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Research

A defensive pathway from NAC and TCP transcription factors activates a BAHD acyltransferase for (Z)-3-hexenyl acetate biosynthesis to resist herbivore in tea plant (*Camellia sinensis*)

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Summary

- Numerous herbivore-induced plant volatiles (HIPVs) play important roles in plant defense. In tea plants (*Camellia sinensis*), (Z)-3-hexenyl acetate (3-HAC) has been characterized as associated with resistance to herbivores. To date, how tea plants biosynthesize and regulate 3-HAC to resist herbivores remain unclear.
- Based on transcriptomes assembled from *Ectropis obliqua*-fed leaves, a cDNA encoding BAHD acyltransferase, namely *CsCHAT1*, was highly induced in leaves fed with *E. obliqua*. Enzymatic assays showed that CsCHAT1 converted (Z)-3-hexenol into 3-HAC. Further suppression of *CsCHAT1* expression reduced the accumulation of 3-HAC and lowered the resistance of tea plants to *E. obliqua*, while 3-HAC replenishment rescued the reduced resistance of *CsCHAT1*-silenced tea plants against *E. obliqua*.
- Two transcription factors (TFs), *CsNAC30* and *CsTCP11*, were co-expressed with *CsCHAT1*. An integrative approach of biochemistry, DNA–protein interaction, gene silencing, and metabolic profiling revealed that the two TFs positively regulated the expression of *CsCHAT1*. The suppression of either one decreased the production of 3-HAC and eliminated the resistance of tea plants to *E. obliqua*. Notably, the suppression of either one considerably impaired JA-induced 3-HAC biosynthesis in tea plant.
- The proposed pathway can be targeted for innovative agro-biotechnologies protecting tea plants from damage by *E. obliqua*.

Introduction

Herbivore-induced plant volatiles (HIPVs) play important roles in protecting plants from herbivore infestation and damage (Turlings *et al.*, 1990). It is well established that herbivore attacks are a major cause of crop loss (Oerke, 2006). To survive the damage caused by herbivores, plants have evolved different preventive or/and protective mechanisms, such as the biosynthesis of preventive phytochemical, including volatile organic compounds (VOCs) (Gong *et al.*, 2023), phenylpropanoids (Alves *et al.*, 2020), and alkaloids (Coquerel *et al.*, 2021), among others. HIPVs are examples of defensive phytochemicals, acting either as natural herbicides or as intraplant and interplant signals that promote plant adaptation toward environmental stresses (Baldwin & Schultz, 1983; Pickett & Khan, 2016; Gong *et al.*, 2023). To date, in order to understand the roles of volatiles in plant communication and defenses, extensive efforts have been undertaken to investigate HIPV compositions in multiple plant species (Hirokazu *et al.*, 2012; Pickett & Khan, 2016). Common HIPVs mainly include monoterpenes (e.g. β-ocimene and linalool), sesquiterpenes (e.g. α-farnesene), and green leaf volatiles (GLVs) (e.g. (Z)-3-hexenol) (Dudareva *et al.*, 2004; Hare, 2011).

To date, a few HIPVs have been identified to associate with tea plant resistance to herbivores. (Z)-3-hexenyl acetate (3-HAC) is a major HIPV in tea plants. Other HIPVs identified from tea plants include (Z)-3-hexenol (Xin *et al.*, 2019; Liao *et al.*, 2021), α-farnesene (Zeng *et al.*, 2017; Wang *et al.*, 2019), β-ocimene (Zeng *et al.*, 2017; Jian *et al.*, 2021; Jing *et al.*, 2021b), nerolidol (Chen *et al.*, 2020), linalool (Mei *et al.*, 2017), (E)-4,8-dimethyl1,3,7-nonatriene (DMNT) (Jing *et al.*, 2021a), and indole (Ye *et al.*, 2021). GLVs repel herbivores, attract natural herbivore enemies, and exert direct toxic effects on fungi and

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bacteria (Engelberth *et al.*, 2004; Ameye *et al.*, 2017; Frontini *et al.*, 2022). 3-HAC primes defense signaling and mediates plant-to-plant communication. Previous studies have shown that 3-HAC is not only involved in activating defensive reactions in numerous plants, but it also promotes communication between neighboring plants upon attack by herbivores and pathogens. The herbivore-induced emission of 3-HAC was reported to activate the defense responses of maize against *Spodoptera littoralis* (Hu *et al.*, 2018) and of sweet potato against weevils (Xiao *et al.*, 2023). Hu *et al.* (2018) also have reported that 3-HAC enhances plant resistance through the induction of jasmonate (JA) accumulation, the upregulation of defense-related genes, and the emission of terpene volatiles. A recent study on tea plants reported a strikingly enhanced 3-HAC emission from tea plants attacked by *Ectropis obliqua* (Jing *et al.*, 2021a).

Although HIPVs are composed of structurally diverse volatiles, the biosynthesis of certain HIPVs is well understood. A BAHD acyltransferase gene family acetyl CoA: (Z)-3-hexen-1-ol acetyltransferase (*CHAT*) cDNA was demonstrated to catalyze (Z)-3-hexenol to 3-HAC conversion in *Arabidopsis thaliana* (D'Auria *et al.*, 2007). A few genes have been cloned for the GLV biosynthetic pathway from tea plants. For example, it was shown that the α-linolenic acid is oxygenated by lipoxygenase 3 (CsLOX3) to produce 13-hydroper-oxy-6Z,9Z,11E-octadecatrienoic acid (13-HPOT) (Zhu *et al.*, 2018), whereafter hydroperoxide lyase (CsHPL) converts 13-HPOT into (Z)-3-hexenal (Deng *et al.*, 2016), and alcohol dehydrogenase (CsADH) catalyzes the reduction of (Z)-3-hexenal to generate (Z)-3-hexenol (Liu *et al.*, 2022). In addition, a *CHAT* homolog has been cloned from tea plants (Jin *et al.*, 2023), although its function remains to be determined. These findings have provided valuable insight into the biosynthesis of HIPVs during plant–herbivore interactions.

Studies have also explored the regulation of HIPV biosynthesis in different plant species. Regulatory mechanisms include signaling molecules and expression changes driven by different classes of transcription factors (TFs). CsMYC2 was found to increase the expression of histone deacetylase 2 (*CsHDA2*), leading to the enhanced production of (*E*)-nerolidol when green leafhoppers fed on tea plant leaves (Gu *et al.*, 2022). Upon tea plant infestation by tea geometrids, CsMYC2, a member of bHLH family, was reported to interact with histone deacetylase 6 (*CsHDA6*) to form a complex, which regulated the expression of α-farnesene synthase (*CsAFS*), thus stimulating α-farnesene production to attract natural geometrid enemies (*E. obliqua*) (Wu *et al.*, 2023). Moreover, when geometrid larvae developed on tea leaves, phytochrome interacting factors-like 1 (CsPIF1-like) and JA signaling-related TF CsMYC2 were found to synergistically activate the expression of *CsCYP79*, leading to benzyl nitrile production for protection (Qian *et al.*, 2023). These findings indicate that TFs are involved in the regulation of HIPV production during plant–herbivore interactions.

In this study, we demonstrate that 3-HAC serves as a defensive HIPV that protects tea plants from damage by *E. obliqua*. To gain insight into its biosynthesis, we cloned a *CsCHAT1* cDNA and characterized its functions in catalyzing the formation of 3-HAC. Exploring the regulatory mechanism of *CsCHAT1*, we screened and identified two TFs, *CsNAC30* and *CsTCP11*. Functional analysis demonstrated that these positively regulated the expression of *CsCHAT1*, promoted the release of 3-HAC, and enhanced the resistance of tea plants to *E. obliqua*. Suppression of either *CsCHAT1*, *CsNAC30*, and *CsTCP11* abolished the JA-induced 3-HAC biosynthesis in tea plants. These findings provide a basis for utilizing *CsNAC30*, *CsTCP11*, and *CsCHAT1* to develop technologies that enhance 3-HAC production for the protection of tea plants against damage by *E. obliqua* in the field.

Materials and Methods

Tea sample and *E. obliqua* preparation

Tea plants (*Camellia sinensis* var. *sinensis* 'Shuchazao') propagated via cutting were grown in the research station. After 2 yr of growth, cutting plants were used for experiments. All plants were uniform in growth and healthy without pathogen infection nor insect infestation. The parental pupae of *E. obliqua* were collected from Dechang Tea Garden (latitude 31.3°N, longitude 117.2°E).

After preliminary screening of collected pupae in a laboratory environment, these were subjected to gender identification under a binocular stereomicroscope. The pupae were separately raised in an intelligent artificial climate incubator under the following conditions: temperature of 26 ± 2°C, 12 h : 12 h, light : dark photoperiod, relative humidity of 75%. After eclosion from pupae to moth, one pair of male and female moth were transferred to a closed and ventilated wooden box for breeding. The breeding frame was placed in an intelligent artificial climate room with consistent conditions. After the egg incubated and raised to 3rd stage, larvae were used for further herbivore damage assay.

Herbivore damage assay

Cutting tea plants were fed to *E. obliqua* (E), as described previously (Chung *et al.*, 2008; Deng *et al.*, 2016). Briefly, the larvae of *E. obliqua* were starved for 8 h, and then placed on the 3rd position leaves of each tea plant. The 20 larvae were removed from leaves after 30% of the leaf area was damaged for 1 h. Meanwhile, plants without feeding larvae were grown under the same condition as controls (CK). The plant growth conditions in the glasshouse included temperature of 28 ± 3°C, a photoperiod of 16 h : 8 h (day : night), and a humidity of 85 ± 5%. Next, the damaged and control leaves were collected at 3, 6, 9, 12, and 24 h and then stored at −80°C for subsequent RNA-Seq.

