A monoterpene synthase regulates geraniol formation and plant defense via alternative splicing in tea plants

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Abstract

Geraniol is an important contributor to the pleasant floral scent of tea products and one of the most abundant aroma compounds in tea plants; however, its biosynthesis and physiological function in response to stress in tea plants remain unclear. Here, we studied eight terpene synthases with expression levels that were correlated with geraniol accumulation in different tissues of tea plants. The proteins encoded by the full-length terpene synthase (*CsTPS1*) and its alternative splicing isoform (*CsTPS1-AS*) could catalyze the formation of geraniol when GPP was used as a substrate *in vitro*, whereas the expression of *CsTPS1-AS* was only significantly induced by *Colletotrichum gloeosporioides* and *Neopestalotiopsis* sp. infection. Silencing of *CsTPS1* and *CsTPS1- AS* resulted in a significant decrease in the geraniol content in tea plants. The geraniol content and antifungal activity of tea plants were compared when *CsTPS1* and *CsTPS1- AS* were silenced. Down-regulation of the expression of *CsTPS1- AS* reduced the accumulation of geraniol, and the silenced tea plants exhibited greater susceptibility to pathogen infection than control plants. However, the geraniol content and pathogen resistance of *CsTPS1*-silenced plants and control plants did not significantly differ. Further analysis showed that silencing of *CsTPS1- AS* led to a decrease in the expression of the defense-related genes *PR1* and *PR2* and the expression of SA pathway-related genes in tea plants, which increased the susceptibility of tea plants to pathogenic fungal infections . Both *in vitro* and *in vivo* results indicated that *CsTPS1* is involved in the regulation of geraniol formation and plant defense via alternative splicing in tea plants. The results of this study provide new insights into geraniol biosynthesis and highlight the role of monoterpene synthases in modulating plant disease resistance via alternative splicing.

1 Introduction

Tea (*Camellia sinensis*) is an important woody economic crop (Xia et al., 2020), and its leaves can be used to produce one of the world's most important beverages (Rietveld and Wiseman, 2003). Tea plants are susceptible to attack by various pathogens and insects during their growth (Chen et al., 2020b). Tea anthracnose disease caused by fungi in the genus *Colletotrichum* , especially *Colletotrichum gloeosporioides* (Jeyaraj et al., 2019) and gray blight disease caused by *Pestalotiopsis* species (Chen et al., 2018), are two of the most destructive foliar diseases of tea plants and are responsible for 30–60% (Wang et al., 2021) and 10–20% of the losses of tea products on an annual basis, respectively (Chen et al., 2018, Jiang et al., 2020). Plants have evolved complex defense mechanisms to defend against pathogens (Sharifi et al., 2018). Plant hormones such as salicylic acid (SA) and jasmonic acid play key roles in defense against pathogens (Sharifi et al., 2018). SA is the primary hormone responsible for plant disease resistance, including the activation of the defense response following pathogen infection (Wang et al., 2021). Previous studies have shown that the release of volatile terpenes is one of the key mechanisms by which plants resist pathogen (Sharifi et al., 2018).

Tea possesses abundant secondary metabolites that are strongly associated with its quality and health benefits (Jiang et al., 2019, Xia et al., 2020). The release of defense-related volatiles plays an important role in mediating both local and systemic responses, as the emission of volatiles primes their defense mechanisms in response to attack by herbivores and pathogens (Bouwmeester et al., 2019; Jiang et al., 2019; Quintana-Rodriguez et al., 2015; Richter et al., 2016; Turlings and Erb, 2018). The exposure of susceptible cultivars to volatiles from resistant cultivars can significantly increase the expression of defense-related genes and confer disease resistance (Castelyn et al., 2015; Eberl et al., 2018; Quintana-Rodriguez et al., 2015; Sharifi et al., 2018). Terpenoids contribute to tea flavor via their low human odor perception thresholds (Yang et al., 2013). Monoterpenes, including linalool and geraniol, enhance the flavor and aroma of tea (Ho et al., 2015). Linalool and geraniol are two of the most abundant and odor-active monoterpenoids in tea plants, and they contribute to the pleasant floral scent of tea products (Han et al., 2016; Yang et al., 2013). Although the biosynthesis of the terpenoid pathway in tea plants has been studied, only a few terpene synthases (TPSs) and *TPS* genes involved in terpenoid synthesis have been identified (Zhou et al., 2017). The key gene involved in linalool formation in tea plants has been isolated and functionally verified (Liu et al., 2018). However, the key enzyme involved in geraniol biosynthesis and its biological function in tea plants remain unclear (Zhou et al., 2020).

