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Overexpression of *MdIPT8* Encodes an Isopentenyl Transferase Enzyme Enhances Resistance to *Colletotrichum Gloeosporioides* in apple (*Malus Domestica*)

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Abstract

Apple (*Malus domestica*) is a delicious fruit and have high economic value. However, numerous destructive fungi affect apple tree and limit the development of apple production. Many researches showed that salicylic acid and jasmonic acid improved plant resistance to fungal disease, but the potential mechanisms between cytokinin and fungal disease were hardly reported. The *IPT* gene family encodes the proteins of synthetizing cytokinin and plays an indispensable role in plant biological stress. In our previous study, we found the disease resistance in autotetraploid apple was much better than 'Hanfu' and we analyze the gene expression in 'Hanfu' and autotetraploid apple. We found the *MdIPT8* gene was significantly different expressed. Therefore, we focused on *MdIPT* genes. We uncovered ten *IPT* genes in the 'Hanfu' whole genome, that are unevenly distributed across five chromosomes. Phylogenetic analysis indicated this family falling into two groups. Moreover, the transcriptional analysis of ten *IPT* genes indicated *MdIPT8* can be induced under *Colletotrichum gloeosporioides* stress stimulating in apple leaves. The MdIPT8 protein coalesced to green fluorescent protein pinpointed to the chloroplast. Finally, we confirmed that the overexpression of *MdIPT8* increased resistance to *C. gloeosporioides*. Additionally, the endogenous cytokinin content of 'Hanfu' leaves increased under the stress of *C. gloeosporioides* and exogenous cytokinin improved the disease resistance of leaves. Our findings supply an overview of the identification of apple *IPT* gene family and increases the understanding of cytokinin in apple anthracnose resistance study.

Key Message

We acquired the overexpression of *MdIPT8* transgenic apple lines and confirmed they have stronger resistance to *Colletotrichum. gloeosporioides*.

1. Introduction

Fruit tree yield is closely related to their growth and development. The pathogen resistance of apple trees determines the quality of fruits(Zhang et al. 2019b). When fungi attack apple trees, pathogens seriously hinder their growing and developing(He et al. 2018). *C. gloeosporioides* is a fungal pathogen that constitutes a serious threat to orchards occurring in tropical and subtropical(Moreira et al. 2021). Chemical treatments are effectively used to withhold fungal diseases(Gur et al. 2017, 2020; Pagès et al. 2020), but aforesaid treatments have negative side effects, for instance, environment unfriendly and food safety reduced(Hu et al. 2017). The most remarkable scenarios for conquering fungal diseases in apple are to select fungal resistant apple sources and cultivating fungal resistant variations through breeding(Chechi et al. 2019). As a result, it is very imperative to delve into the molecular mechanism potential fungal disease resistance in apple.

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Plant defensed fungal disease in many pathways. Plant hormones play a meaningful role in plant fungal resistance signaling pathways(Pieterse et al. 2012). Large numbers of plant immune responses involving salicylic acid(Ding and Ding 2020), jasmonic acid(Zhao et al. 2019), and ethylene(Wang et al. 2020) have been reported. The effects of abscisic acid(Ha et al. 2012) and auxin(Jones et al. 2010) on plant resistance have also been gradually discovered. However, the influences of cytokinin to fungal disease were rarely reported.

Cytokinin, as one of the five important hormones in plants, is a decisive plant hormone that stimulate cell division and cell growth(Choi and Hwang 2007). It regulates plant physiology activities(Sakakibara 2006). It also affect plant disease resistance(Pertry et al. 2009). Researchers found that plant-derived cytokinin promotes *Arabidopsis thaliana* resistance to *Pseudomonas syringae pv. Tomato* DC3000 (Pst) and overexpression of genes encoding isopentenyl transferase enzyme (*AtIPT1*, *2*, *5*, and *7*) raised transgenic *Arabidopsis thaliana* defense to Pst. In the interim, overexpression of cytokinin oxidase gene improved sensitivity of *Arabidopsis thaliana* to infection(Choi et al. 2010). The above outcomes indicated that cytokinin mediated plant to bacterial resistance. But the role of cytokinin in fungal disease resistance is still obscure.

Isopentenyl transferases (IPT) are key enzymes that catalyze the cytokinin biosynthesis and are also important rate-limiting enzymes that affect the types and levels of endogenous cytokinin in plants(Miyawaki et al. 2006). In plants, there are two types of isopentenyl transferase. One is the adenylate isopentenyl transferase (ATP/ADP-IPT) which the main active forms of biosynthesis of cytokinin are isopentenyl adenine and trans-zeatin (tZ). The other is tRNA-IPT, in which another active form involved in the catalytic synthesis of cytokinin is cis-zeatin (cZ). What' s more, tZ was recognized to the most abundant and effective regulators in physiological processes(Hoyerová and Hošek 2020; Chen et al. 2021).

In our previous study, we found the expressed of *IPT* gene was significantly different between disease resistance autotetraploid apple and sensitive 'Hanfu'(Ma et al. 2016). We confirmed that exogenous and endogenous cytokinin can increase apple leaves resistance to *C. gloeosporioides*. In this study, we singled out all potential *IPT* genes in the 'Hanfu' apple genome. Through bioinformatics analysis, physiological data analysis and transgenic technology to find the *IPT* gene which response to *C. gloeosporioides*. The results will contribute on apple resistance breeding.

2. Materials And Methods

2.1. Plant Materials

The material of plant tissue culture for 'GL-3', 'Hanfu' diploid and autotetraploid apple plants were grown at Shenyang Agriculture University (41.49°N, 123.34°E, Liaoning, China). The growth conditions in this study were described by Chen *et al*(Chen et al. 2019) and Zhang *et al*(Zhang et al. 2019a).

2.2. Fungal Culture and Infection

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The fungus *C. gloeosporioides* performed for the experimentations were widened on potato dextrose agar cultures for a week about 28°C. Spores were gingerly placed in water. The suspension population was counted, then controlled to 10^6 spores ml^{-1}. The infected 'HF' plants were placed at 26°C for fungal development. At one day, two days, three days and four days after inoculation, the leaves were collected. They were frozen in pure nitrogen and transferred to -80°C.

2.3. Cytokinin Quantification and Treatments

For trans-zeatin (tZ) measurements, more 50 mg fresh weigh of leaf tissue was weighed. The samples were homogenized by tissue homogenizer below 0 °C. The ratio of homogenization was 10%, which equivalent to 1 g tissue adding 9 ml homogenate. The homogenate was 0.01 M phosphate buffer (PH 7.4). The homogenized samples were centrifuged (5000 rpm, 15 min) and took the supernatant to measure. Cytokinin was measured using an ELISA kit specific for tZ (MEIMIAN, Jiangsu Meimian industrial Co., Ltd).

Thirty healthy diploid apple plants were sprayed with 0.02mg/ml tZ and immediately sprayed with 10^8 spores ml^{-1} suspension. The inoculated plants were eventually incubated at 26°C before evaluation of disease. Three days and five days after inoculation, the leaves were observed and the disease indexes were calculated.

According to the percentage of observed the area of diseased leaf, the disease index of each leaf was rated. The disease conditions were graded as follows. 0 grade: no disease spots on leaves; 1 grade: the diseased leaf area accounts for less than 5% of the entire leaf area; 2 grade: the diseased leaf area accounts for 6%~10% of the entire leaf area; 3 grade: the diseased leaf area accounts for 11%~15% of the entire leaf area; 4 grade: the diseased leaf area accounts for 16%~20% of the entire leaf area; 5 grade: the diseased leaf area accounts for 21%~25% of the entire leaf area; 6 grade: the diseased leaf area accounts for 26%~30% of the entire leaf area; 7 grade: the diseased leaf area accounts for 31%~35% of the entire leaf area; 8 grade: the diseased leaf area accounts for 36%~40% of the entire leaf area; 9 grade: the diseased leaf area accounts for more than 40% of the entire leaf area.

$$\mathrm{Thediseaseindex}(\%) = \left[\sum (\mathrm{A} \times \mathrm{B}) \div (\mathrm{C} \times \mathrm{D})\right] \times 100\%$$

A: Number of diseased leaves at all grade

B: Relative disease grade value

C: Total number of apple leaves

D: Highest disease grade value

2.4. Identification of *IPT* Gene Family

To identify *IPT* family genes in apple, BLAST algorithm-based searches were used to identify members of

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ences were used to perform BLASTP algorithm-

based searches against the GDR(Jung et al. 2019). Additionally, the sequences of *Arabidopsis thaliana* were procured from TAIR (https://www.arabidopsis.org/index.jsp). The rice sequences were downloaded from Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/index.shtml). Gene IDs and CDS of the *MdIPT* family were acquired from the GDR. The length of amino acid sequences of *MdIPT* proteins were obtained by a tool from the ExPasy website (http://web.expasy.org/protparam/).