The larvae of *E. obliqua* were starved for 8 h before damage assay performance. One starved larva and one-third leaf were placed in a Petri dish. After 10 h of feeding treatment, the larva was removed from the Petri dish. The damaged leaf was photographed and recorded, then the its consumed area was and calculated using Fiji Image J software (Legris *et al.*, 2021; McCready *et al.*, 2022).

RNA-Seq

The RNA-Seq and bioinformatics analysis were performed as described previously (Qiao *et al.*, 2021; Zhu *et al.*, 2024). In

brief, total RNA was obtained for cDNA library construction and transcriptome sequencing. Three biological replicates were prepared for each collection time. The raw sequence data have been deposited in the National Center for Biotechnology Information, under accession no. PRJNA901518, which is publicly accessible at https://www.ncbi.nlm.nih.gov/. A description of how RNA-Seq samples were prepared and analyzed is provided in Supporting Information Methods S1.

Exposure of tea plants to 3-HAC and JA treatment

To understand the effect of 3-HAC on plant performance, plants were exposed to an environment with saturated volatiles. Considering that the physiological concentration of 3-HAC released from leaves of tea plants in the glass container after *E. obliqua* attack is 0.175 ng μl^{-1} (Fig. 1a), 3-HAC (purity ≥ 98%, CAS 3681-71-8; Aladdin, Shanghai, China) was quantitatively adsorbed on an equal size cotton ball to make the 3-HAC concentration 0.1, 0.5, and 1 ng μl^{-1} in the 4 l glass container. The cotton ball, having absorbed different concentrations of 3-HAC or methanol, was placed at the headspace of eight tea plants, as described in a previous report (Sugimoto *et al.*, 2015). Eight control tea plants were placed in the same-size container, with a cotton ball adsorbed with an equal amount of 100 µl 100% methanol. All containers were placed in the glasshouse under 28 ± 3°C, a photoperiod of 16 : 8 h (day : night), and a humidity of 85 ± 5%. After 0.5, 1, 3, and 6 h of volatile emission, the leaves at the 3rd position from the methanol control and

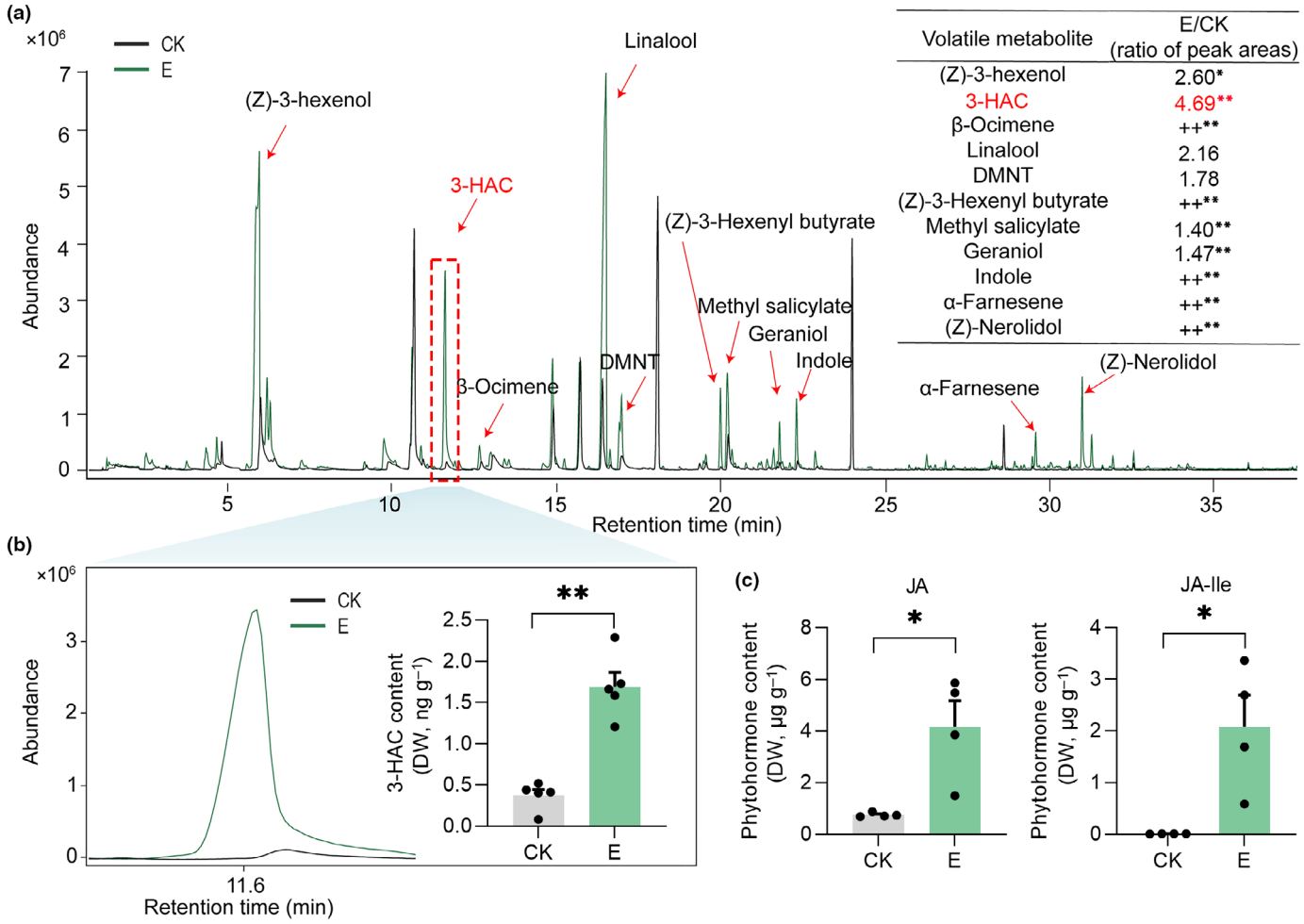

Fig. 1 Identification of volatiles emitted from tea leaves induced by *Ectropis obliqua*. (a, b) The total ion chromatography (TIC) of volatiles released from tea leaves consumed by *E. obliqua*. The ratio of peak areas for volatiles released from tea leaves consumed by *E. obliqua* (a); 3-HAC released from tea leaves consumed by *E. obliqua* (b). The infested samples were consumed by *E. obliqua* for 6 h. E, tea plants were fed to *E. obliqua*. CK, plants without being fed to *E. obliqua*. E/CK represents the ratio of volatile levels from consumed leaves to those from unconsumed control leaves. ++ indicates that the level of compounds with only a trace amount in the control leaves were considerably upregulated by *E. obliqua*. Treatment and control experiments were conducted with five biological replicates, $n = 5$. Data are presented as mean ± SD (Tukey's HSD, **, $P < 0.01$). (c) JA and JA–Ile content in tea plants consumed and not consumed by *E. obliqua*. The infested samples were consumed by *E. obliqua* for 6 h. E, tea plants were fed to *E. obliqua*. CK, plants without being fed to *E. obliqua*. Treatment and control experiments were conducted with four biological replicates, $n = 4$. Data are presented as mean ± SD (Tukey's HSD, *, $P < 0.05$).

3-HAC treatment were collected, respectively. The control and 3-HAC-treated leaves were then fed to *E. obliqua*, as described in 'Herbivore damage assay' in the Materials and Methods section after 1 h of the treatment.

JA treatment of tea plants was performed as previously described (Yang *et al.*, 2013; Li *et al.*, 2022). Briefly, JA (purity ≥ 90%, CAS 77026-92-7; Sigma-Aldrich) was dissolved in methanol to dilute at a concentration of 1 mM with 50% methanol (Yan *et al.*, 2012; Humphrey & Whiteman, 2020). Leaves of healthy tea plants were sprayed with 1 mM JA until the leaves were completely wet. The control plants were sprayed with an equal amount of 50% methanol. All tea plants were placed in the glasshouse under 28 ± 3°C, a photoperiod of 16 : 8 h (day : night), and a humidity of 85 ± 5%. The 3rd leaves were collected at 3, 6, and 12 h after the start of treatment for gene expression analysis and volatile detection.

Volatile extraction and measurement

A solid-phase microextraction (SPME) fiber (50/30 μM DVB/CAR/PDMS; Supelco, Inc., Bellefonte, PA, USA) was used to extract volatiles from tea leaves. The volatile-rich SPME fiber was then used for GC-MS analysis, as described in Methods S2.

Phytohormone content determination

The content of phytohormones jasmonic acid (JA), and jasmonoyl-isoleucine (JA–Ile) in tea leaves was detected via ultra-high performance liquid chromatography (UPLC) as described in Methods S3.

Sequence amplification, qRT-PCR analysis, heterologous protein expression, and purification

The promoter (accession no., OR058582) and ORF sequences of *CsCHAT1* (accession no., OR058579), *CsNAC30* (accession no., OR058580), and *CsTCP11* (accession no., OR058581) were cloned from *Camellia sinensis* var. *sinensis* 'Shuchazao' leaves via PCR. Primers are listed in Table S1. Detailed protocols for sequence amplification, quantitative reverse transcription polymerase chain reaction analysis, heterologous expression, and purification of protein are provided in Methods S4–S6, respectively.

Enzyme activity assay of recombinant CsCHAT1

To evaluate the role of CsCHAT1 in the 3-HAC biosynthesis, MBP-CsCHAT1 fusion protein was purified to test its catalytic activity. The total reaction mixture was 1 ml composed of Tris–HCl buffer (250 mM Tris–HCl and 14 mM β-Mercaptoethanol at pH 7.5), 2.7 mM acetyl CoA, 50 mM (Z)-3-hexenol, and 15 μg purified protein as previously described (Song *et al.*, 2016). The enzymatic reaction was maintained for 30 min and terminated at 100°C for 10 min. Volatiles were collected from the enzymatic mixture under 42°C using SPME fiber for 15 min. The volatile-rich SPME fiber was subjected to GC-MS analysis.