Alternative splicing (AS) can generate different mRNA splicing isoforms from a single mRNA precursor via different splicing sites (Li et al., 2020), and this can result in diverse protein isoforms(Laloum et al., 2018). An increasing number of studies have shown that AS plays an important role in the growth, development, and abiotic and biotic stress tolerance of plants (Mi et al., 2021; Posé et al., 2013). AS is also key in the biosynthesis of secondary metabolites (Zhao et al., 2014) and the response to pathogen infection (Liu et al., 2016). AS also figures prominently in abiotic stress tolerance, especially in ABA-mediated responses (Laloum et al., 2018). More than 41% of genes undergo AS during cold acclimation, and the four main types of AS events in tea plants are intron retention, exon skipping, alternative 5' splice site, and alternative 3' splice site (Li et al., 2020). AS isoforms of the*CsLOX2, CsLOX9* , *a* nd *CsLOX10* genes can be induced under low-temperature treatment (Zhu et al., 2018a). AS in tea plants plays an important role in regulating the synthesis of secondary metabolites (Zhu et al., 2018b), including the synthesis of anthocyanins (Chen et al., 2020a), linalool (Liu et al., 2018), and volatile fatty acid derivatives(Xu et al., 2019). However, whether AS plays a role in the regulation of geraniol formation and biotic stress responses in tea following pathogen infection remains unclear.

Here, the first geraniol synthase (*CsGES*) was identified, cloned, and functionally characterized in tea plants. The expression level of the AS isoform *CsTPS1 -AS* , but not the full-length*CsTPS1* , was significantly increased following *C. gloeosporioides* and *Neopestalotiopsis* sp. infection, and the function of *CsTPS1 -AS* in planta was assessed. Silencing of*CsTPS1 -AS* led to a decrease in the expression of defense-related and SA biosynthesis-related genes and an increase in the susceptibility of tea plants to *C. gloeosporioides* and*Neopestalotiopsis* sp. infection. The findings of this study enhance our understanding of geraniol formation in tea plants following fungal infection and provide new insights into the functions of AS isoforms during pathogen infection in plants.

2 Materials and methods

2.1 Plant material

Several cultivars of tea plants, including *C. sinensis* var. sinensis "Shuchazao (SCZ)," "Zhongcha108 (ZC108)," "Longjingchangye (LJCY)," "Fudingdabai (FDDB)," and "Yingshuang (YS)," were collected from the Tea Plant Cultivar and Germplasm Resource Garden of Anhui Agricultural University (Guohe Town, China) and immediately frozen in liquid nitrogen. All the tea samples were stored at –80degC until use.

2.2 Chemicals and reagents

Standards of geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), geraniol, (Z)-β-ocimene, and (E)-β-ocimene were purchased from Sigma-Aldrich (St. Louis, MO, USA). SYBR1 Premix ExTaqII and

PrimeScript RT Reagent Kit were purchased from TaKaRa (Dalian, China).

2.3 RNA isolation, cDNA cloning, and sequence analysis

Total RNA from leaves of *C. sinensis* (SCZ) was isolated using a Fast Pure Plant total RNA Isolation Kit (Vazyme Cat, RC401-01, Nanjing, China) according to the manufacturer's instructions. The cDNA was synthesized by reverse transcription of the total RNA using Prime Script RT Master Mix (Vazyme, China). The open reading frame sequences were amplified using Phusion ® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA); primers for the cloned *CsTPS1/1 -AS* gene are shown in Table S1. The PCR products were purified using a Gel Extraction Kit (CWBIO, Jiangsu, China). The resulting target cDNA fragment was ligated into the pGEX-4T1 vector and then subsequently transformed into Trans 1-T1 competent cells.

2.4 GC–MS Analysis of geraniol and other volatiles in tea samples

Geraniol and other volatiles in samples were analyzed using solid-phase microextraction (SPME) combined with gas chromatography (Thermo Scientific TRACE 1300)/mass spectrometry (Thermo Scientific ISQ 7000 GC/MS). Briefly, the tea samples in liquid nitrogen were ground to a fine powder and transferred to a 20-mL headspace bottle. Two μl of ethyl caprate (1 ppm in methyl alcohol) was used as an internal standard. The samples were incubated at 60°C for 1 h, and the volatiles were adsorbed by SPME during the entire process. A fused-silica GC column (DB-5, 60 m × 0.25 mm, film thickness 0.25 μm, J&W Scientific, Folsom, CA, USA) was used to separate chemical compounds. Pure helium was used as the carrier gas at a flow rate of 1 mL/min. The GC injector had a split ratio of 10:1. The GC oven was maintained at 40°C for 3 min and was increased by 5°C/min to 80°C; it was then increased to 160°C at 2°C/min and then to 240°C at a rate of 10°C/min. After being held at 240°C for 5 min, the detector temperature was set to 250°C. The mass spectrometry data were acquired in full-scan mode with an m/z range of 300–600 after a solvent delay of 0 s. All compounds were identified by comparison with a mass spectrometry library (NIST) and compounds with known retention times. Geraniol, (Z)-β-ocimene, and (E)-β-ocimene were identified using standards.