2.5. Phylogenetic Analysis

For phylogenetic analysis, IPT orthologs in *Arabidopsis thaliana* and Rice were obtained at TAIR and RGAP. The protein sequences of ten MdIPTs, nine AtIPTs, and ten OsIPTs were aligned through ClustalW program using the format parameters at MEGA X. A phylogenetic tree was then established by using the neighbor-joining plan with 1000 bootstrap replicates(Kumar et al. 2018).

2.6. Chromosomal Locations

Information on the *MdIPT* genes, including chromosomal location and DNA sequences, was obtained from the GDR. MapInspect (https://www.plantbreeding.wur.nl/) was used to map the distribution of the *MdIPT* genes.

2.7. Total RNA Extraction and cDNA Synthesis

Total RNA were derived from apple tissues with the modified CTAB technique(Chang et al. 2007). The cDNAs were reverse transcribed by using the TaKaRa RT reagent kit and maintained at − 20℃ until use.

2.8. RT-qPCR Assays

The quantitative measurement of genes expression was performed on an ABI QuantStudio™12K Flex real-time PCR instrument. The reaction system and procedure refer to the instruction manual of SYBR Premix Ex Taq. The melting curve was analyzed and the related expression of genes were counted according to the $2^{-\Delta\Delta Ct}$ guideline after the reaction was completed. The apple *EF-1a* (Accession No. DQ341381) was used as a normalization for analysis. The primers used are filled in Supplemental Table.

2.9. Promoter Sequence Analysis of *MdIPT* Genes

Promoter sequences (2000 bp) of apple *IPT* genes were obtained from the GDR. They were analyzed in ten members of the *MdIPT* family by inputting the nucleic acid sequences in FASTA format into the Plant CARE, and the protocols were set to default(Lescot et al. 2002).

2.10. Subcellular Localization

The complete length *MdIPT8* (MD13G1182800) cDNA were duplicated into the *Nde* Ⅰ and *BamH* Ⅰ sites in the pRI101-GFP to engender 35S::*MdIPT8-GFP*, and pRI101-GFP plasmid was used as a control. Different *Agrobacterium* solutions were transformed into *Nicotiana benthamiana* leaves. After 60 h of darkness, the signal of green fluorescent was scrutinized under a Leica TCS confocal microscope.

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2.11. Construction of Expression Vector and Obtained Transgenic Plants

The full-length *MdIPT8* cDNA were cloned and inserted into pRI101-AN binary vector plasmid so that *MdIPT8* was under the regulation of 35S promotor. The gene was transformed into 'GL-3' based on the established protocol described by Chen *et al*(Chen et al. 2019).

2.12. Leaf Agroinfiltration and Fungal Inoculation

'Hanfu' diploid apple leaves were utilized for agroinfiltration, and *Agrobacterium*-attended transformation was executed by the injection progress(Meng et al. 2018). Two days after injection, spore suspension (10^6/mL) of *Colletotrichum gloeosporioides* strain was cultivated on each site of injection after the apple leaves were disassembled, then they were hatched at 27 ℃ in damp dishes for 3 days in the darkness. Thirty leaves were used in each treatment. The 'GL-3' and overexpressing *MdIPT8* lines were used for fungal inoculation. Spore suspension was injected to leaves in vitro and sprayed in the whole apple plants. After 3 and 5 days, they were observed.

3. Results

3.1. Cytokinin Positively Regulates *C. gloeosporioides* Resistance in 'Hanfu' Apple

Previous research demonstrated that 'Hanfu' autotetraploid apple exhibited significantly differences in resistance to fungi, compared with 'Hanfu' apple(Chen et al. 2017) and cytokinin plays a momentous character in plant biotic stress(Choi et al. 2010). To determine the role of cytokinin in 'Hanfu' apple defense against *C. gloeosporioides*, we measured tZ content in 'Hanfu' and autotetraploid apple leaves. The concentration of tZ was significantly elevated in autotetraploid apple leaves compared to 'Hanfu' apple leaves (Figure. 1a). Subsequently, we also measured the tZ content of 'Hanfu' apple leaves in the *C. gloeosporioides* stress. We found that the concentration of tZ was elevated in the *C. gloeosporioides* treatment period (Figure. 1b). This observation suggests that cytokinin could regulate *C. gloeosporioides* resistance. To confirm this, we pre-treated the apple plants with exogenous cytokinin before infection with *C. gloeosporioides*. The results showed that exogenous cytokinin treatment significantly enhanced *C. gloeosporioides* resistance of the apple plants compared with the mock treatment (Figure. 1c, 1d). Taken together, cytokinin positively regulates *C. gloeosporioides* resistance in 'Hanfu' apple.

3.2. Identification of The *IPT* Gene Family in 'Hanfu' Apple

Previous research has shown that the cytokinin are mainly influenced by the balance between biosynthesis and catabolism. Isopentenyl transferases (IPT) are key enzymes that catalyze the cytokinin biosynthesis. In recent times, a high-quality assembly in 'Hanfu' were published(Zhang et al. 2019b). In

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this study, ten *MdIPT* genes (Table 1) were obtained using 'HFTH1 Genome v1.0.a1 chromosomes' database in GDR to examined the protein sequences of AtIPTs and OsIPTs. The *IPT* genes were named as *MdIPT1* to *MdIPT10*, respectively, based on the chromosomal locations of the 'Hanfu' apple. The genomic distribution of chromosomal locations of *MdIPT* across seventeen chromosomal positions (Md01 – Md17) was figured out using MapInspect. A total of ten *MdIPT* genes were allotted to five chromosomal locations ranging from 1 to 3 genes per chromosome. Chromosomal location analyses showed that this family are dispersed on chromosome 16, chromosome 13, chromosome 11, chromosome 6 and chromosome 3 (Fig. S1). Chromosome 3 contains three *MdIPT* loci. Chromosome 6 contains one *MdIPT* loci. The rest chromosomes (Chr 11, 13 and 16) each harbors two loci. To validate the accuracy of the BLASTP results, these *IPT* family genes were further confirmed through BLASTN search at GenBank.

To understand and categorize the evolutionary relationships between MdIPTs and IPTs from other plants, a phylogenetic tree was organized using the amino acid sequences of ten MdIPTs, nine AtIPTs from *Arabidopsis thaliana*, and ten OsIPTs from rice (Fig. 2). This analysis indicated that apple IPTs could be grouped into two classifications based on information from *Arabidopsis thaliana* and rice. There are seven ATP/ADP-IPTs, including MdIPT1, MdIPT3, MdIPT6, MdIPT7, MdIPT8, MdIPT9 and MdIPT10, and three tRNA-IPTs, including MdIPT1, MdIPT2 and MdIPT5. By comparing the evolution of the three plants, interesting features were identified. All IPT homologs showed similar clustering patterns among *Arabidopsis thaliana*, rice, and apple.

Table 1
MdIPT gene family

Gene name	Gene ID	CDS length(bp)	Protein length(aa)
MdIPT1	HF40675-RA	1551	516
MdIPT2	HF03183-RA	1350	449
MdIPT3	HF02931-RA	960	320
MdIPT4	HF37306-RA	876	291
MdIPT5	HF28031-RA	2841	946
MdIPT6	HF28291-RA	897	298
MdIPT7	HF42175-RA	948	315
MdIPT8	Not Given	1011	336
MdIPT9	HF07762-RA	948	315
MdIPT10	HF13830-RA	1245	414

3.3. *MdIPT8* Act as A *C. gloeosporioides* Responsive Gene

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We found that *C. gloeosporioides* can induce apple to produce cytokinin (Fig. 1b) and exogenous cytokinin may reduce disease incidence (Fig. 1c). Thus, an application of *C. gloeosporioides* was further investigated the expression of *MdIPT* genes. As shown in Fig. 3, eight genes (*MdIPT3*, *MdIPT4*, *MdIPT5*, *MdIPT6*, *MdIPT7*, *MdIPT8*, *MdIPT9*, and *MdIPT10*) were unquestionably up-regulated at 2 days or 3 days after inoculating *C. gloeosporioides*. On the contrary, *MdIPT2*, *MdIPT3* and *MdIPT6* were distinctly down-regulated at 1 day after *C. gloeosporioides* treatment. The expression of *MdIPT3* and *MdIPT8* gene increased in response to *C. gloeosporioides* treatment until 3 days. In contrast, *MdIPT2* were considerably down-regulated after fungal treatment. *MdIPT8* was up-regulated after *C. gloeosporioides* application at all subsequent time points. As a result, *MdIPT8* act as a *C. gloeosporioides* responsive gene. Therefore, we focused our analysis on *MdIPT8*.