To determine the enzymatic kinetics of CsCHAT1, the optimization of reaction temperatures was tested with a range from 20 to 45°C at a pH of 7.0, and the optimization of pH values was tested with a range from 5 to 9. Furthermore, seven different (Z)-3-hexenol concentrations were tested from 0.15–4 mM at the optimal temperature and pH. The 3-HAC content of the resulting enzymatic extracts was determined for GC-MS analysis. All enzymatic reactions were repeated at least three times.

Phylogenetic tree construction

A phylogenetic tree was constructed for 52 CHAT CDS protein sequences from 29 different plant species using the NJ (Neighbor–Joining) method in the software MEGA 6.0 with the following parameters: bootstrap method (1000 replicates), Poisson model, Uniform rates, Complete deletion. The accession information of the 52 CHAT amino acid sequence was listed in Table S2.

Subcellular localization of CsCHAT1

The subcellular localization of CsCHAT1 was examined by observing the CsCHAT1-GFP transiently expressed in *Nicotiana benthamiana* leaves. Detailed protocols are described in Methods S7.

Protein–DNA interaction assays

Yeast one-hybrid assay, electrophoresis mobility shift assay (EMSA), and dual-luciferase (dual-LUC) assay were conducted to test the binding of CsNAC30 and CsTCP11 to the *CsCHAT1* promoter as described previously (Zhang *et al.*, 2018). For yeast one-hybrid assay experiments, the *CsCHAT1* promoter was cloned into a pHis2.1 vector to generate the pHis2.1-CsCHAT1 construct. The ORF sequences of CsNAC30 and CsTCP11 were cloned into pGADT7 to generate the pGADT7-CsNAC30 and pGADT7-CsTCP11 constructs, respectively. Primers were listed in Table S1. After co-transformation of pHis2.1-CsCHAT1 with pGADT7-CsNAC30 and pGADT7-CsTCP11 into the competent *Saccharomyces cerevisiae* strain Y187, respectively. The resulting transformed yeast cells were plated onto medium lacking Trp and Leu (SD/−Trp/−Leu) or Trp, Leu, and His (SD/−Trp/−Leu/-His). Subsequently, the positive colonies were inoculated on SD/−Trp/−Leu/-His medium supplemented with 30 mM 3-amino-1,2,4-triazole (3-AT) and grown for 3 d at 28°C. EMSA and dual-LUC assays are described in detail in Methods S8 and S9.

Silencing of *CsCHAT1*, *CsNAC30*, and *CsTCP11* in tea plants using AsODNs

An antisense oligonucleotides (AsODNs)-mediated gene suppression was employed to repress the expression of *CsCHAT1*, *CsNAC30*, and *CsTCP11*, as previously described (Dinc *et al.*, 2011). Based on the SOLIGO online software (http://sfold.wadsworth.org/cgi-bin/soligo.pl), the ORF sequences of *CsCHAT1*, *CsNAC30*, and *CsTCP11* were used to design the AsODN probe, respectively. Sequences used to synthesize

AsODNs are listed in Table S1. The experimental details of *CsCHAT1*, *CsNAC30*, and *CsTCP11* expression suppression in tea plants using AsODNs are described in Methods S10. Additionally, the gene-suppressed tea plants were treated with 0.5 ng μl^{-1} 3-HAC and 1 mM JA, as described in Methods S10.

Statistical analysis

Data were analyzed using SPSS Statistics 17.0 (http://ccm.net/download/download-1522-spss) and presented as the mean ± SD of at least three biological replicates. Significance was determined at $P < 0.05$ by ANOVA followed by Tukey's HSD test.

Results

Induction of 3-HAC and JA production by *E. obliqua*

HIPVs released from *E. obliqua*-consumed leaves were analyzed via GC-MS. The results showed that 3-HAC was a major HIPV emitted from the tea leaves (Fig. 1a,b). Furthermore, 3-HAC was also detected in trace amounts from unconsumed control leaves. The levels of 3-HAC from consumed leaves were 4.7-fold greater than those from plants without feeding larvae. Thus, *E. obliqua* potently induced 3-HAC production. In addition to 3-HAC, GC-MS analysis also detected higher levels of (Z)-3-hexenol, β-ocimene, linalool, DMNT, (Z)-3-hexenyl butyrate, methyl salicylate, geraniol, indole, α-farnesene, and (Z)-nerolidol in consumed levels compared to in controls.

UPLC-MS analysis was performed to determine the profiles of JA and JA–Ile in tea plants consumed by *E. obliqua* (Fig. 1c). JA and JA–Ile levels were significantly increased in the consumed tea plants compared to the control plants. It should be noted that JA content increased > 3-fold in consumed leaves relative to control leaves. These results indicated that *E. obliqua* significantly induced JA formation in tea plants.

3-HAC enables the resistance of tea plants to *E. obliqua*

Ectropis obliqua are the main herbivore of tea plants (J. Zhang *et al.*, 2024). To understand whether 3-HAC could enhance the resistance of tea plants to *E. obliqua*, plants were placed in a glass container that was full of 3-HAC or methanol (control group) for 1 h (Fig. 2a). Then, the tea leaves absorbed with 3-HAC and methanol were fed by *E. obliqua* under normal air conditions, respectively. The areas of consumed leaves in 0.5 ng μl^{-1} 3-HAC-treated tea plants was significantly lower than that in methanol-treated plants (Fig. 2b,c). This result indicated that 3-HAC enhances the resistance of tea plants to *E. obliqua*.

Expression profiling of herbivore-resistant genes was carried out to understand potential molecular mechanisms underlying the 3-HAC-induced resistance of leaves to *E. obliqua*. *CsLOX1* and *CsLOX3*, two genes involved in the biosynthesis of JA, were significantly upregulated in leaves treated with 3-HAC for 1 h (Fig. 2d,e). *CsPI1* (protease inhibitor), a gene related to direct defense, was significantly upregulated by 3-HAC treatment (Fig. 2f). *CsADH2* and *CsHPL*, two genes associated with GLV biosynthesis (Deng *et al.*, 2016; Liu *et al.*, 2022), were also significantly upregulated by 3-HAC treatment (Fig. 2g,h).

UPLC-MS analysis was conducted for leaves treated with 0.5 ng μl^{-1} 3-HAC and methanol for 0.5, 1, 3, and 6 h. After 3-HAC treatment, JA and JA–Ile levels increased at all of four time points, being significantly induced within 1 h (Fig. 2i,j). These results suggested a potential role of 3-HAC in inducing the JA response in tea plant. Moreover, we treated tea plants with 1 mM JA to explore its effect on 3-HAC emission (Fig. 2k). GC-MS analysis showed a significant increase of 3-HAC in JA-treated leaves relative to in untreated leaves at all of three time points, reaching a 2.2-fold upregulation after 3 h. These results indicated that 3-HAC stimulated JA accumulation to activate the tea plant defense response against *E. obliqua*, while JA also influenced 3-HAC biosynthesis.

Identification of CsCHAT1 in *E. obliqua*-consumed leaf transcriptomes

To understand the mechanism of 3-HAC biosynthesis in tea plants, the amino acid sequence of the CHAT protein, an acetyltransferase in *Arabidopsis thaliana*, was used for a BLASTP. The blasting results yielded eight homologous CHAT proteins, including *CSS0006320.1*, *CSS0032557.1*, *CSS0013992.1*, *CSS0044776.1*, *CSS0036810.1*, *CSS0006791.1*, *CSS0049855.1*, and *CSS0012054.1*. A phylogenetic analysis was conducted for all of BAHD family members from 29 different plant species, with five major clades clustering (Fig. 3a; Table S2). Eight homologous CHAT proteins of tea plants were clustered into the fifth subgroup of the BAHD acyltransferase family, all harboring the conserved domains (HXXXD and DFGWG motifs) (Fig. S1a) (Ma *et al.*, 2005).

Furthermore, data mining of *E. obliqua*-consumed leaf transcriptomes (accession PRJNA901518) was performed to obtain FPKM values for these eight CHAT genes (Fig. 3b). Notably, the fold change of three CHAT genes, *CSS0044776.1*, *CSS0032557.1*, and *CSS0006320.1* was higher in consumed leaves than in control leaves (Fig. S1b). *CSS0044776.1* expression was significantly different among tea plants consumed by *E. obliqua* for 3, 6, and 12 h, being *c.* 3.3-fold higher in consumed leaves at 3 h compared to in unconsumed leaves (Fig. S1b). Therefore, *CSS0044776.1* was selected as a candidate, termed *CsCHAT1*. Further quantitative reverse transcription polymerase chain reaction analysis verified the expression profiles of the *CsCHAT1* and showed an upregulation of *c.* 9.3-fold in *E. obliqua*-consumed leaves at 3 h compared to in control plants (Fig. 3c). Moreover, 1 mM of JA treatment also significantly induced *CsCHAT1* expression in leaves (Fig. 3d). Based on these data, we hypothesized that the *CsCHAT1* gene might be associated with 3-HAC biosynthesis in tea plants.