2.5 Heterologous protein expression and purification

Heterologous protein expression and purification were carried out following the methods of a previous study (Chen et al., 2020c) with slight modifications. The full-length sequence of *CsGES* was digested with *Bam* H1 and *Sma* l1, and the resulting gene fragments were cloned into the expression vector pGEX-4T-1 (Amersham Biosciences, Freiburg, Germany). The recombinant plasmids were transformed into *E. coli* strain BL21 (DE3) pLysS cells. The empty expression vector pGEX-4T-1 was transformed into *E. coli* BL21 (DE3) pLysS cells, which served as the negative control. After incubation at 37°C in Luria–Brentani liquid medium containing ampicillin (50 ug ml^{-1}) and chloramphenicol (50 ug ml^{-1}) for 20 to 24 h, the culture was diluted and grown until the optical density (OD_{600}) of the cultured cells reached 0.6–0.8. Protein expression was induced by adding isopropyl-ß-D-thio-galactopyranoside to a final concentration of 1 mM. The cultures were incubated at 16°C with oscillation at 150 rpm for 22 h. Following IPTG induction, the cells were harvested at 4000 × g for 10 min; the expressed protein was then isolated and refolded as described in a previous study (Jing et al., 2019). The fusion proteins were purified by GST-binding resin (Novagen, Darmstadt, Germany) following the manufacturer's protocol. The protein concentration was determined using a photometric method (Bradford, 1976) with bovine serum albumin as a standard. The correct size of the proteins was confirmed by SDS–PAGE.

2.6 Enzyme assay for *CsGES*

Enzyme activity assays were carried out in 1-mL reaction buffer (pH 7.2, 0.1 M PBS, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 100 mM KCI, and 1 mM DTT, 10% glycerol (v/v)) containing 50–100 μg of crude recombinant protein and 5 μg of FPP and GPP substrate in a 20-mL glass tube (Martin et al., 2010a). The reactions were incubated at 30°C for 1 h and then at 42°C for 15 min (Zhou et al., 2017), and the volatiles were collected by SPME. At least three biological replicates (different preparations) were carried out. The reaction products

were identified using GC-MS per the method described above. Enzyme activity products, geraniol, (Z)-β-ocimene, and (E)-β-ocimene were identified using comparison standards.

2.7 Gene suppression of *CsGES* in *C. sinensis* using AsODNs

Functional assays of *CsGES*(*CsTPS1* , *CsTPS1-AS* , and *CsTPS1/1 -AS*) in tea plants were carried out by suppressing the expression of *CsGES* in *C. sinensis* following a previously described method (Zhao et al., 2020b). Candidate sequences (Table S1) of the antisense oligonucleotide (AsODN) with complementarity to the segment of target gens (*CsTPS1* , *CsTPS1-AS* , and *CsTPS1/1 -AS*) were selected using Soligo software (*http://sfold.wadsworth.org/cgi-bin/index.pl*), respectively. AsODNs were synthesized by TSINGKE Biological Technology Co., Ltd. (Anhui, China). The target gene in the tea leaves was silenced using AsODN following a previously described method (Zhao et al., 2020a; Zhao et al., 2020b). Briefly, 1 mL of 40 μM AsODN- *CsTPS1/1 -AS* solution (to suppress both *CsTPS1* and *CsTPS1 -AS*) and AsODN-*CsTPS1* solution (to suppress *CsTPS1*), or AsODN-*CsTPS1 -AS* solution (to suppress *CsTPS1 -AS*) was injected into whole tea leaves. The sense oligonucleotides (sODN) were injected into tea leaves as a control treatment. At least six experimental replicates were conducted for each treatment. After treatment, the tea leaves were harvested, immediately frozen in liquid nitrogen, and then kept at –80°C before analysis. The content of geraniol in the treated tea was detected as described above.

2.8 Quantitative real-time PCR analysis

The leaves of *C. sinensis* var. *sinensis* were used for gene expression analysis. The tea samples were ground into powder under liquid nitrogen and collected in an RNA-free centrifuge tube for RNA extraction. Total RNA from tea plant leaves was used as a template. Fluorescent quantitative cDNA templates were synthesized by reverse transcription using Hifair® III First-Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) kit. Real-time PCR was performed according to previously published protocols (Chen et al., 2020c) using the gene-specific primer sequences (Table S1). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal reference gene, and relative expression levels were calculated using the $2^{-\Delta^{\sim}T}$ method (Jing et al., 2020). All reactions were carried out using the CFX96 Real-Time System (Bio-Rad, USA). The two-step temperature program was as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 62°C for 30 s in 96-well optical reaction plates.

2.9 Pathogen cultivation and infection of tea plants

The pathogenic fungi *Neopestalotiopsis* sp. and *C. gloeosporioides* were cultivated in PDA medium in Petri dishes and grown in an incubator at 25°C±3 with a humidity of 75 ± 5%. The pathogenic infection experiment was carried out as follows. Briefly, one-year-old *C. sinensis* (SCZ) seedlings were selected, and the leaves in each treatment were wounded with a sterile needle. Five-mm diameter mycelial discs of *C. gloeosporioides* and *Neopestalotiopsis* sp. grown on PDA were inoculated into the test leaves. The control leaves were treated with 5-mm diameter pure PDA. Finally, the seedlings were grown in a greenhouse at 25°C and 70% relative humidity. At least six biological replicates were carried out.