3.4. Multiple Organs Expression Analysis of *MdIPT8* and Subcellular Localization of MdIPT8

'Hanfu' apple tissues were applied to recognize the expression levels of *MdIPT8* by real-time polymerase chain reaction (Fig. S2). *MdIPT8* was probed in all tissues, including root, fruit, stem, leaf, and flower. In the leaf, the highest expression level was spotted (Fig. S2).

To examine the subcellular localization of MdIPT8, we impermanently expressed MdIPT8-GFP constructs in *Nicotiana benthamiana* leaves. These synthesis protein signals were overseen by Leica microscopy. Green brightness was apparently monitored in the chloroplast (Fig. 4). But, in the control group, the luminance signal of GFP was observed all over the tobacco cell (Fig. 4).

3.5. *MdIPT8* Positively Regulates *C. gloeosporioides* Resistance in Apple

For further analysis, we cloned *MdIPT8* by using PCR. To determine if *MdIPT8* works in defense against *C. gloeosporioides* in apple, a 35S::*MdIPT8* vector was transiently expressed in apple leaves via agroinfiltration, followed by inoculation with *C. gloeosporioides* (Fig. 7b). After inoculation 3 days, necrosis was monitored round the injection site on leaves (Fig. 7c). Overexpression of *MdIPT8* decreased the area of necrosis, demonstrating that *MdIPT8* gene enables reduce the fungus of virulence to apple leaves (Fig. 7c, 7d). In summary, these results indicated that *MdIPT8* positively regulates *C. gloeosporioides* resistance in apple.

To preliminarily explore how *IPT* genes are regulated, a 2 kb promoter region for all *IPT* genes was identified, and the Plant CARE website was used to identify cis-elements. The promotor sequences of *MdIPTs* was accessed through the JBrowse tool in GDR. The prediction results show that the promoters of *IPT* genes contain multiple regulatory elements: phytohormone responses elements (ERE, TGA-element, ABRE, AuxRR-core, P-box, TATC-element, etc.), stress responses elements (DRE1, G-box, ARE, W-box, TGACG-motif, GC-motif, etc.), tissue-specific expression elements (CAT-box, CCGTCC motif) and light

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). Most genes in the family can participate in

stress resistances and are regulated by other plant hormones. Of the family, ten genes are ubiquitously controlled by stress responses.

3.6 Overexpression *MdIPT8* improves resistance to *Colletotrichum gloeosporioides* in transgenic apple

To elucidate the role of *MdIPT8* in *C. gloeosporioides* resistance, we overexpressed *MdIPT8* in 'GL-3' using *Agrobacterium*-mediated method. We created three *MdIPT8*-OE lines. Then, we identified three putative transgenic plants by qRT-PCR. Apple RNA were extracted from 'GL-3' (WT) and three independent *MdIPT8* transgenic lines for quantitative real-time PCR. Analysis of *MdIPT8* expression levels showed that the expressing level of *MdIPT8* in three transgenic lines were significantly higher than in WT (Fig. 6a). *MdIPT8*-OE line #3 have highest expression levels, and the relative expression levels were 14.9-flod higher than they were in the 'GL-3' plants. Meanwhile, we measured tZ content in two high expressing level lines and tZ content in transgenic plants were higher than in WT plants (Fig. 6b). Thus, we obtained overexpressing *MdIPT8* apple plants.

To characterize the role of *MdIPT8* in defense against *C. gloeosporioides* in apple, we tested the effects of *C. gloeosporioides* to 'GL-3', *MdIPT8*-OE line #2 and *MdIPT8*-OE line #3 plants. Treatment with *C. gloeosporioides* for 3 days and 5 days accelerated diseased areas development in 'GL-3' leaves and plants, but only slightly influenced infected areas in overexpression *MdIPT8* leaves and plants (Fig. 7a and c). Analysis of disease index showed that apple transgenic plants to *C. gloeosporioides* resistance were much better than WT palnts (Fig. 7b and d).

4. Discussion

In fruit trees, apple is one of the most influential economic value(Zhang et al. 2019b). Nevertheless, apple production is diminished via many kinds of fungal diseases(He et al. 2018). Apple anthracnose, caused by *Colletotrichum* spp., causes significant yield losses to the growers(Moreira et al. 2021). Therefore, analysis of the apple response mechanism is of great significance for increasing apple yield. Some plant hormones (such as jasmonate and abscisic acid)(Dugé De Bernonville et al. 2012; Su et al. 2020) can activate the transcription of many defense-related genes and help regulate plant stress responses. A few reports scrutinized the roles of cytokinin against fungal disease. Overall, the mechanism by which cytokinin regulates apple fungal stress is poorly understood. Here, we found that exogenous spraying cytokinin increased apple resistance to *C. gloeosporioides* (Fig. 1c). Endogenous cytokinin content were significant higher in the *C. gloeosporioides* treated plants than in the untreated plants and showed a positive correlation (Fig. 1b). The results show that cytokinin improve apple disease resistance.

IPT genes are cytokinin biosynthesis genes. In plants, it play a paramount function in fine-turning cytokinin(Ha et al. 2012). Previously, plentiful studies have analyzed the functional of *IPT* genes in the model plants(Miyawaki et al. 2004) and non-model plant(Žižková et al. 2015) using forward techniques of bioinformatics. *AtIPT* genes have been more functionally verified(Galichet et al. 2008). In *Arabidopsis*, and contain more sufficient cytokinin(Guo and

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Gan 2011). The overexpression of *MdIPT3a* inhibits root development, a delay in flowering, and enhanced outgrowth of axillary buds(Zhu et al. 2012). However, previous research results mostly focused on how *IPT* affects plant growth and development, and there are few reports on the regulation processes involved in fungal response. In order to acquire information about *MdIPT* potential function after inoculating *C. gloeosporioides*, we kept a genetic expression experiment to analyze 'Hanfu' *IPT* family members. Thus, we found that the expression of *MdIPT8* can be activated by fungus treatments (Fig. 3), supposing that *MdIPT8* may be involved in hormone-induced apple growing and developing even respond to various pathogen stresses (Figs. 5 and 7). This is basically consistent with the results of previous research reports that its homologous gene *AtIPT1* can significantly respond to pathogen stress in *Arabidopsis*(Choi et al. 2010). All in all, this research disclosed a novel mechanism wherein cytokinin regulate fungal stress tolerance in apple by the expression of *MdIPT8*.

Promoter sequence analysis disclosed the presence of many cis-acting elements correlated with hormone and stress responses in the *MdIPT* gene promoter (Fig. S3). In other researcher's study, *MdIPT5b* expression maintains high cytokinin levels, leading to improve salt tolerance(Feng et al. 2019). Therefore, some abiotic stress response factors may be critical for regulating the expression of *MdIPT* and speculate that biological stress may also play a fundamental role in conducting *MdIPT* genes expression. WRKY is one of the most enormous transcription factor families. The proteins of WRKY family are relevant with many fungal stress defense pathways. WRKY proteins can function due to the highly conserved WRKY domain that binds the W-box region(Rushton et al. 2010). The 2000bp promoter region of *MdIPT8* have W-box (Fig. S3), so we suggested that the expression of *MdIPT8* probably is regulate by WRKY proteins so that *MdIPT8* enhance the resistance of apple fungal disease.

Hence, our research demonstrated that *MdIPT8* regulates *C. gloeosporioides* stress in apple by improving cytokinin content. It was the first to report the cytokinin response to *C. gloeosporioides* in apple by molecular methods. Our findings provide genetic resources and theoretical bases for the molecular breeding of fungal stress in apple.