Catalytic activity of CsCHAT1 responsible the biosynthesis of 3-HAC and its subcellular localization

To evaluate the involvement of CsCHAT1 in 3-HAC biosynthesis *in vitro*, CsCHAT1 was fused with MBP, and the MBP-CsCHAT1 fusion protein was purified to test its catalytic activity

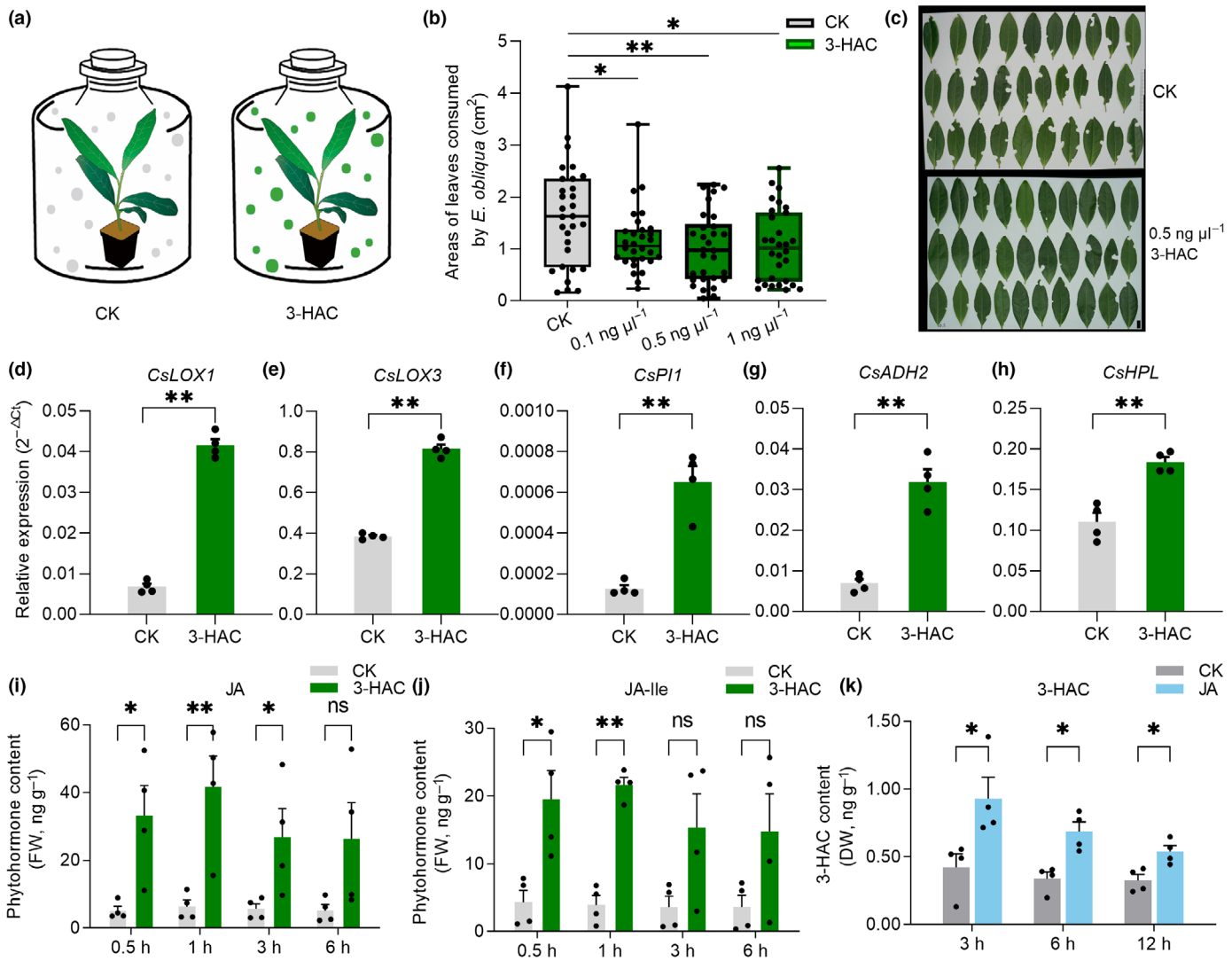

Fig. 2 3-HAC enhances the resistance of tea plants to *Ectropis obliqua*. (a) Experimental design of tea plant exposure in a 3-HAC-rich container. 3-HAC, tea plants were treated with 3-HAC for 1 h; CK, tea plants were treated with methanol for 1 h. (b, c) *Ectropis obliqua-consumed* areas of leaves in presence of 0.1, 0.5, and 1 ng μl^{-1} 3-HAC (b). Data are presented as boxplots (minima, 25th percentile, median, 75th percentile, and maxima); Phenotype of *E. obliqua-consumed* leaves in presence of 0.5 ng μl^{-1} 3-HAC and methanol (c). 3-HAC and CK, 0.5 ng μl^{-1} 3-HAC- and methanol-treated tea leaves were fed to *E. obliqua* for 10 h, respectively. Thirty *E. obliqua* were applied to 30 treated tea leaves. Control and 3-HAC treatment was performed with 30 biological replicates (n = 30). Bar, 1 cm. Data are presented as mean ± SD (one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$). (d–h) The expression patterns of *CsLOX1*, *CsLOX3*, *CsPI1*, *CsADH2*, and *CsHPL*. 3-HAC, leaves were collected after plants were exposed to 0.5 ng μl^{-1} 3-HAC for 1 h; CK, leaves were collected after plants were exposed to 0.5 ng μl^{-1} methanol for 1 h. Control and 3-HAC treatment was performed with four biological replicates (n = 4). Data are presented as mean ± SD (Tukey's HSD, **, $P < 0.01$). (i, j) Contents of JA and JA–Ile in control and 0.5 ng μl^{-1} 3-HAC-exposed leaves. 3-HAC, leaves were collected after 0.5, 1, 3, and 6 h of 0.5 ng μl^{-1} 3-HAC exposure; CK, leaves were collected after 0.5, 1, 3, and 6 h of 0.5 ng μl^{-1} methanol exposure. Control and 3-HAC treatment was performed with four biological replicates (n = 4). Data are presented as mean ± SD (one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$; ns, no significant difference). (k) The 3-HAC content in tea plants after 1 mM of exogenous JA. JA, leaves were collected at 3, 6, and 12 h after JA application; CK, leaves were collected at 3, 6, and 12 h after methanol application. Control and 3-HAC treatment was performed with four biological replicates (n = 4). Data are presented as mean ± SD (one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$; ns, no significant difference).

(Fig. S2a). GC-MS detection of the enzymatic reaction showed that MBP-CsCHAT1 converted (Z)-3-hexenol and acetyl CoA to 3-HAC (Peak 1), while 3-HAC was not detected in the control (MBP) enzymatic reaction mixture (Figs 4a, S2b). Reaction condition optimization determined that the optimal pH and reaction temperature were 7.5 and 35°C, respectively (Fig. 4b,c). Subsequent evaluation of 3-HAC production in the presence of different concentrations of (Z)-3-hexenol at optimal pH and reaction temperature revealed that the K_{cat}/K_m values of CsCHAT1 was 18.96 mM^{-1} s^{-1} (Figs 4d, S2c).

In addition, CsCHAT1 was fused with GFP to determine its subcellular localization. Confocal microscopy revealed that the CsCHAT1-GFP fusion protein was constitutively expressed in the epidermis cell of *Nicotiana benthamiana* leaves (Fig. 4e).