2.10 Contact antifungal activity of geraniol *in vitro*

The contact antifungal activity of geraniol against *Neopestalotiopsis* sp. and C. *gloeosporioides* was determined following a procedure described in a previous study (Jiang et al., 2020). The median inhibitory concentration (MIC_{50}) of geraniol was determined using a serial two-fold dilution method. Based on preliminary experiments, the stock solution was serially diluted in 30 mL of PDA medium at 45–50°C at different concentrations (1 μl/mL, 0.5 μl/mL, 0.25 μl/mL, 0.125 μl/mL, and 0.0625 μl/mL) to evaluate inhibitory activity against *Neopestalotiopsis* sp. and *C. gloeosporioides.* The negative control received the same quantity of acetone mixed with PDA. Ten mL of toxic medium was poured onto aseptic 9-cm plastic Petri dishes. A 5-mm diameter fungal disc of *Neopestalotiopsis* sp. and *C. gloeosporioides* was immediately inoculated in the center of each PDA plate, and plates were incubated in the dark at 25°C. After 5 days, colony growth diameter (mm) was measured using digital calipers. Each test was repeated three times.

2.11 Pathogen infection of tea plants treated with AsODNs

C. sinensis (SCZ) leaves showing no signs of disease and insect damage were used in experiments. The target gene of each treated tea leaf was silenced using the gene suppression method described above. Briefly, AsODN- *CsTPS1/1 -AS,* AsODN-*CsTPS1* , and AsODN-*CsTPS1 -AS* solution was injected into the tea leaves of different treatments. The treated tea leaves were then immediately inoculated with mycelial discs (5 mm diameter) of *Neopestalotiopsis* sp. and *C. gloeosporioides* . In the control treatment, each treated tea leaf was injected with the same volume of sODN solution and immediately inoculated with mycelial discs of the two pathogens. All treated tea plants were cultured in a greenhouse at 25 ± 3°C with 70 ± 5% relative humidity and a 16/8 hr (day/night) photoperiod. Treated tea leaves were collected for analysis after 72 h when they showed signs of disease. There were six biological replicates for each treatment.

2.12 WGA staining and microscopic observation of pathogenic hyphae

The growth status of pathogens in tea leaves was assessed using a stereoscopic fluorescence microscope (Olympus SZX16, Tokyo Japan), and the total infected area was measured using image analysis software (Olympus Cellsens Standard, Tokyo, Japan). Tea leaves inoculated with*Neopestalotiopsis* sp. and *C. gloeosporioides* were placed in 4-mL centrifuge tubes with FAA fixed solution (G1103, Servicebio®, Wuhan China); sent to Wuhan Seville Biotechnology Co., Ltd. for fluorescent wheat germ agglutinin (WGA) staining; and photographed with a fluorescence microscope.

2.13 Subcellular localization analysis of CsTPS1 and CsTPS1-AS proteins

Subcellular localization assays of CsTPS1 and CsTPS1-AS proteins were performed following the procedure described in a previous study (Wang et al., 2019a). Briefly, binary vectors (pCHNP-eYFP/mCherry) were constructed with several elements on the pCAMBIA1300 backbone (CAMBIA, Canberra, Australia). The amplified fragments were introduced into pCHNP-eYFP with the NcoI site using in-fusion technology. The empty vector pCHNP-mCherry was used as a negative control. *Agrobacterium tumefaciens* strain GV3101 carrying the construct for the transient expression of individual mCherry and CsTPS1 EYFP and CsTPS1-AS EYFP fusion proteins was mixed and infiltrated into the leaves of tobacco. Images were taken using a laser confocal fluorescent microscope (Lecia DMi8, Germany). The EYFP, mCherry fluorescence, and chloroplast autofluorescence were analyzed at excitation wavelengths of 488 nm, 561 nm, and 561 nm and emission wavelengths of 500–530 nm, 580–620 nm, and 680–720 nm, respectively.

3 Results

3.1 Geraniol synthase candidates identified by analysis of gene expression levels and geraniol accumulation in tea plants

We identified *TPS* genes in tea plants from recently published tea genome sequences in the Tea Plant Information Archive (TPIA,*http://tpia.teaplant.org*). Gene expression levels and terpenoid abundances permitted the identification of geraniol synthase (*CsGES*) genes in tea plants. Geraniol synthase candidates were obtained based on the correlations between all *TPS* genes and the content of geraniol in the different tissues of tea plants as well as a gene-to-metabolite network (Figure 1A). A total of 27 *TPS* genes were screened, and eight geraniol synthase candidates (*CsTPS1–CsTPS8*) positively associated with the geraniol content (indicated by dark green dots in Figure 1A) were selected for further study.