Declarations

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Conflict of interests

The authors declare that they have no conflicts of interest to report.

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Author contributions

Conceptualization: Yue Ma and Feng Wang, Funding acquisition: Yue Ma and Zhigang Wang, Formal analysis: Jiajun Shi and Feng Zhang, Project administration: Yue Ma and Jiajun Shi, Resources: Yue Ma and Feng Wang, Supervision: Yue Ma and Feng Wang, Validation: Feng Wang, Jiajun Shi, Feng Zhang, Qiu Jiang, Yuhong Yuan, Xiaolin Nie and Ying Zhou, Visualization: Jiajun Shi and Feng Zhang, Supervision: Yue Ma and Feng Wang, Writing - original draft: Jiajun Shi and Feng Zhang, Writing - review & editing: Yue Ma and Jiajun Shi.

References

1. Chang L, Zhang Z, Yang H et al (2007) Detection of Strawberry RNA and DNA Viruses by RT-PCR Using Total Nucleic Acid as a Template. J Phytopathol 155:431–436. https://doi.org/10.1111/j.1439-0434.2007.01254.x
2. Chechi A, Stahlecker J, Dowling ME, Schnabel G (2019) Diversity in species composition and fungicide resistance profiles in Colletotrichum isolates from apples. Pestic Biochem Physiol 158:18–24. https://doi.org/10.1016/j.pestbp.2019.04.002
3. Chen K, Song M, Guo Y et al (2019) MdMYB46 could enhance salt and osmotic stress tolerance in apple by directly activating stress-responsive signals. Plant Biotechnol J 17:2341–2355. https://doi.org/10.1111/pbi.13151
4. Chen L, Zhao J, Song J, Jameson PE (2021) Cytokinin glucosyl transferases, key regulators of cytokinin homeostasis, have potential value for wheat improvement. Plant Biotechnol J 19:878–896. https://doi.org/10.1111/pbi.13595
5. Chen M, Wang F, Zhang Z et al (2017) Characterization of fungi resistance in two autotetraploid apple cultivars. Sci Hortic 220:27–35. https://doi.org/10.1016/j.scienta.2017.03.034
6. Choi J, Huh SU, Kojima M et al (2010) The Cytokinin-Activated Transcription Factor ARR2 Promotes Plant Immunity via TGA3/NPR1-Dependent Salicylic Acid Signaling in Arabidopsis. Dev Cell 19:284–295. https://doi.org/10.1016/j.devcel.2010.07.011
7. Choi J, Hwang I (2007) Cytokinin: perception, signal transduction, and role in plant growth and development. J Plant Biol 50:98–108. https://doi.org/10.1007/BF03030617
8. Ding P, Ding Y (2020) Stories of Salicylic Acid: A Plant Defense Hormone. Trends Plant Sci 25:549–565. https://doi.org/10.1016/j.tplants.2020.01.004
9. Dugé De Bernonville T, Gaucher M, Flors V et al (2012) T3SS-dependent differential modulations of the jasmonic acid pathway in susceptible and resistant genotypes of Malus spp. challenged with Erwinia amylovora. Plant Sci 188–189:1–9. https://doi.org/10.1016/j.plantsci.2012.02.009
10. Feng Y, Liu J, Zhai L et al (2019) Natural variation in cytokinin maintenance improves salt tolerance in apple rootstocks. Plant Cell Environ 42:424–436. https://doi.org/10.1111/pce.13403
11. Galichet A, Hoyerová K, Kamínek M, Gruissem W (2008) Farnesylation directs AtIPT3 subcellular Arabidopsis. Plant Physiol 146:1155–1164.

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https://doi.org/10.1104/pp.107.107425

12. Guo Y, Gan S (2011) AtMYB2 regulates whole plant senescence by inhibiting cytokinin-mediated branching at late stages of development in Arabidopsis. Plant Physiol 156:1612–1619. https://doi.org/10.1104/pp.111.177022
13. Gur L, Reuveni M, Cohen Y (2020) Control of Alternaria fruit rot in “Pink Lady” apples by fungicidal mixtures. Crop Prot 127:104947. https://doi.org/10.1016/j.cropro.2019.104947
14. Gur L, Reuveni M, Cohen Y (2017) Phenology-Based Management of Alternaria Fruit Rot in Pink Lady Apples. Plant Dis 102:1072–1080. https://doi.org/10.1094/PDIS-05-17-0735-RE
15. Ha S, Vankova R, Yamaguchi-Shinozaki K et al (2012) Cytokinins: metabolism and function in plant adaptation to environmental stresses. Trends Plant Sci 17:172–179. https://doi.org/10.1016/j.tplants.2011.12.005
16. He L, Li X, Gao Y et al (2018) Characterization and Fungicide Sensitivity of Colletotrichum spp. from Different Hosts in Shandong, China. Plant Dis 103:34–43. https://doi.org/10.1094/PDIS-04-18-0597-RE
17. Hoyerová K, Hošek P (2020) New Insights Into the Metabolism and Role of Cytokinin N-Glucosides in Plants. Front Plant Sci 11:741. https://doi.org/10.3389/fpls.2020.00741
18. Hu X, Zhong Y, Huang K et al (2017) Differential expression of 12 NBS-encoding genes in two apple cultivars in response to Alternaria alternata f. sp. mali infection. Can J Plant Sci 98:279–287. https://doi.org/10.1139/cjps-2017-0117
19. Jones B, Gunnerås SA, Petersson SV et al (2010) Cytokinin regulation of auxin synthesis in Arabidopsis involves a homeostatic feedback loop regulated via auxin and cytokinin signal transduction. Plant Cell 22:2956–2969. https://doi.org/10.1105/tpc.110.074856
20. Jung S, Lee T, Cheng C-H et al (2019) 15 years of GDR: New data and functionality in the Genome Database for Rosaceae. Nucleic Acids Res 47:D1137–D1145. https://doi.org/10.1093/nar/gky1000
21. Kumar S, Stecher G, Li M et al (2018) MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol 35:1547–1549. https://doi.org/10.1093/molbev/msy096
22. Lescot M, Déhais P, Thijs G et al (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res 30:325–327. https://doi.org/10.1093/nar/30.1.325
23. Ma Y, Xue H, Zhang L et al (2016) Involvement of Auxin and Brassinosteroid in Dwarfism of Autotetraploid Apple (Malus × domestica). Sci Rep 6:26719–26719. https://doi.org/10.1038/srep26719
24. Meng D, Li C, Park H-J et al (2018) Sorbitol Modulates Resistance to Alternaria alternata by Regulating the Expression of an NLR Resistance Gene in Apple. Plant Cell 30:1562–1581. https://doi.org/10.1105/tpc.18.00231
25. Miyawaki K, Matsumoto-Kitano M, Kakimoto T (2004) Expression of cytokinin biosynthetic isopentenyltransferase genes in Arabidopsis: tissue specificity and regulation by auxin, cytokinin,