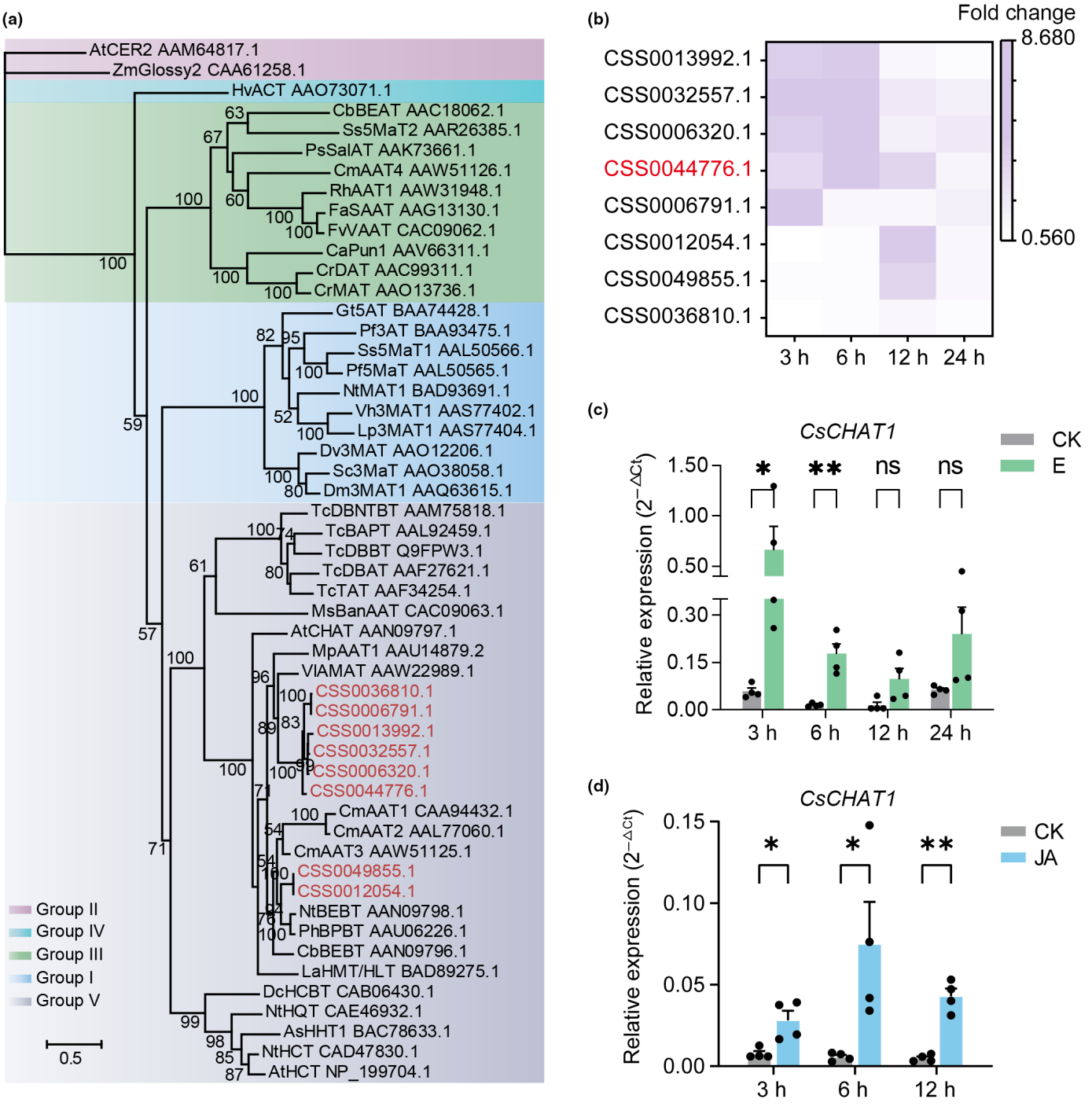

Fig. 3 Phylogenetic tree and expression profiles of *CsCHAT1*. (a) An unrooted phylogenetic tree based on the amino acid sequences of *CsCHAT1* and its homologs. The accessions numbers in red indicated CsCHAT candidate proteins. Numbers below the branch indicate bootstrap values. At, *Arabidopsis thaliana*; Zm, *Zea mays*; Hv, *Hordeum vulgare*; Cb, *Clarkia breweri*; Ss, *Salvia splendens Ker Gawl*; Ss, *Salvia splendens Ker Gawl*; Cm, *Cucumis melo*; Rh, *Rosa hybrid cultivar*; Fa, *Fragaria x ananassa*; Fv, *Fragaria vesca*; Ca, *Capsicum annuum*; Cr, *Catharanthus roseus*; Gt, *Gentiana triflora Pall*; Pf, *Perilla frutescens*; Nt, *Nicotiana tabacum*; Vh, *Verbena x hybrida*; Lp, *Lamium purpureum*; Dv, *Dermacentor variabilis*; Sc, *Pericallis cruenta*; Dm, *Dendranthema morifolium*; Tc, *Taxus canadensis*; Ms, *Musa sapientum*; Mp, *Malus x domestica*; Vl, *Vitis labrusca*; Cs, *Camellia sinensis*; Ph, *Petunia x hybrida*; La, *Lupinus albus*; Dc, *Dianthus caryophyllus*; As, *Avena sativa*. (b) The fold change in *CsCHAT* candidate expression in tea leaves was consumed by *E. obliqua* at 3, 6, 12, and 24 h. The *CsCHAT1* gene was highlighted in red. (c) Quantitative reverse transcription polymerase chain reaction results showing *CsCHAT1* expression in *E. obliqua-consumed* tea plant leaves at 3, 6, 12, and 24 h after treatment. E, tea plants were fed to *E. obliqua*. CK, plants without being fed to *E. obliqua*. Treatment and control experiments were conducted with four biological replicates, $n = 4$. Data are presented as mean ± SD (one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$; ns, no significant difference). (d) The expression level of *CsCHAT1* in tea plants after 1 mM of exogenous JA at 3, 6, and 12 h. Treatment and control experiments were conducted with four biological replicates, $n = 4$. Data are presented as mean ± SD (one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$).

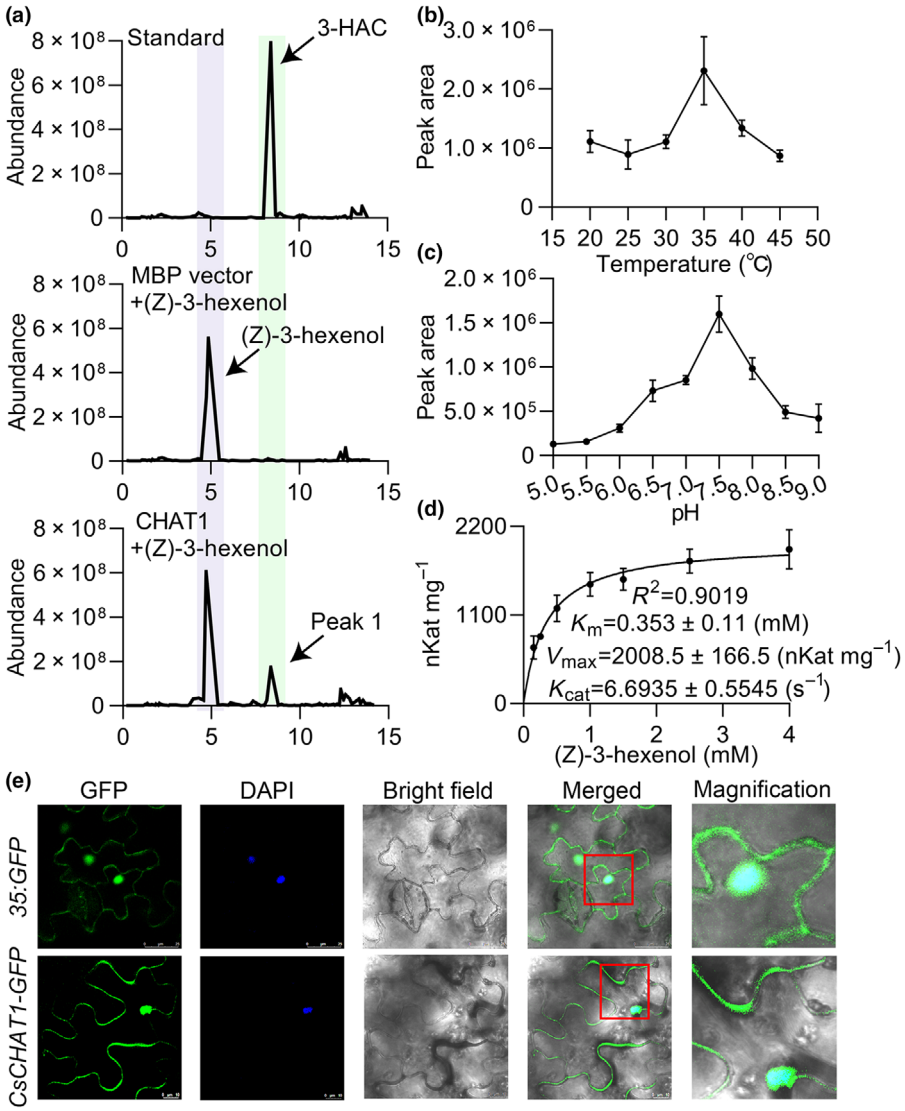

Fig. 4 *In vitro* enzymatic activity and subcellular localization of CsCHAT1. (a) Total ion chromatography of GC-MS showing a product (Peak 1) formed *in vitro* via catalysis by recombinant CsCHAT1 proteins using (Z)-3-hexenol and acetyl CoA as the substrates. (b) Optimum reaction pH of the CsCHAT1 enzymatic reaction. Data are presented as mean ± SD of three repetitions. (c) Optimum reaction temperature of the CsCHAT1 enzymatic reaction. Data are presented as mean ± SD of three repetitions. (d) Enzymatic kinetics of recombinant CsCHAT1. Data are presented as mean ± SD of three repetitions. (e) Subcellular localization of GFP-tagged CsCHAT1 proteins in leaves of *Nicotiana benthamiana* at 48 h after infusion. DAPI staining marks nuclei. The bars are 25 and 10 μm in leaves expressing *35S: GFP* and *CsCHAT1-GFP*, respectively. The magnification was set to 3.57 and 2.55 in leaves infected with 35S: GFP and CsCHAT1-GFP, respectively.

Further magnification indicated that CsCHAT1-GFP exhibited similar subcellular localization with GFP driven by 35S promoter, which was localized in both the nucleus and cytoplasm.

Suppression of *CsCHAT1* expression leading to reduction of 3-HAC in plants

AsODN-mediated gene silencing was performed to suppress *CsCHAT1* in tea leaves. Based on the sequence of *CsCHAT1*, gene-specific AsODN probes, AsODN-*CsCHAT1* were designed to completely supplement its CDS sequence (Table S1), and the probes were then injected into the third mature leaves of tea plants (Fig. 5a). The results showed that AsODN-*CsCHAT1* efficiently suppressed *CsCHAT1* transcripts and 3-HAC production 12 h after injection in tea plants (Fig. 5b,c). To assess the resistance of *CsCHAT1*-silenced tea plants against *E. obliqua* feeding, the *CsCHAT1*-silenced and control leaves were fed to the larvae of *E. obliqua*. The feeding results showed that more *CsCHAT1*-silenced leaves were consumed by the *E. obliqua* (Figs 5d, S3a).

To further explore whether 3-HAC was the main factor driving the resistance of *CsCHAT1*-silenced tea plants against *E. obliqua* feeding, exogenous 3-HAC replenishment was applied to *CsCHAT1*-silenced and control (water and ODN) leaves, respectively (Fig. 5e). After 1 h of 3-HAC treatment, the *CsCHAT1*-silenced and control leaves were fed to larvae, respectively. The feeding results showed that there was no difference in the areas of consumed leaves between *CsCHAT1*-silenced and control leaves (Figs 5f, S3b), suggested that 3-HAC treatment rescued the weak resistance of *CsCHAT1*-silenced tea plants against *E. obliqua*.