3.2 Expression levels of eight candidate *CsGES* genes in pathogen-infected tea plants

Given that geraniol has been reported to function as an antifungal compound (Li et al., 2017; Kalagatur et al., 2018; Tang et al., 2018), changes in the abundance of geraniol in response to *C. gloeosporioides* and *Neopestalotiopsis* sp. infection were characterized using GC–MS. The geraniol content in the infected leaves significantly increased after 24 and 48 h of infection (Figure 1B), indicating that geraniol might play a role in activating defense-signaling pathways following fungal attack in tea plants. To determine which candidates are involved in the biosynthesis of geraniol, gene-specific primers (Table S1) of these eight genes were designed, and the expression of these genes in response to pathogen infection was analyzed 0, 12,

24, and 48 h after infection with *C. gloeosporioides* and *Neopestalotiopsis* sp. (Figure 1C,D). To verify the specificity of the primers, the abundances of the transcripts of the eight candidate genes were analyzed, and their products were verified by agarose gel electrophoresis (Figure 1E). One clear band was observed for seven genes (*CsTPS2–CsTPS8*), whereas three clear bands were observed for *CsTPS1* (Figure 1E), which indicates the presence of an AS form of *CsTPS1* in tea plants that is expressed in response to fungal attack.

To verify the presence of the AS forms of *CsTPS1,* the full-length sequences and the shorter AS forms of *CsTPS1* were obtained from young leaves of *C. sinensis* var. *sinensis cv.*Shuchazao using gene-specific primer pairs (Table S1) (Wei et al., 2018). The whole-length *CsTPS1* contains a 1758-bp open reading frame (Figure S1) that encoded 585 amino acids (Figure S2); there were 83 fewer amino acids in the AS form (referred to as *CsTPS1-AS*) (Figure 2A and Figure S2). The AS form of *CsTPS1* was confirmed based on an AS database for tea plants (TeaAS,*http://www.teaas.cn/index.php*) (Mi et al., 2021). The expression of *CsTPS1* and its AS isoform (*CsTPS1 -AS*) was quantified in response to pathogen infection. To further verify whether *CsTPS1 -AS* is expressed in tea plants in response to pathogen infection. The new specific quantitative primers for *CsTPS1 -AS* and*CsTPS1* were redesigned (Table S1and Figure S2). The expression of*CsTPS1 -AS* and *CsTPS1* was quantified using RT-PCR, respectively. With the exception of *CsTPS1 -AS* , the expression of none of the eight candidates was induced in tea plants following pathogen infection (Figure 1C,D,F). The expression of*CsTPS1-AS* was significantly induced in response to infection with both *C. gloeosporioides* and *Neopestalotiopsis* sp.,which is consistent with changes in the content of geraniol in infected leaves. Therefore, the roles of *CsTPS1* and its AS forms in geraniol biosynthesis and the response to pathogen infection were studied.

To verify that *CsTPS1-AS* is involved in regulating geraniol biosynthesis and disease resistance in tea plants, the expression of *CsTPS1 -AS* in infected tea plants was determined at various points after infection in repeated experiment (Figure 2B). The expression of *CsTPS1-AS* was significantly increased under pathogen infection compared with the control, especially at 24 and 48 h, which is consistent with changes in the content of geraniol in leaves infected with the two pathogens (Figure 1B). Overall, these findings indicate that*CsTPS1 -AS* might be involved in the biosynthesis of geraniol in response to pathogen infection in tea plants.

3.3 *CsTPS1* and its AS forms can catalyze the formation of geraniol *in vitro*

To determine whether *CsTPS1* and its AS form*CsTPS1 -AS* are involved in the formation of geraniol in tea plants, *CsTPS1* and its AS splicing form *CsTPS1 -AS*were expressed in *E. coli* Rosetta (DE3) cells, and the enzymatic activity of the recombinant proteins was assessed using GPP as substrate. The products of the enzymes were adsorbed by SPME during the reaction process, and GC–MS was used to analyze the enzyme products. The recombinant proteins of *CsTPS1* and its AS splicing forms were involved in monoterpene formation when GPP was used as substrate (Figure 2). The main product was identified as geraniol based on commercial standards; however, (E) β-ocimene and (Z) β-ocimene were also observed (Figure 2C and 2D). No products were identified when FPP was used as substrate. These *in vitro* data suggest that *CsTPS1* and its AS forms are involved in the formation of geraniol in tea plants.