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26. Miyawaki K, Tarkowski P, Matsumoto-Kitano M et al (2006) Roles of Arabidopsis ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. Proc Natl Acad Sci U S A 103:16598–16603. https://doi.org/10.1073/pnas.0603522103
27. Moreira RR, Zielinski EC, Castellar C et al (2021) Study of infection process of five species of Colletotrichum comparing symptoms of glomerella leaf spot and bitter rot in two apple cultivars. Eur J Plant Pathol 159:37–53. https://doi.org/10.1007/s10658-020-02138-y
28. Pagès M, Kleiber D, Violleau F (2020) Ozonation of three different fungal conidia associated with apple disease: Importance of spore surface and membrane phospholipid oxidation. Food Sci Nutr 8:5292–5297. https://doi.org/10.1002/fsn3.1618
29. Pertry I, Václavíková K, Depuydt S et al (2009) Identification of Rhodococcus fascians cytokinins and their modus operandi to reshape the plant. Proc Natl Acad Sci U S A 106:929–934. https://doi.org/10.1073/pnas.0811683106
30. Pieterse CMJ, Van der Does D, Zamioudis C et al (2012) Hormonal Modulation of Plant Immunity. Annu Rev Cell Dev Biol 28:489–521. https://doi.org/10.1146/annurev-cellbio-092910-154055
31. Rushton PJ, Somssich IE, Ringler P, Shen QJ (2010) WRKY transcription factors. Trends Plant Sci 15:247–258. https://doi.org/10.1016/j.tplants.2010.02.006
32. Sakakibara H (2006) CYTOKININS: Activity, Biosynthesis, and Translocation. Annu Rev Plant Biol 57:431–449. https://doi.org/10.1146/annurev.arplant.57.032905.105231
33. Su W, Huang L, Ling H et al (2020) Sugarcane calcineurin B-like (CBL) genes play important but versatile roles in regulation of responses to biotic and abiotic stresses. Sci Rep 10:167–167. https://doi.org/10.1038/s41598-019-57058-7
34. Wang J-H, Gu K-D, Han P-L et al (2020) Apple ethylene response factor MdERF11 confers resistance to fungal pathogen Botryosphaeria dothidea. Plant Sci 291:110351. https://doi.org/10.1016/j.plantsci.2019.110351
35. Zhang F, Wang F, Yang S et al (2019a) MdWRKY100 encodes a group I WRKY transcription factor in Malus domestica that positively regulates resistance to Colletotrichum gloeosporioides infection. Plant Sci 286:68–77. https://doi.org/10.1016/j.plantsci.2019.06.001
36. Zhang L, Hu J, Han X et al (2019b) A high-quality apple genome assembly reveals the association of a retrotransposon and red fruit colour. Nat Commun 10:1494–1494. https://doi.org/10.1038/s41467-019-09518-x
37. Zhao X-Y, Qi C-H, Jiang H et al (2019) Functional identification of apple on MdHIR4 in biotic stress. Plant Sci 283:396–406. https://doi.org/10.1016/j.plantsci.2018.10.023
38. Zhu YD, Jin YS, Wei S et al (2012) Functional analysis of the isopentenyltransferase gene MdIPT3a from apple (Malus pumila Mill.). J Hortic Sci Biotechnol 87:478–484. https://doi.org/10.1080/14620316.2012.11512898
39. Žižková E, Dobrev PI, Muhovski Y et al (2015) Tomato (Solanum lycopersicum L.) SlIPT3 and SlIPT4 isopentenyltransferases mediate salt stress response in tomato. BMC Plant Biol 15:85–85.

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Figures

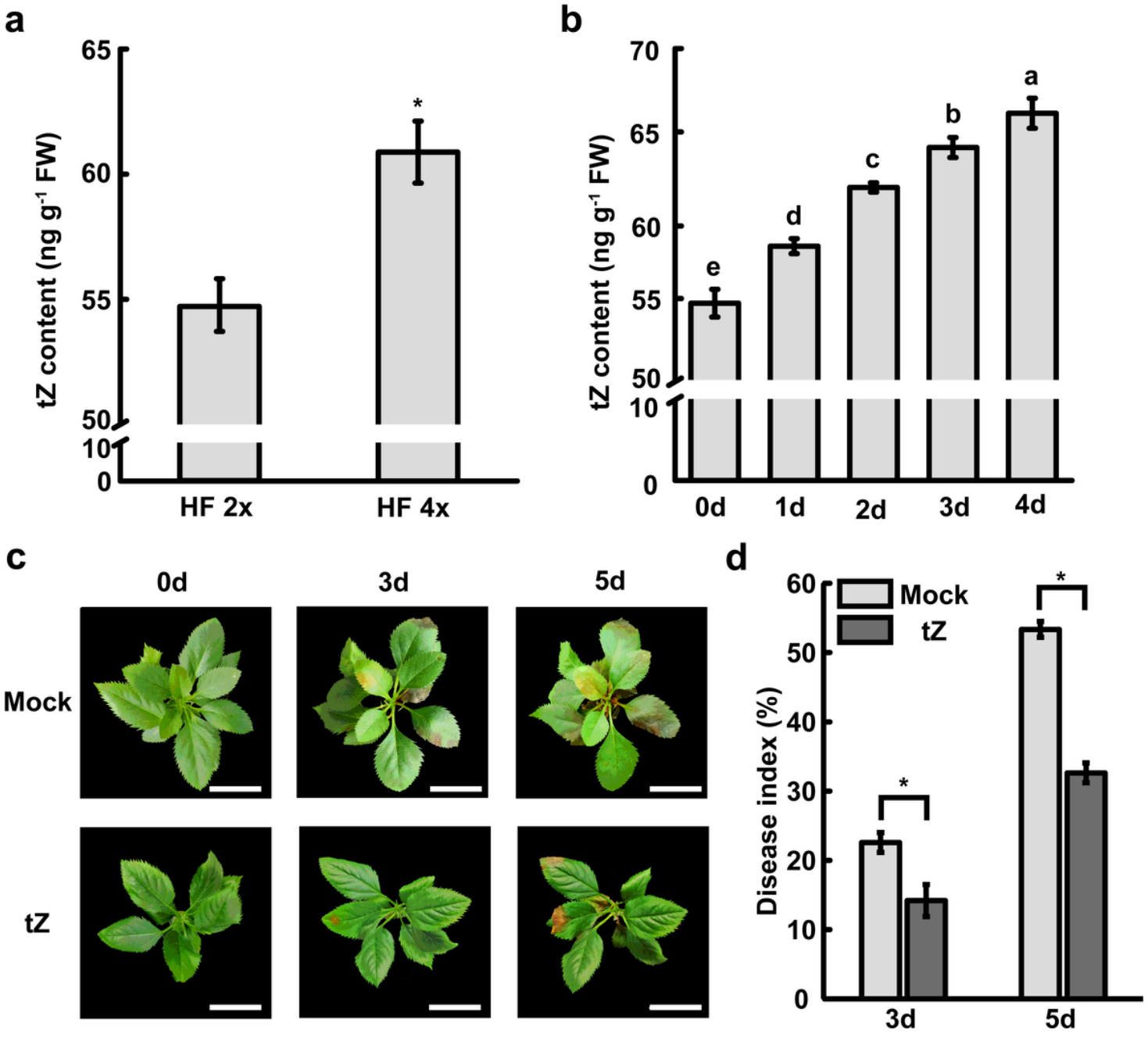

Figure 1

tZ in ‘Hanfu’ leaves enhances resistance to C. gloeosporioides a. tZ content in the leaves of apple. HF 2x represents ‘Hanfu’ diploid apple and HF 4x represents autotetraploid apple. The vertical bars represent the SDs from three replicates. * P < 0.05 (Student’s t-test). b. tZ content in the apple leaves after the C. gloeosporioides treatment. Significant differences were indicated by different letters between treatments (P < 0.05, ANOVA). The vertical bars represent the SDs from three replicates. c. Disease feedback phenotypes from typical untreated and tZ treated ‘Hanfu’ apple plants, bars = 1 cm. d. Disease index for

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untreated and tZ treated apple plants. * P < 0.05 (Student's t-test). The vertical bars represent the SDs from three replicates.