Furthermore, to investigate the effect of JA on CsCHAT1 in 3-HAC biosynthesis, exogenous JA and methanol (control group) were applied to *CsCHAT1*-silenced, water-, and ODN-treated tea plants for 3 h, respectively (Fig. 5g). Notably, JA significantly accelerated the 3-HAC accumulation in water- and ODN-treated tea leaves, which was 2.1- and 1.8-fold that of the control group, respectively (Fig. 5h). However, 3-HAC content only increased to 1.4-fold in *CsCHAT1*-silenced tea plants after JA treatment. The results implied that *CsCHAT1* suppression

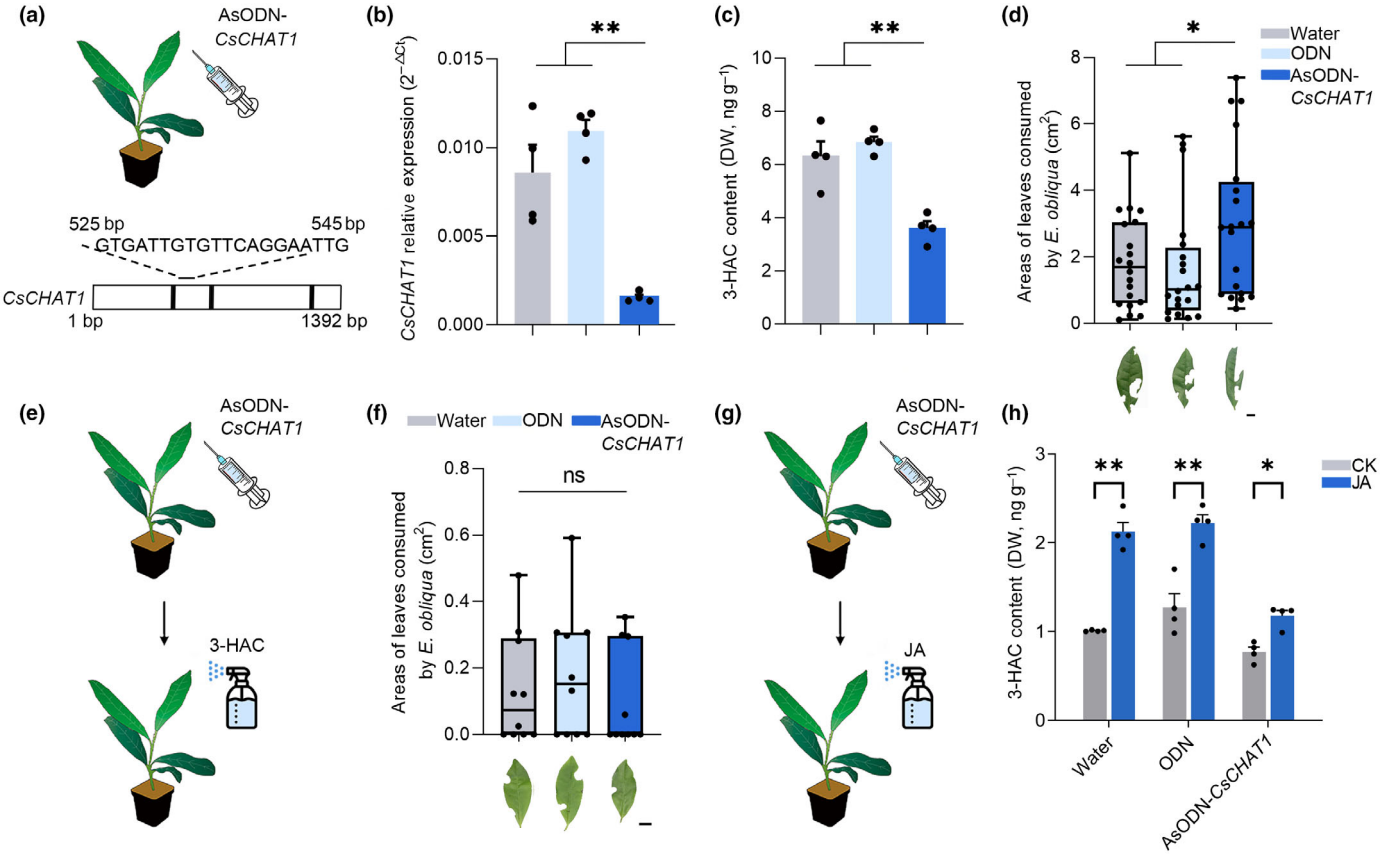

Fig. 5 Silencing *CsCHAT1* expression reduced 3-HAC content and *Ectropis obliqua* resistance in tea plants. (a) A scheme illustrating transient suppression of *CsCHAT1* transcripts by the antisense of *CsCHAT1* (AsODN-*CsCHAT1*). The 525–545 bp nucleotide sequence from the CDS region of *CsCHAT1* was used to synthesize AsODN-*CsCHAT1*. The black rectangle represents the three conserved motifs of CsCHAT1, namely HXXXD1, HXXXD2, and DFGWG. (b) Transcriptional suppression of *CsCHAT1* in *CsCHAT1*-silenced tea plants. AsODN-*CsCHAT1*, water, and ODN treatments were conducted with four biological replicates, $n = 4$. Data are presented as mean ± SD (one-way ANOVA: **, $P < 0.01$). (c) Decrease of 3-HAC in leaves of *CsCHAT1*-silenced tea plants. AsODN-*CsCHAT1*, water, and ODN treatments were conducted with four biological replicates, $n = 4$. Data are presented as mean ± SD (one-way ANOVA: **, $P < 0.01$). (d) Areas of *E. obliqua-consumed* leaves in *CsCHAT1*-silenced tea plants. Data are presented as boxplots (minima, 25th percentile, median, 75th percentile, and maxima). *CsCHAT1*-silenced, water-, and ODN-treated leaves of tea plants were collected 10 h after *E. obliqua* feeding. Twenty leaves were consumed by *E. obliqua*, which were considered as 20 biological replicates, $n = 20$. Bar, 1 cm. Data are presented as mean ± SD (one-way ANOVA: *, $P < 0.05$). (e) Scheme illustrating supplementation of 3-HAC in *CsCHAT1*-silenced tea plants. (f) Areas of *E. obliqua-consumed* leaves in 3-HAC-supplied *CsCHAT1*-silenced tea plants. Data are presented as boxplots (minima, 25th percentile, median, 75th percentile, and maxima). The samples were collected 10 h after being fed to *E. obliqua* on 0.5 ng μl^{-1} 3-HAC-supplied *CsCHAT1*-silenced, water-, and ODN-treated leaves for 1 h. Ten leaves were consumed by *E. obliqua*, which were considered as 10 biological replicates, $n = 10$. Bar, 1 cm. Data are presented as mean ± SD (one-way ANOVA, ns, no significant difference). (g) Scheme illustrating of supplementation of JA in *CsCHAT1*-silenced tea plants. (h) Content of 3-HAC in JA-treated *CsCHAT1*-silenced tea plants. The samples were collected 3 h after 1 mM JA-treated *CsCHAT1*-silenced, water-, and ODN-treated leaves of tea plants. Treatments and controls were conducted with four biological replicates, $n = 4$. Data are presented as mean ± SD (one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$).

considerably impaired JA-induced 3-HAC biosynthesis in tea plants. Collectively, these results showed that *CsCHAT1* served as the major factor modulating resistance against *E. obliqua* by facilitating 3-HAC biosynthesis.

CsNAC30 and CsTCP11 positively regulate *CsCHAT1* expression

To identify potential TFs that influence *CsCHAT1* expression, we analyzed the transcriptomes of *E. obliqua*-consumed leaves. Mining of transcriptome data revealed that 3659 differentially expressed genes associated with *CsCHAT1* expression in *E. obliqua*-consumed leaves (Table S3), including 309 differentially expressed TFs (Table S4). Furthermore, Pearson correlation analysis identified that 12 TFs significantly positively correlated to the expression of *CsCHAT1*, of which six TFs (*CsMYB4*, *CsbHLH35a*, *CsNAC30*, *CsbHLH35b*, *CsDREB3*, and *CsTCP11*) expression were upregulated 3-fold in *E. obliqua*-consumed leaves relative to in unconsumed leaves (Fig. 6a; Table S5). Notably, promoter analysis identified a characteristic NAC (ACGTG motif, −900 bp to −906 bp) and TCP (GCCCR motif, −559 bp to −565 bp) transcription factor binding *cis*-elements within the 1202 bp *CsCHAT1* promoter sequence (Fig. 6b) (Li *et al.*, 2005; Shamimuzzaman & Vodkin, 2013). Based on this,

