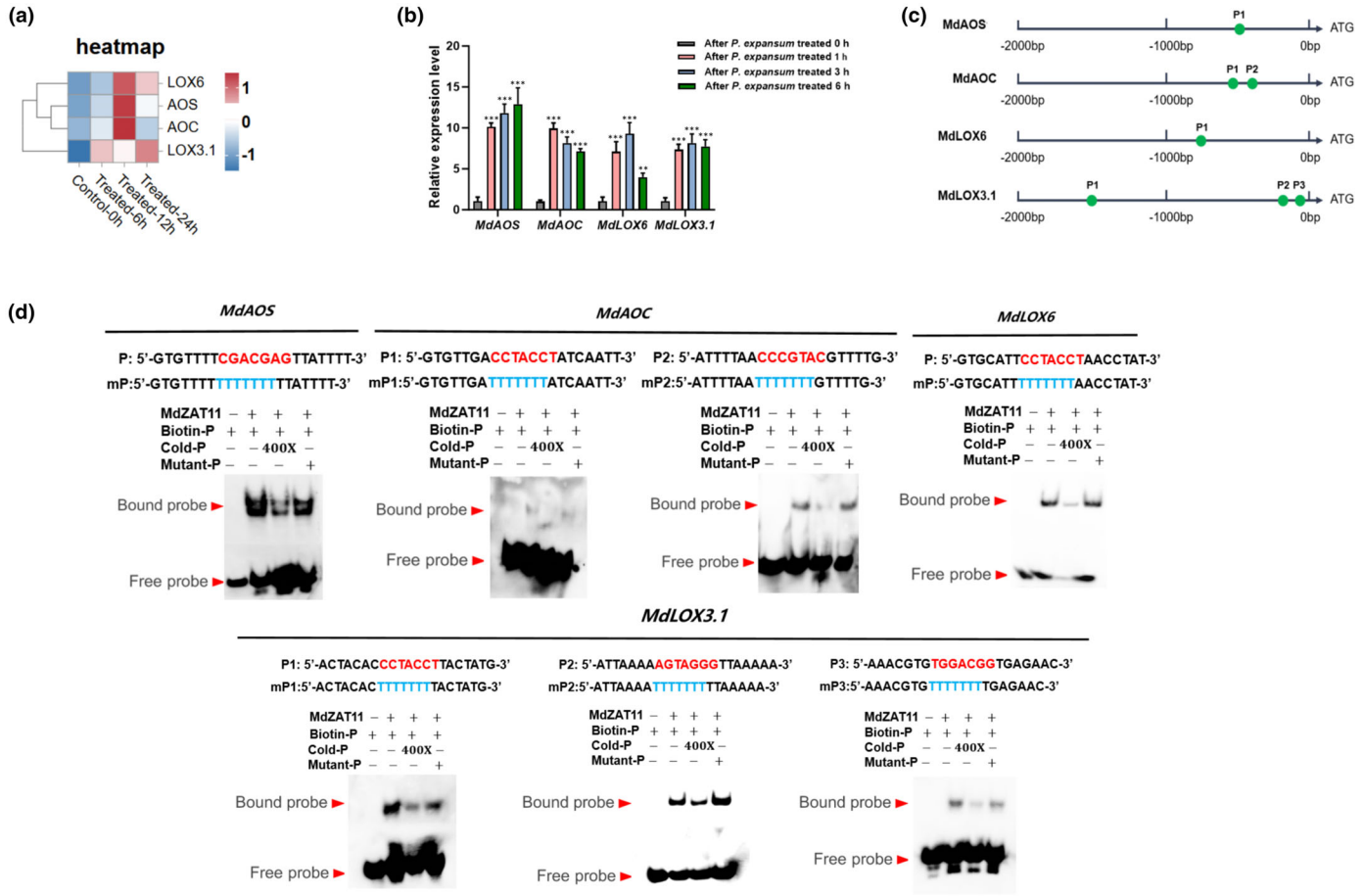

Fig. 5 MdZAT11 directly binds to the promoters of *MdAOS*, *MdAOC*, *MdLOX6*, and *MdLOX3.1*. (a) Heatmap of gene expression for the four JA biosynthetic genes based on RNA-seq data after *Penicillium expansum* infection. (b) RT-qPCR validation of the expression levels of the four genes shown in (a) at 0, 1, 3 and 6 h after inoculation. Expression levels were normalized to the 0 h control. (c) The distribution of binding sites in the promoters of the target genes. (d) Electrophoretic mobility shift assay (EMSA) showing the binding of MdZAT11 to the promoters of the target genes. The unlabeled probes served as competitors. The symbols '−', '+', and '400×' indicate the absence, presence, and 400-fold probe concentration, respectively. Data are presented as mean ± SD (n = 3). **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA.