3.4 Geraniol inhibits the mycelial growth of fungi *in vitro*

Experiments were carried out to evaluate the ability of geraniol to inhibit the growth of *Neopestalotiopsis* sp. and *C. gloeosporioides in vitro* . Geraniol inhibited the mycelial growth of the two pathogenic fungi. The mycelial growth of both fungi was dose-dependent *in vitro* (Figure 3). Geraniol concentrations from 0.125 μL/mL to 1.0 μL/mL limited the mycelial growth of *C. gloeosporioides* (Figure 3A and 3C). The mycelial growth of*Neopestalotiopsis* sp. was strongly inhibited by geraniol concentrations from 0.0625 μL/mL to 0.5 μL/mL (Figure 3B and 3D). In addition, the MIC_{50} of geraniol against*Neopestalotiopsis* sp. and *C. gloeosporioides* was 0.29 μL/mL and 0.42 μL/mL, respectively (Figure 3E), indicating that geraniol more strongly inhibited the mycelial growth of*Neopestalotiopsis* sp. compared with *C. gloeosporioides* .

3.5 Silencing of *CsTPS1* and *CsTPS1-AS* reduces the geraniol content and pathogen resistance of tea plants

The expression of *CsTPS1 and CsTPS1-AS* was simultaneous suppressed in tea leaves using a shared AsODN according to a previously described procedure (Zhao et al., 2020b). The expression of*CsTPS1/1-AS* transcripts in tea leaves treated with AsODN-*CsTPS1/1-AS* for 24 h was significantly reduced compared with that in the control leaves (Figure 4A).Consistent with the gene expression patterns, the abundance of geraniol was significantly reduced in *CsTPS*- silenced leaves compared with control leaves (Figure 4B and 4C), indicating that *CsTPS1/1-AS* plays a key role in the formation of geraniol in tea plants.

Because the content of geraniol was increased in response to pathogen infection, we asked whether the formation of geraniol mediated by*CsTPS1/1-AS* plays a role in pathogen infection. To address this question, we silenced the expression of *CsTPS1/1-AS* in tea leaves, and silenced and control leaves were infected with *C. gloeosporioides* and *Neopestalotiopsis* sp. The leaves of*CsTPS1/1-AS*- silenced and control tea plants showed typical disease symptoms 72 h post-infection (hpi) (Figure 4D and 4F). However, the average surface area of disease spots in *CsTPS1/1-AS*- silenced leaves was significantly larger than that in control leaves (Figure 4E and 4G). These results suggested that tea leaves became more susceptible to infection to both fungi when *CsTPS1/1-AS* was silenced. Overall, our results indicate that *CsTPS1/1-AS* plays a key role in the biosynthesis of geraniol and pathogen resistance of tea plants.

3.6 *CsTPS1* and its AS forms confer different levels of disease resistance

To compare the function of *CsTPS1* and its AS forms in regulating geraniol formation and pathogen resistance in tea plants**,**gene-specific AsODNs were designed to silence *CsTPS1* and its AS forms (Table S1). The geraniol content was lower in tea leaves in which the expression of *CsTPS1-AS* was suppressed compared with that in control plants at 12, 24, and 48 h, respectively (Figure 5A and 5B). As expected, *CsTPS1 -AS* -silenced tea plants were more susceptible to infection with both *C. gloeosporioides* and*Neopestalotiopsis* sp. (Figure 5C) at 72 hpi, as the average surface area of disease spots on the tea leaves was larger in*CsTPS1-AS*-silenced tea plants compared with that in control plants (Figure 5D). By contrast, when *CsTPS1* was successfully suppressed in tea leaves (Figure 5E), the geraniol content was not change in tea leaves (Figure 5F) in which the expression of*CsTPS1* was suppressed compared with that in control plants at 12, 24, and 48 h, respectively. Meanwhile, no difference in the susceptibility of tea leaves to pathogen infection was observed between*CsTPS1* -silenced tea leaves and control tea leaves at 72 hpi (Figure 5G and 5H).

WGA staining was used to observe the hyphal growth of*Neopestalotiopsis* sp. and *C. gloeosporioides* on tea leaves. After WGA staining, the hyphae emitted a green fluorescence under the microscope. The green fluorescence intensity of*CsTPS1-AS* -silenced tea leaves was higher than that of control tea leaves (Figure 6A). The extent of mycelial growth on*CsTPS1-AS* -silenced tea leaves was higher than that on control leaves (Figure 6B).

These findings indicate that *CsTPS1* and its AS forms perform distinct functions in both geraniol formation and pathogen resistance in tea plants and that *CsTPS1* plays a role in regulating geraniol biosynthesis and pathogen resistance via AS.

3.7 The distribution and subcellular localization of CsTPS1 and CsTPS1-AS differ

Monoterpenes are synthesized exclusively by plastids in higher plants; thus, plant monoterpene synthases are localized to the chloroplast. To verify this prediction, the two CsTPS1 and CsTPS1-AS proteins were fused to the N-terminal of eYFP, and the fusion proteins were transiently expressed in tobacco leaves. The eYFP signals of CsTPS1 and CsTPS1-AS fusion proteins were consistent with chlorophyll autofluorescence and showed no overlap with the cytosolic mCherry signals from the negative controls (Figure 6C). These findings confirmed that the two CsTPS1 and CsTPS1-AS proteins are localized to the chloroplast. However, the distribution and localization of the CsTPS1 and CsTPS1-AS proteins in the chloroplast varied (Figure 6C). The CsTPS1 protein is likely localized in the stroma of the chloroplast and exhibits a highly homogeneous distribution. Conversely, the CsTPS-AS protein might be localized to the outer membrane of the chloroplast and exhibit a sporadic distribution (Figure 6C). The distribution and localization of*CsTPS1* and its AS forms in the chloroplast differ, and this might explain the distinct levels of disease resistance that they confer to tea plants.