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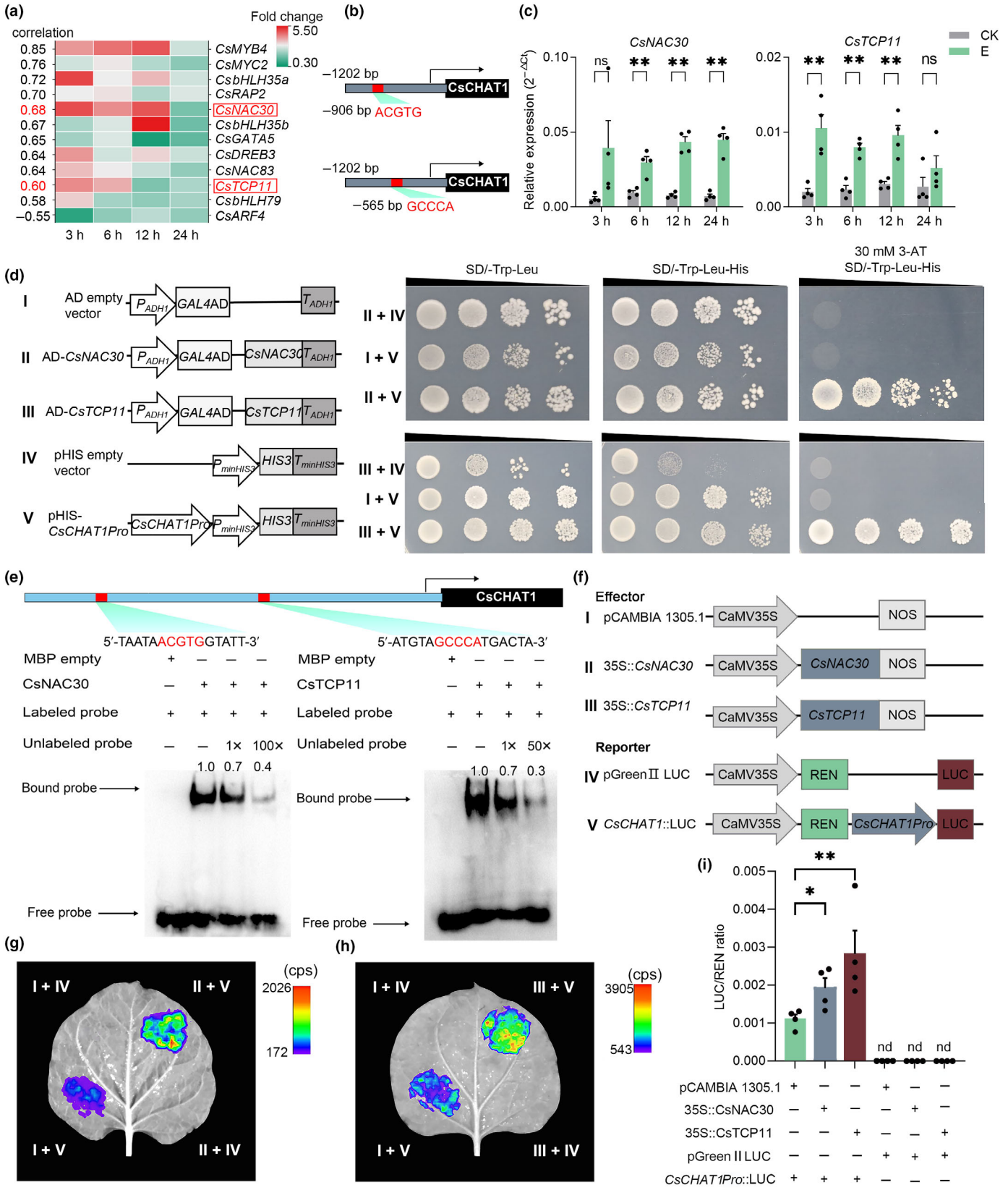

Fig. 6 Identification of transcription factors CsNAC30 and CsTCP11 as key regulators of *CsCHAT1* expression. (a) Pearson correlation analysis and fold change of the 12 candidate TFs and *CsCHAT1*. Original FPKM values were calculated based on the transcriptome of tea leaves consumed by *E. obliqua*. (b) Structure of the *CsCHAT1* promoter regions. The *cis*-elements of *CsNAC30* and *CsTCP11* were identified at the −906 bp and − 565 bp promoter positions, respectively. (c) Quantitative reverse transcription polymerase chain reaction results showing the expression profiles of *CsNAC30* and *CsTCP11* in *E. obliqua-consumed* leaves of tea plants at 3, 6, 12, and 24 h after treatments, respectively. E, tea plants were fed to *E. obliqua*. CK, plants without being fed to *E. obliqua*. Treatment and control experiments were conducted with four biological replicates, $n = 4$. Data are presented as mean ± SD (one-way ANOVA: **, $P < 0.01$; ns, no significant difference). (d) Yeast one-hybrid assays showing that both CsNAC30 and CsTCP11 bound to the promoter of *CsCHAT1*. The black triangles indicate the gradient dilutions (1, 1/10, 1/100, 1/1000) of yeast. (e) EMSA results showing the binding of both CsNAC30 and CsTCP11 to the promoter of *CsCHAT1*. The labeled and unlabeled probe represents the putative binding motif with and without biotin modification. The numbers above the protein-probe complex indicated their relative intensity using ImageJ software. (f) Schemes of three effector and two reporter vectors developed for dual-LUC assay. (g, h) Luminescence images after 48 h of co-expression of effector and reporter in a *Nicotiana benthamiana* leaf. (i) The *CsCHAT1Pro* luminescence intensity was enhanced by CsNAC30 and CsTCP11 expression, respectively. REN and LUC activities were measured to estimate the expression-promoting activities of CsNAC30 and CsTCP11. nd, not detected. Treatment and control experiments were conducted with four biological replicates, $n = 4$. Data are presented as mean ± SD (one-way ANOVA: *, $P < 0.05$; **$P < 0.01$).

CsNAC30 and CsTCP11 were selected as candidate TFs associated with the *CsCHAT1* expression.

Furthermore, quantitative reverse transcription polymerase chain reaction analysis showed that *CsNAC30* and *CsTCP11* expression was significantly higher in *E. obliqua*-consumed leaves than in the control leaves within 3–24 h, corroborating transcriptome data (Figs 6c, S4a). Meanwhile, similarly to the *CsCHAT1* expression induced by JA, *CsNAC30* and *CsTCP11* expression were upregulated by 1 mM JA treatment (Fig. S4b). These results suggest a correlation of *CsNAC30* and *CsTCP11* with *CsCHAT1* expression in *E. obliqua*-consumed leaves.

To assess the function of CsNAC30 and CsTCP11 in *CsCHAT1* expression, Y1H assays were performed. The results showed that CsNAC30 and CsTCP11 interacted with the *CsCHAT1* promoter on SD/−Trp-Leu-His medium (contains 30 mM 3-amino-1,2,4-triazole, 3-AT), but not with the control pHIS vector (Fig. 6d). In addition, an EMSA was completed to test whether CsNAC30 and CsTCP11 directly bound to the *CsCHAT1* promoter, respectively (Fig. 6e). The recombinant CsNAC30 and CsTCP11 fused to MBP-tag proteins were expressed in *E. coli* and purified using affinity chromatography. Core NAC and TCP binding *cis*-elements within the *CsCHAT1* promoter were modified with biotin for EMSA analysis. EMSA results revealed that CsNAC30 and CsTCP11 bound to the core *cis*-element of the *CsCHAT1* promoter, respectively.

Dual-LUC assay was further performed in *Nicotiana benthamiana* leaves to investigate the regulatory effect of CsNAC30 and CsTCP11 on *CsCHAT1* expression. Both *CsNAC30* and *CsTCP11* were cloned immediately downstream of the 35S promoter in the pCAMBIA1305.1 vector. The promoter sequence of *CsCHAT1* was cloned to the pGreenII 0800-LUC vector to establish a pGreenII-*CsCHAT1pro* recombinant reporter, while the pGreenII 0800-LUC empty vector served as a negative reporter (Fig. 6f). *Nicotiana benthamiana* leaves were infected with two pairs of effector and reporter, pGreenII-*CsCHAT1pro* co-expressed with pCAMBIA1305.1-CsNAC30 and pCAMBIA1305.1-CsTCP11, respectively. pGreenII 0800-LUC co-infection with pCAMBIA1305.1-CsNAC30 and pCAMBIA1305.1-CsTCP11 served as the negative controls, respectively. Another negative control was the co-infection of pCAMBIA1305.1 and pGreenII-*CsCHAT1pro*. The LUC activity imaging results showed that the fluorescence intensity of pGreenII-*CsCHAT1pro* co-expressed with pCAMBIA1305.1-CsNAC30 or pCAMBIA1305.1-CsTCP11 was significantly higher than that observed after co-expression with pCAMBIA1305.1 (Fig. 6g,h). No fluorescence was detected in the two control groups. Moreover, the intensity of the LUC and Renilla (Ren) was quantified to estimate the LUC/REN ratios. The results showed that the LUC/REN ratio of pCAMBIA1305.1-CsNAC30 and pGreenII-*CsCHAT1pro* or pCAMBIA1305.1-CsTCP11 and pGreenII-*CsCHAT1pro* were significantly higher than those in controls (Fig. 6i). These data demonstrated that CsNAC30 and CsTCP11, respectively, bound to the promoter of *CsCHAT1* to positively regulate its expression.

Silencing of both *CsNAC30* and *CsTCP11* expression decreases 3-HAC levels and the resistance of tea leaves to *E. obliqua*

Silencing of both *CsNAC30* and *CsTCP11* was performed using AsODNs to investigate their role in 3-HAC biosynthesis and tea plant resistance against *E. obliqua*. Five specific AsODN probes for *CsNAC30* and *CsTCP11* were designed (Table S1), and the AsODNs were injected into the third mature leaves of tea plants for 12 h. Quantitative reverse transcription polymerase chain reaction analysis indicated that AsODN-*CsNAC30*-P2 and AsODN-*CsTCP11*-P3 (hereinafter referred to as AsODN-*CsNAC30* and AsODN-*CsTCP11*, respectively) were considered as optimal AsODN probes with the highest suppression efficiency for *CsNAC30* and *CsTCP11* when compared with the other test probes (Fig. S5a,b). To evaluate the involvement of CsNAC30/CsTCP11 in 3-HAC biosynthesis in response to *E. obliqua* feeding, tea leaves were injected with AsODN-*CsNAC30*/*CsTCP11* for 12 h and were then fed to *E. obliqua* for 0.1 h (Fig. 7a). Meanwhile, water- and ODN-treated leaves fed to *E. obliqua* were used as controls.