MdZAT11 is closely related to PcZAT11, MsZAT11, and PaZAT11 from pear, wild apple, and sweet cherry, forming a distinct clade (bootstrap values 96–100%) within *Rosaceae* (Fig. 1c). These proteins share two C2H2 zinc fingers and a conserved EAR motif, suggesting a conserved role in this family. Subcellular localization and transcriptional activation assays indicated that MdZAT11 functions as a nucleus-localizing protein without self-activation (Fig. 1f,g). Using an *Agrobacterium*-mediated transient expression assay, we found that *MdZAT11* boosts apple resistance to *P. expansum*, supporting its role as a positive regulator of disease resistance (Fig. 2).

In peaches, PpZAT5 facilitates the activation of *PpMYB10.1* expression by directly binding to the A(G/C)T core element (e.g. ACTGTTACT) within its promoter region. PpZAT5 and PpBBX32 synergistically boost anthocyanin accumulation through the JA signaling pathway (Huang *et al.*, 2024). In apples, *MdZAT10* expression is upregulated by MeJA, and its overexpression leads to the upregulation of key genes involved in JA biosynthesis, such as *MdAOS* and *MdAOC1* (Yang *et al.*, 2021). These findings suggest that C2H2-ZFPs may play a role in JA biosynthesis or signaling in plants. The present study demonstrated that the transient expression of *MdZAT11* in apples significantly enhanced resistance to *P. expansum*. C2H2-ZFPs modulate the expression of downstream genes through specific interactions with *cis*-elements located in promoter regions. In this study, we performed TF-centered Y1H assays and revealed that MdZAT11 specifically binds to nine target DNA sequences in yeast. Consistent with the findings of Bao *et al.* (2022), MdZAT11 and MdZAT5 exhibit no intrinsic self-activation activity in yeast; however, Y1H assays are still capable of detecting their binding to specific DNA motifs. Upon exposure to environmental signals, including stress, post-translational modifications, or interactions with cofactors, these proteins may be able to activate target gene expression. These motifs predominantly contain the core sequence MYBPZM, along with other *cis*-acting elements, including CURECORECR, CGACGOSAMY3,