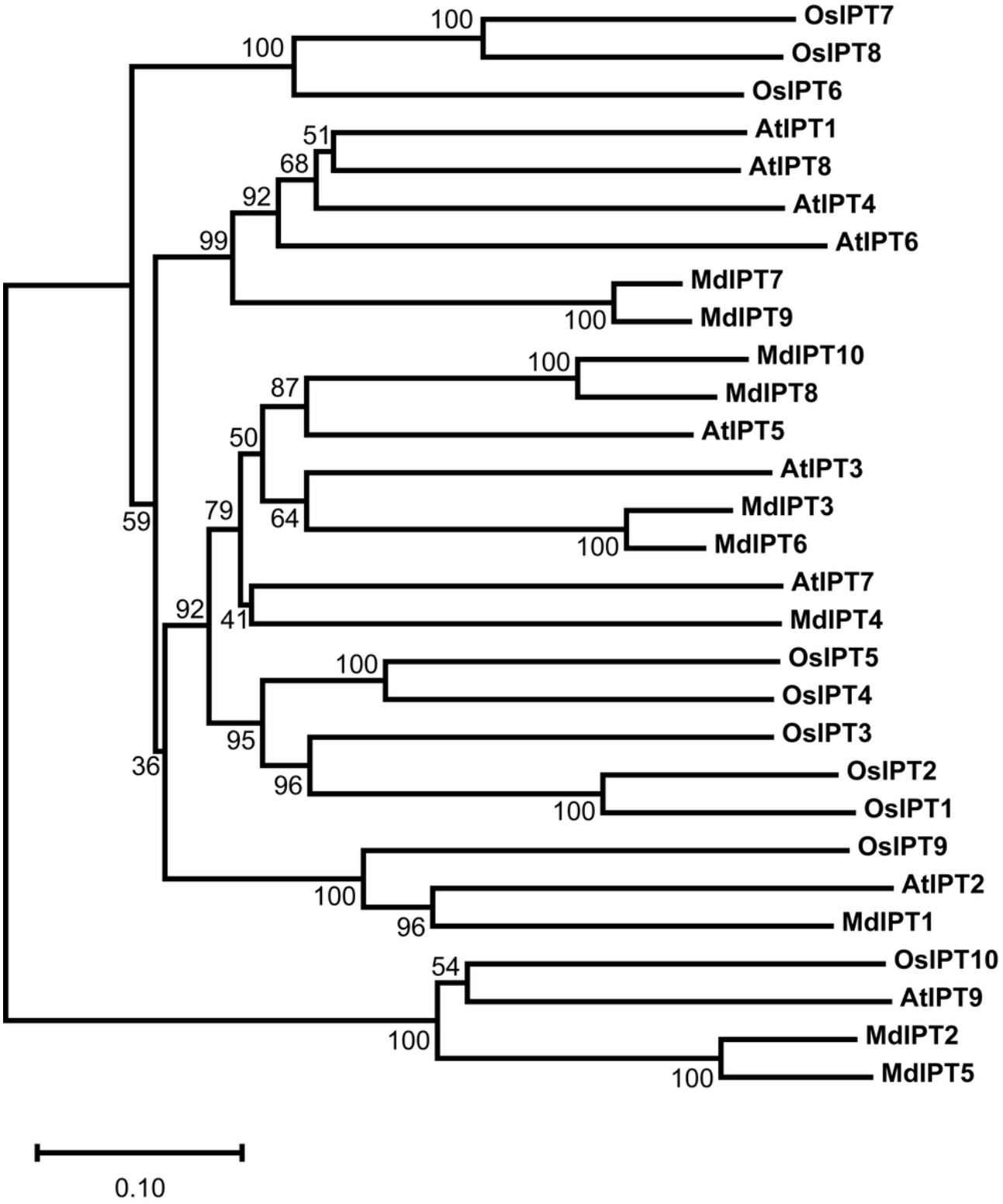

Figure 2

Phylogenetic tree of MdIPT, AtIPT and OsIPT proteins Ten of the MdIPT proteins, nine of AtIPT proteins and ten of OsIPT proteins were used to form the neighbor-joining tree. Identity documents of AtTPT1-9 AT5G19040, AT1G25410, AT3G23630,

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AT3G19160 and AT5G20040. Identity documents of OsTPT1-10 were LOC_Os03g24440, LOC_Os03g24240, LOC_Os05g24660, LOC_Os03g59570, LOC_Os07g11050, LOC_Os07g09220, LOC_Os05g47840, LOC_Os01g49390, LOC_Os01g73760 and LOC_Os06g51350.

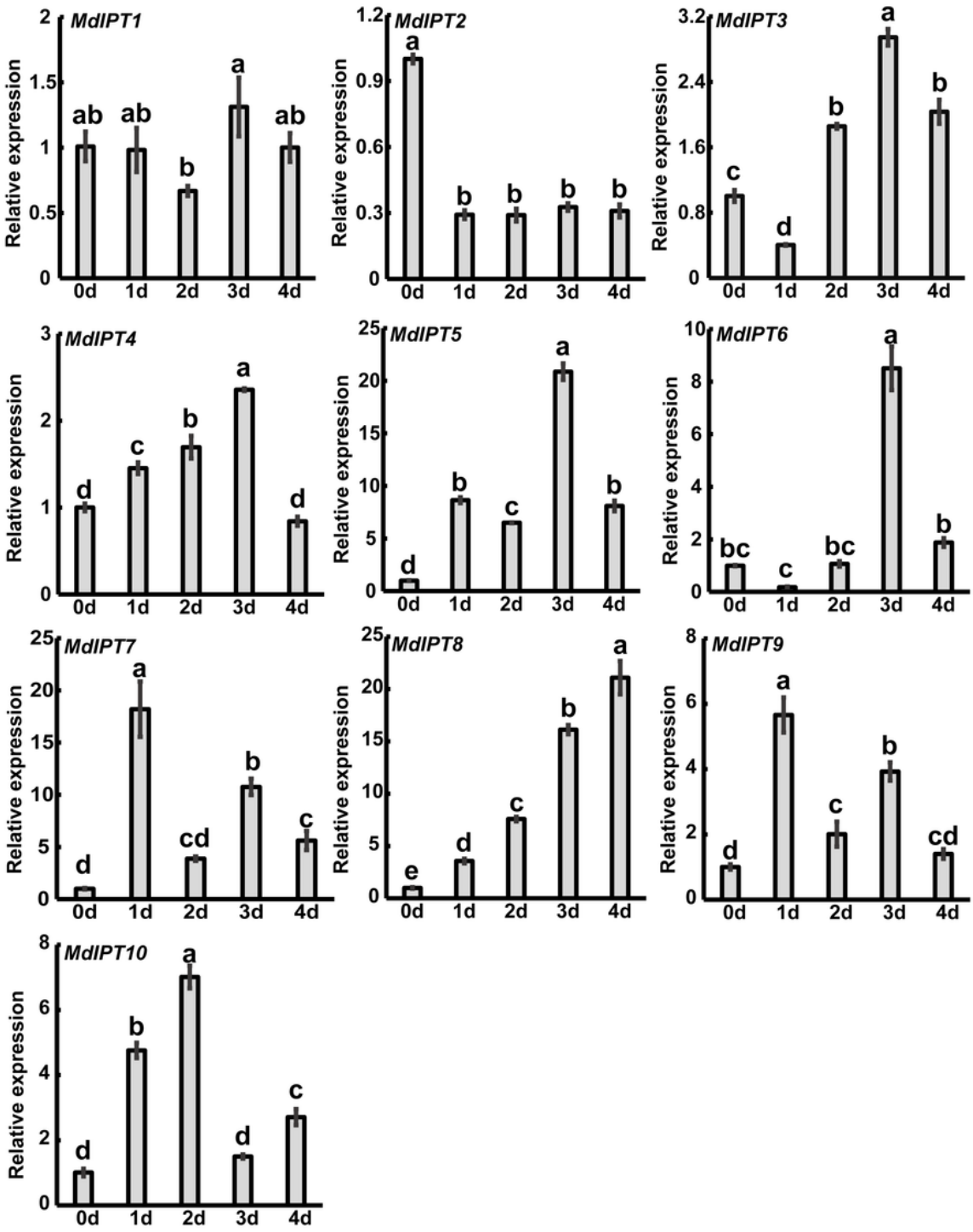

Figure 3

Quantitative RT-PCR analysis of MdIPTs after inoculating C. gloeosporioides in ‘Hanfu’ leaves Non-treated

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nificant differences were indicated by different

letters between treatments (P < 0.05, ANOVA). The vertical bars represent the SDs from three replicates.