3.8 *CsTPS1*-AS affects the expression of defense-related genes in the SA pathway in infected tea leaves

In plants, SA plays a crucial signaling role in activating defense pathways in plants, including systemic acquired resistance (SAR) and related immune responses. To verify the role of *CsTPS1-AS* -mediated disease resistance via activation of the expression of downstream-related defense genes in the SA pathway, we characterized the expression of defense-related genes in the SA pathway in *CsTPS1 -AS* -silenced tea plants and control tea plants. The expression of *PR1* and *PR2* in CsTPS1-*AS*- silenced tea leaves was significantly lower than that in control plants (Figure 6D).

PAD4 (*Phytoalexin-deficient 4*) is an important signaling gene involved in activating the expression of downstream-related defense genes in the plant immune system. The expression of *PAD4* was not detected in control tea plants; however, its expression was significantly increased in infected tea plants (Figure 6E). The expression of *PR1* and *PR2* in tea plants infected with *C* . *gloeosporioides* was approximately 2-fold higher than that in uninfected control tea plants (Figure 6D and 6E). When tea leaves were infected with *C. gloeosporioides* , the expression of *PR1* , *PR2* , and *PAD4* was significantly reduced in *CsTPS1-AS* -silenced tea leaves compared with control plants (Figure 6E). Overall, these findings indicate that *CsTPS1-AS* can affect the expression of genes in the SA pathway in infected tea leaves.

4 Discussion

4.1 CsTPS1 is a geraniol synthase in tea

Geraniol has a sweet, floral aroma similar to that of roses, and it contributes to the characteristic floral aroma and flavor of many fruits. Tea plants are important evergreen crops that are grown in temperate and subtropical regions. In response to herbivore and pathogen invasion, tea plants release volatiles, such as 3-hexenol, geraniol, β-ocimene, β-caryophyllene, and α-farnesene (Zhou et al., 2019). Tea green leafhopper, a major pest of tea plants, can significantly induce the emission of geraniol from tea leaves (Zhou et al., 2019). Other studies have shown that the higher content of geraniol in tea plants might be responsible for their stronger resistance to the pathogen causing tea leaf blight (Zhang et al., 2006). In addition, geraniol is considered one of the most abundant terpenes in tea, and it contributes greatly to its aroma (Yang et al., 2013). Geraniol is an important defense-inducing substance in tea plants; however, the biosynthesis of geraniol in tea leaves has not yet been clarified.

Although geraniol synthase genes have been reported in *Vitis vinifera* , *Glycine max* , *Coffea arabica* , and other plants (Martin et al., 2010b; Liu et al., 2014), geraniol synthase genes have not yet been identified in tea plants. Only a few *TPS* genes have been identified in tea trees to date (Zhou et al., 2020), such as *CsNES* , nerolidol synthase (Zhou et al., 2017), *CsLIS/NES* , linalool/nerolidol synthase (Liu et al., 2018), *CsAFS* , α-farnesene synthase (Wang et al., 2019b), *CsOCS* , and β-ocimene synthase (Xu et al., 2018). *CsTPS1* was first identified by analysis of gene expression levels and geraniol accumulation in tea plants, and both *in vitro* and *in vivo* analysis showed that it functions as a geraniol synthase in tea plants (Figure 2C and 4B).

Plant *TPS* s are divided into seven families (TPS-a to TPS-g) (Nieuwenhuizen et al., 2013). Although phylogenetic analyses of terpenes can provide insights into the function of TPSs, *TPS* s on the same branch might have different functions. In our study, *CsGES*(*CsTPS1/1-AS*) and *CsOCS* were in the same branch (Figure 6F); their homologous sequence alignments were similar, but their functions were quite different. Phylogenetic analysis showed that *CsTPS1* clustered with *CsOCS2* , which belongs to the *TPS-b* gene family (Figure 6F). The *TPS-b* subfamily is the second largest in *C. sinensis* , and it includes approximately 37.5% of all *TPS* genes in tea (Zhou et al., 2020).*CsOCS* specifically catalyzes the synthesis of β-ocimene from GPP (Xu et al., 2018), and *CsGES* (*CsTPS1/1-AS*) catalyzes the conversion of GPP to both geraniol and β-ocimene, and mainly catalyzed the synthesis geraniol. Therefore, the latter gene thus encodes the main enzyme that catalyzes the synthesis of geraniol (Figure 2C and 2D).