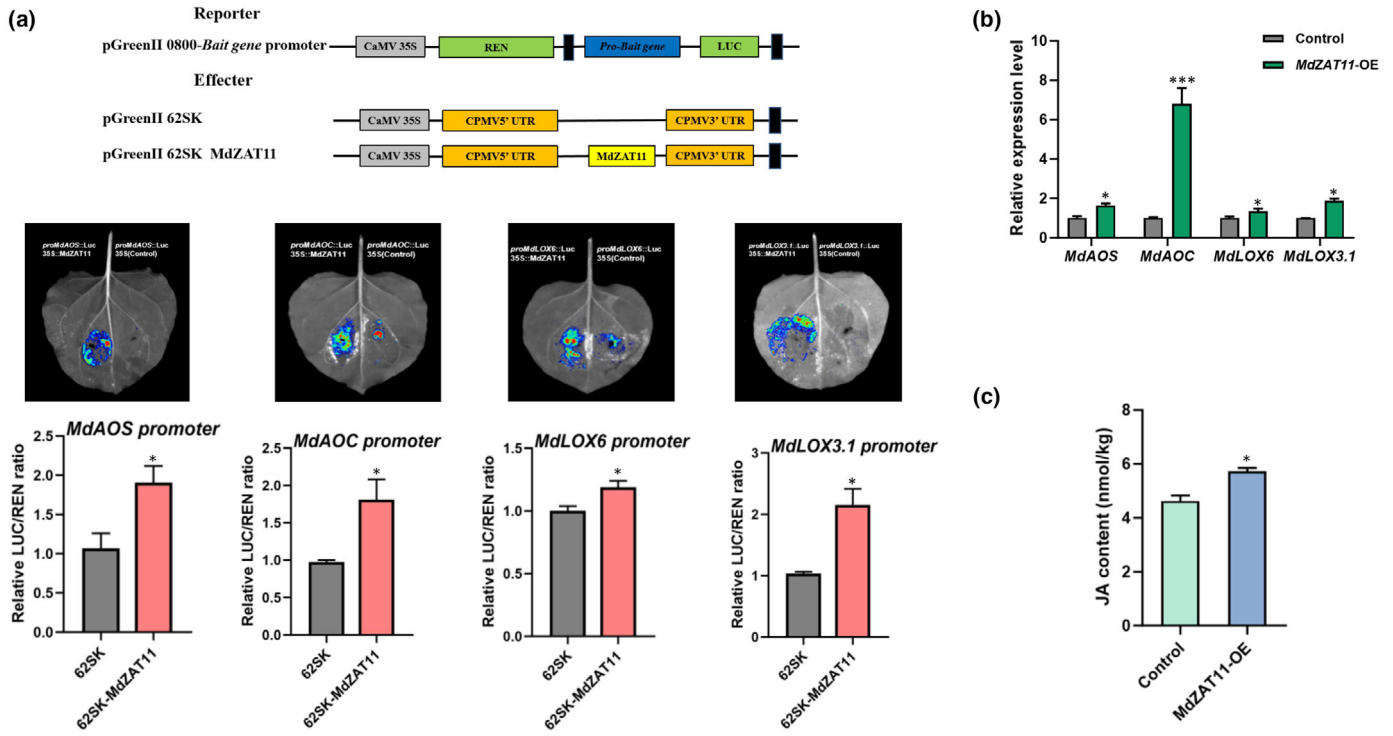

Fig. 6 Transcriptional activation of JA biosynthetic genes by MdZAT11 and its effect on JA content. (a) Structural diagrams of effector and reporter constructs. Representative LUC chemiluminescence images are shown below. The LUC/REN ratio was normalized to the empty effector control (62SK). Data are presented as mean ± SE (n = 3). (b) Expression levels of *MdAOS*, *MdAOC*, *MdLOX6*, and *MdLOX3.1* in apple fruit transiently expressing *MdZAT11* at 3 d after inoculation with *Penicillium expansum*. The empty pCAMBIA1300 vector was used as the control. Data are presented as mean ± SD (n = 3). (c) Determination of JA content in apple after transient expression of *MdZAT11* at 3 d after inoculation with *P. expansum*. The empty pCAMBIA1300 vector was used as the control. The data are expressed as mean ± SE (n = 3). An asterisk indicates a statistically significant difference between the control group and the treatment group, with *, $P < 0.05$; ***, $P < 0.001$ (Student's t-test).

CACTFTPPCA1, and PALBOXAPC (Fig. 4a). A combined analysis of genes containing these target sequences in the apple genome, along with differentially expressed genes during the early stages of *P. expansum* infection, indicated, through KEGG enrichment analysis, that MdZAT11 target genes are significantly enriched in JA biosynthesis-related pathways in apple fruits (Fig. 4). The regulators under investigation comprised four genes (*AOS*, *AOC*, *LOX6*, and *LOX3.1*) encoding enzymes that catalyze successive steps in JA biosynthesis from α-linolenic acid. Lipoxygenases, including LOX6 and LOX3.1, initiate this pathway by oxygenating α-linolenic acid, followed by AOS and AOC (Wasternack & Hause, 2013; Monte, 2023). EMSAs and DLR assays showed that MdZAT11 binds directly to the promoters of *MdAOS*, *MdAOC*, *MdLOX6*, and *MdLOX3.1*, and activates their expression. Overexpressing *MdZAT11* in apple fruit significantly increased the expression of these JA biosynthetic genes and endogenous JA levels, supporting MdZAT11 as a positive regulator of JA-mediated defense in apples (Figs 5, 6).