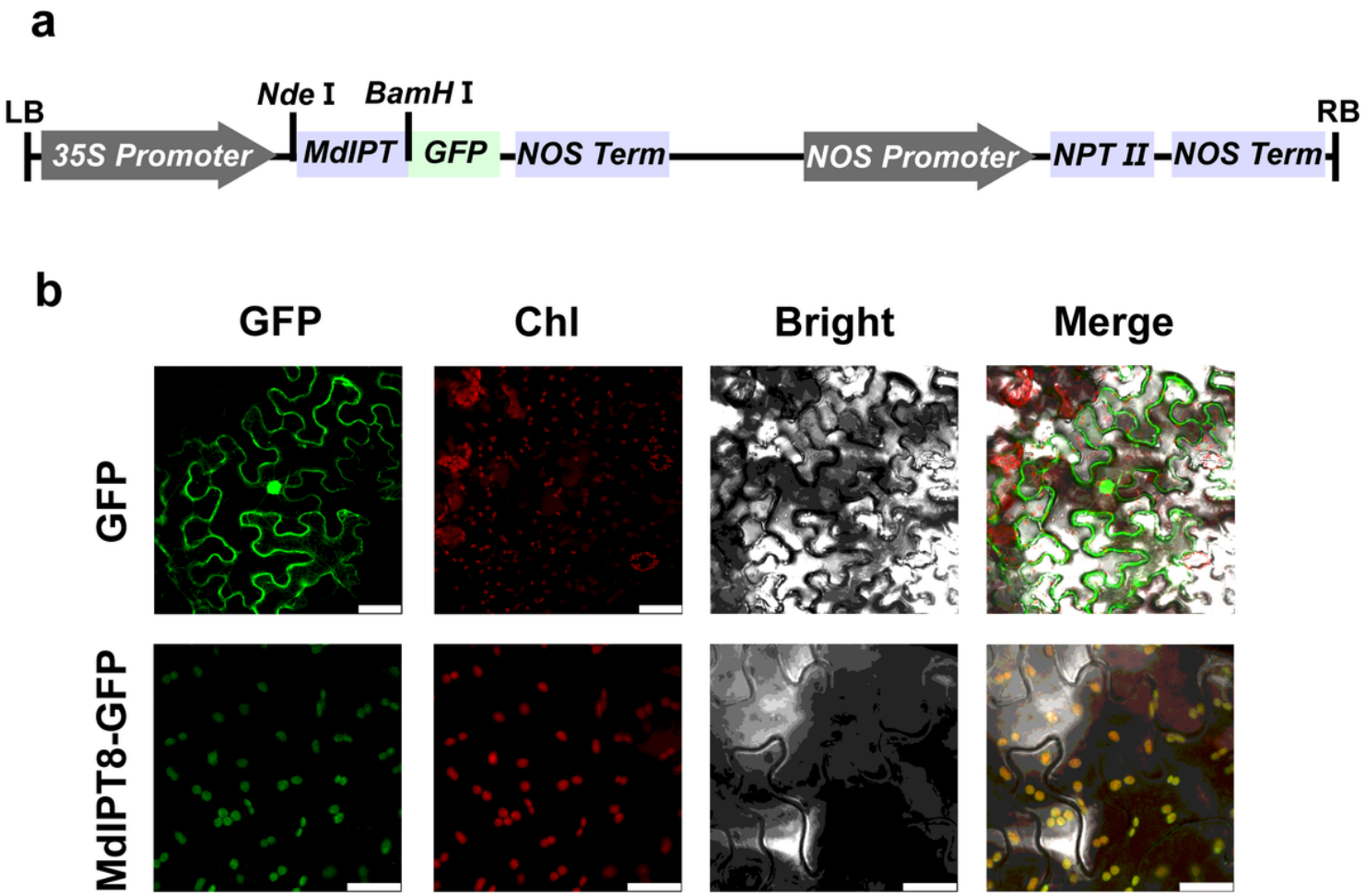

Figure 4

Subcellular localization of MdIPT8 a. Structural diagram of the pRI101GFP-MdIPT8 construct. b. Nicotiana benthamiana leaves were used as the transformation target of 35S::GFP, bars = 50 μm (top). Nicotiana benthamiana leaves were used as the transformation target of 35S::MdIPT8-GFP, bars = 25 μm (bottom). Nicotiana benthamiana cell chloroplasts were observed by their autofluorescence.

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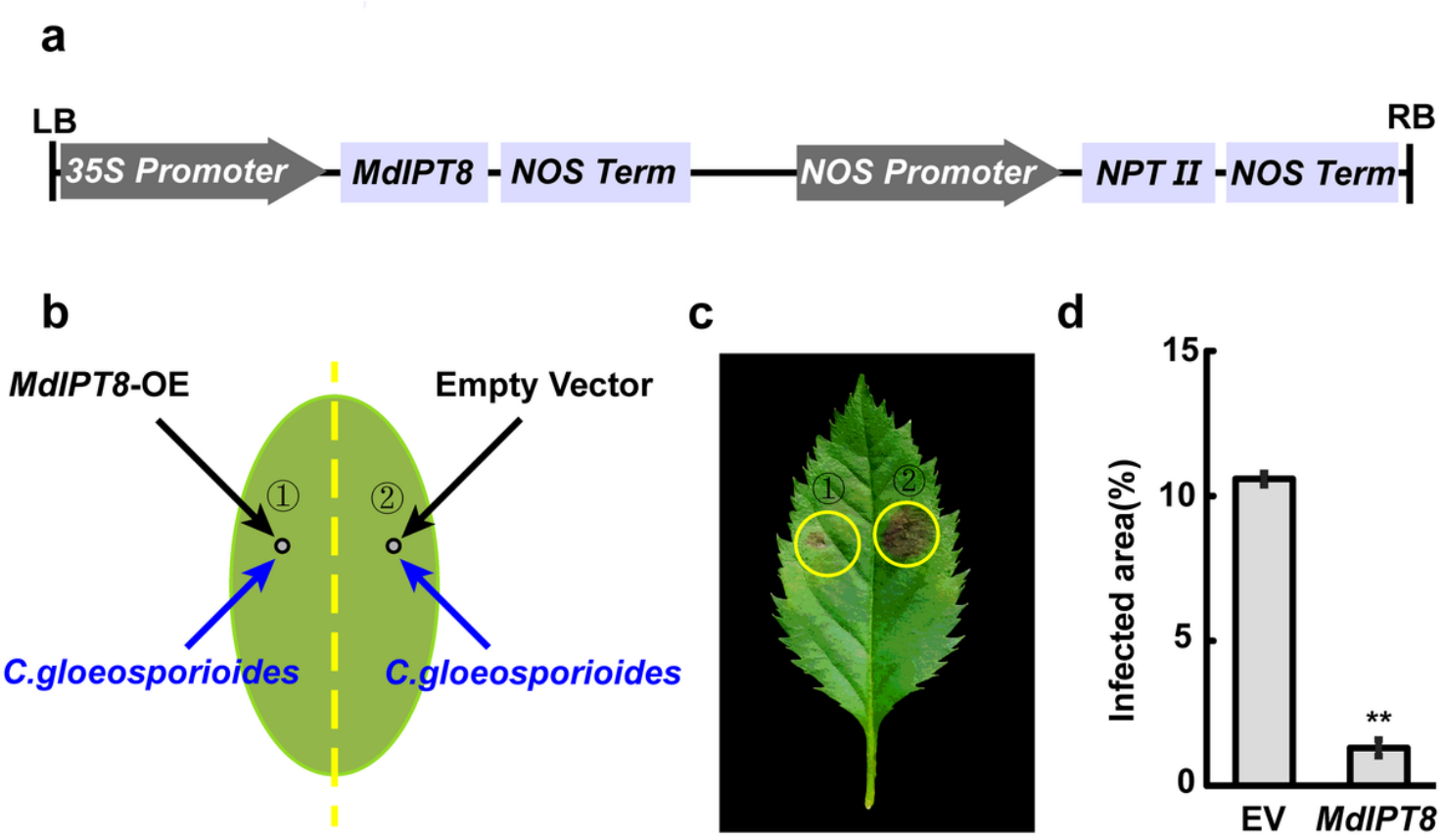

Figure 5

Overexpression of MdIPT8 in ‘Hanfu’ leaves strengthens resistance to C. gloeosporioides a. Structural diagram of pRI101-MdIPT8. b. Agroinfiltration and inoculation of leaves. Black arrows point to sites of infiltration of Agrobacterium containing pRI101-MdIPT8 vector mixture into subculture ‘Hanfu’ apple leaves, with Agrobacterium containing empty pRI101 vector mixture as controls. Blue arrows indicate the C. gloeosporioides spore suspension was utilized for the injection site. Numbers represent different injection sites. c. Leaf phenotypes after inoculation. The center of each yellow circle is the injection site. d. Infected area represents the ratio of the lesion area to the area of the half apple leaf. The vertical bars represent the SDs from three replicates. ** indicate significant differences ($P < 0.01$) as determined by Student’s t-test.