4.2 *CsTPS1* is involved in regulating the defense response via AS in tea plants

The transcriptional regulation of *TPS* genes is critically important for volatile terpenoid biosynthesis (Nage-

gowda, 2010. The substrate and product specificity of *TPS* s can regulate terpenoid biosynthesis at the enzyme level (Fischer et al., 2013). In addition to regulating transcriptional processes such as splicing, *TPS* genes also regulate other complex aspects of transcription. AS, which produces multiple mRNA subtypes from a single gene, is widespread in plants and often produces a variety of transcripts with diverse functions (Reddy et al., 2013). The full-length sequences and short AS forms of*CsTPS1* were obtained from the young leaves of tea plants. Although both *CsTPS1* and its AS forms could catalyze the formation of geraniol *in vitro* , *CsTPS1* and its AS forms confer different levels of disease resistance. The expression of*CsTPS1-AS* , but not the full-length sequences of *CsTPS1* , was induced in response to pathogen infection (Figure 5C and 5G). This might explain differences in the distribution and localization of CsTPS1 and CsTPS1-AS in the chloroplasts.

The silencing of *CsTPS1-AS* significantly decreased the content of geraniol and the resistance of tea plants to infection by the two pathogens (Figure 5B and 5C); however, no changes in disease symptoms were observed when *CsTPS1* was silenced (Figure 5G and 5H). Hence, the shorter AS form *of CsTPS1* plays a critical role in enhancing the resistance of tea plants to pathogen infection.

4.3 *CsTPS1-AS* enhances the resistance of tea plants to pathogen infection by regulating geraniol formation and the expression of SA-related genes

Plant pathogens can activate SA pathways, which enhance the resistance of plants to pathogen infection (Eberl et al., 2018). The pathogenesis-related defense genes *PR1* and *PR2* are typical markers of the SA-mediated defense system (Zhang et al., 2020). The expression of *PR1* and *PR2* was significantly increased in pathogen-infected tea plants (Figure 6D and 6E). This indicates that pathogens can induce the expression of pathogenesis-related genes in the SA-mediated pathway, which enhances the resistance of plants to pathogen infection. The expression of *PR1* and *PR2* was significantly lower in *CsTPS1-AS*- silenced plants than in control plants (Figure 6D). This suggests that *CsTPS1-AS* mediates the response to pathogen infection by up-regulating the expression of pathogenesis-related genes.

PAD4 is known to play a key role in SAR through SA-dependent and SA-independent pathways (Cui et al., 2017; Hu et al., 2022). To further clarify the role of *CsTPS1-AS* in plant defense, the expression of*PAD4* was assessed after pathogen infection in tea plants. As expected, silencing of*CsTPS1-AS* significantly decreased the expression of *PAD4*in infected tea plants (Figure 6E), suggesting that *CsTPS1* -AS might enhance SAR in tea plants by activating the expression of*PAD4* . Overall, these findings indicate that *CsTPS1-AS*might play a role in pathogen resistance by regulating the expression of*PR1* , *PR2* , and *PAD4* .

Silencing of *CsTPS1 -AS* also significantly decreased the content of geraniol (Figure 5B) and the amount of mycelial growth on CsTPS1-AS-silenced tea leaves was more than that on control leaves (Figure 6A and 6B). Meanwhile, geraniol shown that more strongly inhibited the mycelial growth of *Neopestalotiopsis* sp. and*C. gloeosporioides in vitro* (Figure 3). These findings indicate that geraniol plays an important role in enhancing resistance to infection by both of these fungal pathogens. Our findings are consistent with the results of a previous study showing that (E)-β-caryophyllene mediates the defense response of *A. thaliana*flowers to pathogen infection by directly inhibiting bacterial growth (Huang et al., 2012). Our findings indicate that the function of*CsTPS1 -AS* was to enhance the resistance of tea plants to pathogen infection by up-regulating the biosynthesis of geraniol. Thus,*CsTPS1 -AS* enhances the resistance to pathogen infection in tea plants by regulating geraniol formation and the expression of SA-related genes. Based on these results, we propose a putative working model for the function of *CsTPS1/1 -AS* in pathogen infection (Figure 7).

In conclusion, we identified a key *TPS* gene that functions as a geraniol synthase (*CsGES*) in tea plants, and both *in vitro*and *in vivo* studies indicated that this geraniol synthase is involved in regulating geraniol formation and plant defense via AS. The results of this study provide new insights into geraniol biosynthesis and clarify the role of monoterpene synthases in modulating the disease resistance in plants via AS.

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Conflict of Interests

The authors declare that there are no conflict of interests.

Author Contributions

H.J., W.S., X.W.,and C.S. conceptualized the initial study; H.J.,M.Z., and F. Y. were involved in the experimental layout and carried out experiment and analyzed experiment results; H.J., X.L., J.J., Y.Z., Y.W., and T.J. were analyzed experiment results; Q.W., and M.Z. performed the subcellular localization experiments; H.J. drafted original manuscript and provided funding; C.S. provided funding and edited the manuscript.

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