Consistent with this, elevated JA levels were observed in *MdZAT11* transient expression assays, and the JA-responsive defense gene *MdPR4* showed significantly increased expression (Fig. 2e). This increase was concomitant with higher levels of endogenous JA, indicating that JA signaling may be involved in the upregulation of *MdPR4*. Whether this occurs downstream of JA accumulation or through additional mechanisms remains to be investigated. JA is a crucial plant hormone for defense against pathogens (Du *et al.*, 2017; Macioszek *et al.*, 2023; Li *et al.*, 2025). While traditionally considered antagonistic to the SA pathway in dicots, recent studies have shown more complex interactions (Thaler *et al.*, 2012; Wasternack & Hause, 2013; Zhao *et al.*, 2020). A parallel study involving other TFs demonstrated that, although the birch transcription factor BpWRKY6 lacks self-activation activity in yeast, it directly upregulates the expression of JA synthesis-related genes (*BpLOX15*, *BpAOC4*, and *BpAOS1*) in plants, thereby augmenting resistance to the gypsy moth. Additionally, the interaction between BpMAPK6 and BpWRKY6 enhances the activation of these target genes (Xie *et al.*, 2025). Our research supports that MdZAT11 acts as a positive regulator of JA biosynthesis in apple fruit, thereby broadening the regulatory network of C2H2-ZFPs in the context of postharvest disease resistance. This study raises the question of whether MdZAT11 interacts with other proteins that could modulate its regulation of target genes, either by promotion or inhibition.

In apple, *MdNPR1*, *MdWRKYs*, and *MdERFs* genes are already known to regulate disease resistance through SA, ethylene, or broad transcriptional signaling mediated signaling pathways (Malnoy *et al.*, 2007; Zhao *et al.*, 2019; Wang *et al.*, 2020). Our