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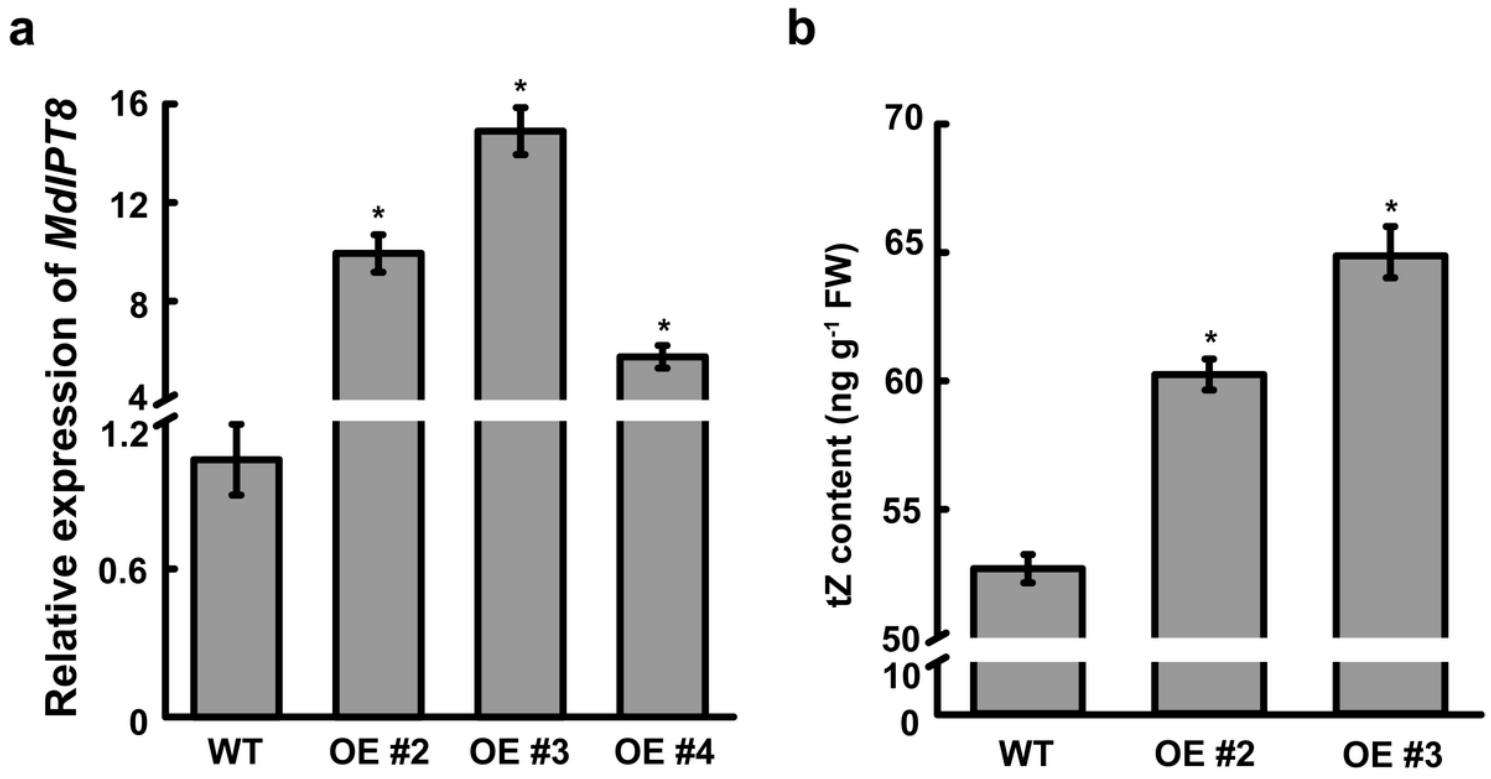

Figure 6

Confirmation of overexpressing MdIPT8 transgenic lines a. RT-qPCR analysis of the MdIPT8 expression level in WT plants and three MdIPT8 transgenic lines. b. tZ content in the leaves of WT, MdIPT8-OE #2 and #3. * $P < 0.05$ (Student's t-test). The vertical bars represent the SDs from three replicates.

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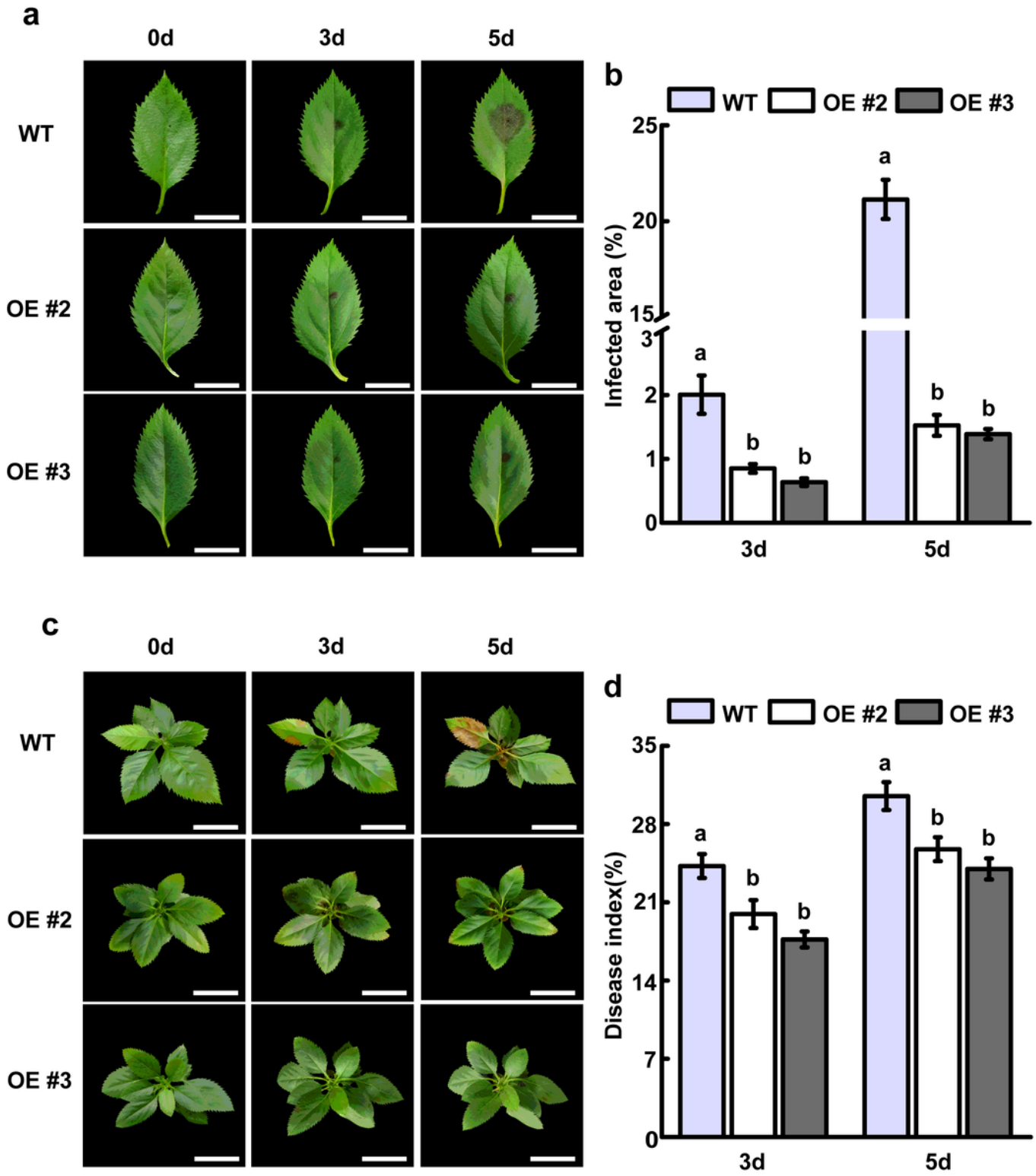

Figure 7

MdIPT8 enhances resistance to C. gloeosporioides in transgenic apple a. Phenotypes of WT, MdIPT8-OE #2 and #3 leaves after C. gloeosporioides treatment, bars = 1cm. b. Infected area represents the ratio of the lesion area to the area of the whole apple leaf. c. Phenotypes of WT, MdIPT8-OE #2 and #3 plants after C. gloeosporioides treatment, bars = 1cm. d. Disease index for WT plants and two MdIPT8

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transgenic lines. Significant differences were indicated by different letters between treatments ($P < 0.05$, ANOVA). The vertical bars represent the SDs from three replicates.

Supplementary Files

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