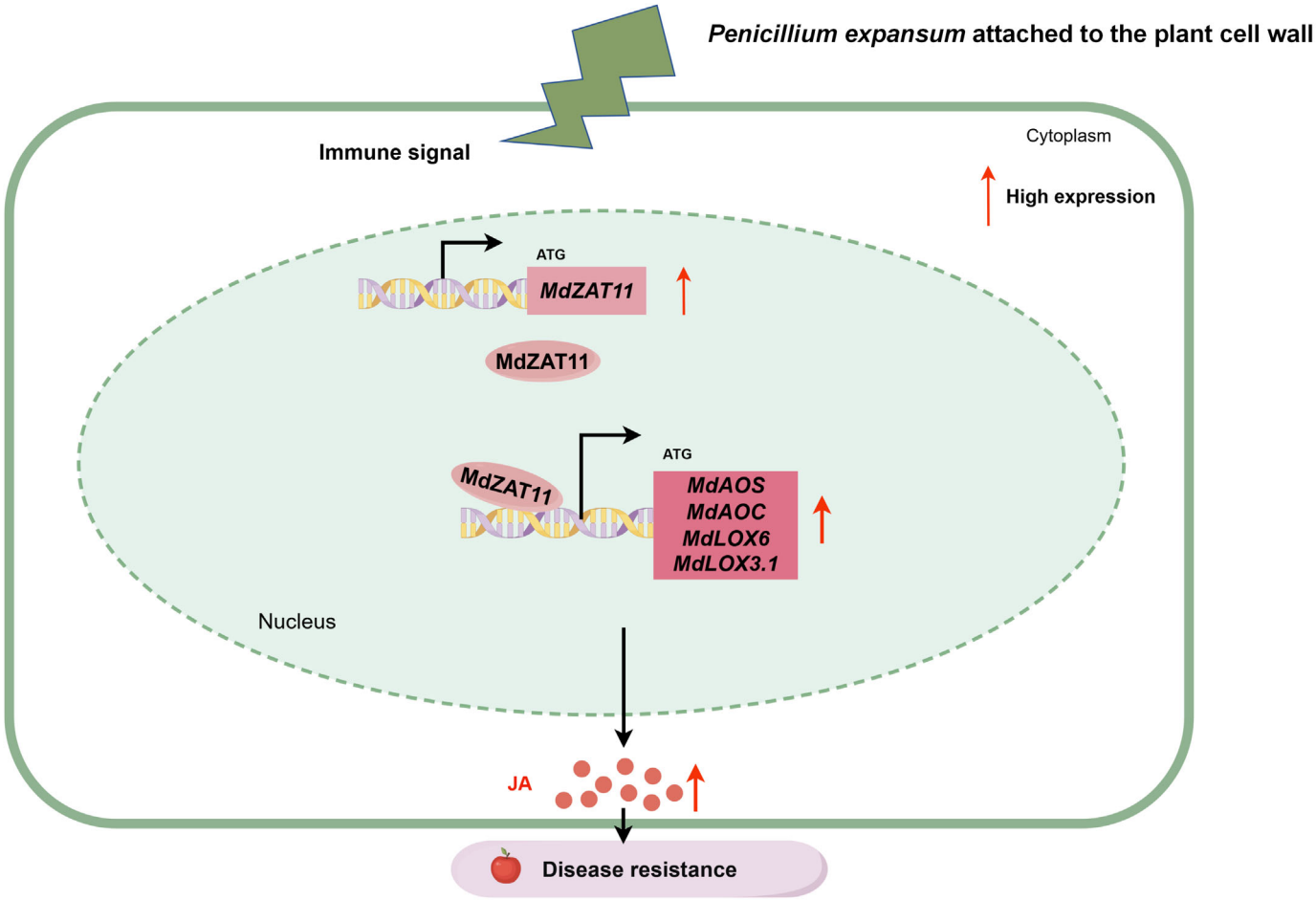

Fig. 7 A working model illustrating the role and mechanism of MdZAT11 in response to *Penicillium expansum* infection. Red arrows represent high levels of gene expression. The image was created using Figdraw (https://www.figdraw.com/#/).

study shows that MdZAT11 mainly works by directly activating JA biosynthesis genes, *MdAOS*, *MdAOC*, *MdLOX6*, and *MdLOX3.1*. Although we did not examine how MdZAT11 interacts with these other resistance-related genes, our findings suggest that MdZAT11 forms a JA-mediated defense module that may function alongside other known pathways. Our functional characterization of *MdZAT11* was based on transient expression assays in apple fruit, which provide robust initial evidence for its regulatory role. At the same time, we recognize that sustained *MdZAT11* expression in stably transformed lines would further strengthen this conclusion, and this is a direction we intend to pursue. Future studies are needed to explore how MdZAT11 fits into the wider resistance network in apple.

In conclusion, we show that MdZAT11 functions as a positive regulator of apple resistance to *P. expansum* by directly activating JA biosynthesis (Fig. 7). MdZAT11 is upregulated in response to *P. expansum* infection and directly binds to and activates the promoters of *MdAOS*, *MdAOC*, *MdLOX6*, and *MdLOX3.1*, enhancing their transcription, increasing JA accumulation, and ultimately, strengthening defense responses. These findings advance our understanding of the transcriptional regulation of hormone-mediated defense in postharvest fruit and suggest that MdZAT11 represents a promising target for enhancing postharvest disease resistance in apple.

Acknowledgements

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Competing interests

None declared.

Author contributions

YL and HZ conceived the research and designed the experiments. YL, KW and YS performed the experiments. SD and QY

analyzed the data. YL wrote the manuscript. All authors read and approved the final version of the manuscript.

ORCID

Solairaj Dhanasekaran https://orcid.org/0000-0003-4434-2240
Yuchun Lu https://orcid.org/0009-0006-1686-3259
Yingying Su https://orcid.org/0000-0003-2185-8746
Kaili Wang https://orcid.org/0000-0001-5405-8097
Qiya Yang https://orcid.org/0000-0003-2196-4063
Hongyin Zhang https://orcid.org/0000-0001-6906-4713

Data availability

The data supporting this study are available in Supporting Information Tables S1–S4. All relevant data can be found within the manuscript and its supporting materials. Sequence data from this article can be found in GDR under the following accession nos.: MdActin (*MD11G1110300*) and MdZAT11 (*MD04G1119600*), All the sequencing data in this study have been submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the project ID PRJNA1394627 (RNA-Seq).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Table S1 Genes and primers selected for PCR.

Table S2 Summary of RNA-seq data quality metrics for all samples.

Table S3 Genes and primers selected for RT-qPCR.

Table S4 Primer validation of the vector.

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