The quantitative reverse transcription polymerase chain reaction data showed that the expression level of *CsNAC30* and *CsTCP11* was significantly lower in *CsNAC30*- and *CsTCP11*-silenced leaves than in control leaves, respectively (Fig. 7b). Notably, suppression of *CsNAC30* and *CsTCP11* significantly reduced

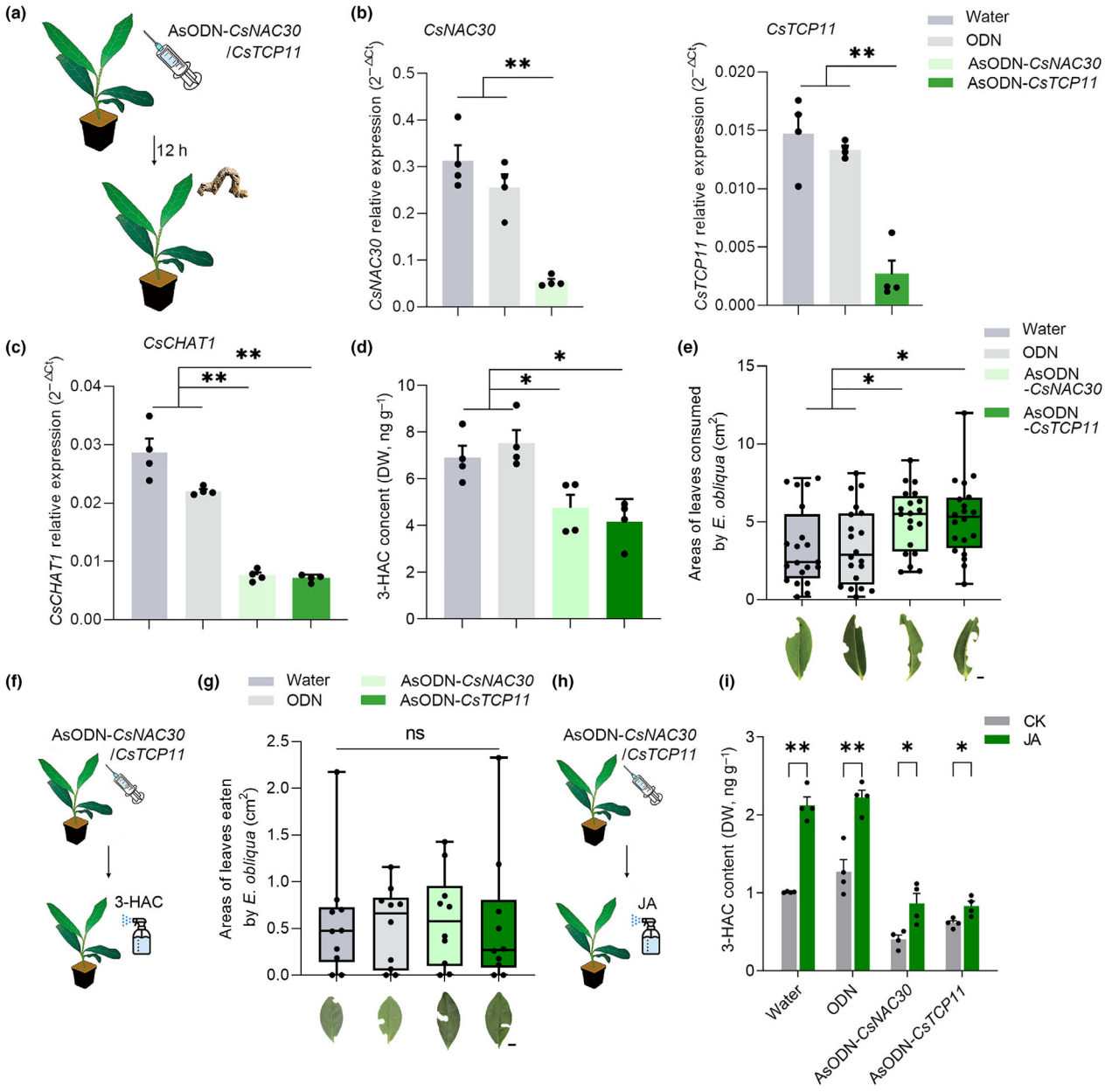

Fig. 7 Silencing *CsNAC30 and CsTCP11* expression reduced 3-HAC content and *Ectropis obliqua* resistance in tea plants. (a) A schematic illustrating transient suppression of *CsNAC30 and CsTCP11* expression using an antisense of AsODN-*CsNAC30/ CsTCP11*. (b) Relative expression of *CsNAC30*, and *CsTCP11* in leaves of *CsNAC30/CsTCP11*-silenced tea plants. AsODN-*CsNAC30/CsTCP11*, water, and ODN treatments were conducted with four biological replicates, $n = 4$. Data are presented as mean ± SD (one-way ANOVA: **, $P < 0.01$). (c) Relative expression of *CSCHAT1* in leaves of *CsNAC30/CsTCP11*-silenced tea plants. AsODN-*CsNAC30/CsTCP11*, water, and ODN treatments were conducted with four biological replicates, $n = 4$. Data are presented as mean ± SD (one-way ANOVA: **, $P < 0.01$). (d) Decrease of 3-HAC in the leaves of *CsNAC30/CsTCP11*-silenced tea plants. AsODN-*CsNAC30/CsTCP11*, water, and ODN treatments were conducted with four biological replicates, $n = 4$. Data are presented as mean ± SD (one-way ANOVA: *, $P < 0.05$). (e) Areas of leaves consumed by *E. obliqua* in *CsNAC30/CsTCP11*-silenced tea plants. Data are presented as boxplots (minima, 25th percentile, median, 75th percentile, and maxima). *CsNAC30/CsTCP11*-silenced, water-, and ODN-treated leaves of tea plants were collected 10 h after *E. obliqua* feeding. Twenty leaves were consumed by *E. obliqua*, which were considered as 20 biological replicates, $n = 20$. Bar, 1 cm. Data are presented as mean ± SD (one-way ANOVA: *, $P < 0.05$). (f) Scheme illustrating supplementation of 3-HAC in *CsNAC30/CsTCP11*-silenced tea plants. (g) Areas of *E. obliqua-consumed* leaves in 3-HAC-supplied *CsNAC30/CsTCP11*-silenced tea plants. Data are presented as boxplots (minima, 25th percentile, median, 75th percentile, and maxima). The samples were collected 10 h after being fed to *E. obliqua* on 0.5 ng μl^{-1} 3-HAC-supplied *CsNAC30/CsTCP11*-silenced, water-, and ODN-treated leaves for 1 h. Ten leaves were consumed by *E. obliqua*, which were considered as 10 biological replicates, $n = 10$. Bar, 1 cm. Data are presented as mean ± SD (one-way ANOVA, ns, no significant difference). (h) Scheme illustrating supplementation of JA in *CsNAC30/CsTCP11*-silenced tea plants. (i) Content of 3-HAC in JA-treated *CsNAC30/CsTCP11*-silenced tea plants. The samples were collected 3 h after 1 mM JA-treated *CsNAC30/CsTCP11*-silenced, water-, and ODN-treated leaves of tea plants. Treatment and control experiments were conducted with four biological replicates, $n = 4$. Data are presented as mean ± SD (one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$).

CsCHAT1 expression (Fig. 7c). GC-MS analysis of volatiles showing that 3-HAC content exclusively significantly decreased in *CsNAC30*- and *CsTCP11*-silenced leaves relative to control leaves (Fig. 7d), while the contents of other HIPVs, including (Z)-3-hexenol, β-ocimene, linalool, (Z)-3-butyrate, methyl salicylate, geraniol, α-farnesene, and (Z)-nerolidol, were not significantly changed (Fig. S6). Moreover, UPLC analysis indicated that JA and JA–Ile levels were not significantly changed in *CsNAC30*- and *CsTCP11*-silenced leaves compared to in control leaves (Fig. S7). Importantly, more leaves of *CsNAC30*- and *CsTCP11*-silenced plants were consumed by *E. obliqua* compared to control leaves, suggesting that the suppression of *CsNAC30* and *CsTCP11* resulted in tea plant susceptibility to *E. obliqua* attack (Figs 7e, S8a). These results implied that CsNAC30 and CsTCP11 contributed to tea plant resistance against *E. obliqua* attack through enhanced 3-HAC biosynthesis.

To further explore whether the susceptibility of *CsNAC30*/*CsTCP11*-silenced tea plant to *E. obliqua* resulted the decrease in 3-HAC *in vivo*, 0.5 ng μl^{-1} 3-HAC was exogenously applied to *CsNAC30*/*CsTCP11*-silenced and control (water and ODN) plants (Fig. 7f). After 1 h of 3-HAC treatment, the *CsNAC30*/*CsTCP11*-silenced and control leaves were fed to *E. obliqua* larvae. Area analysis of consumed leaves showed that there was no difference in the consumed leaves between *CsNAC30*/*CsTCP11*-silenced and control tea plants (Figs 7g, S8b), suggesting that disrupted 3-HAC biosynthesis was the main factor leading to the susceptibility of *CsNAC30*/*CsTCP11*-silenced tea plant to *E. obliqua*.

Furthermore, to investigate the effect of CsNAC30 and CsTCP11 on JA-induced 3-HAC biosynthesis, 1 mM JA and methanol (control group) were exogenously applied to *CsNAC30*/*CsTCP11*-silenced, water-, and ODN-treated tea plants for 3 h, respectively (Fig. 7h). JA- and methanol-treated leaves were collected to detect 3-HAC content through GC-MS analysis. The resulting GC-MS indicated that 3-HAC content was significantly increased in water- and ODN-treated tea plants relative to that in *CsNAC30* and *CsTCP11*-silenced tea plants after JA treatment (Fig. 7i). These results suggested that the JA-induced 3-HAC biosynthesis was regulated by *CsNAC30* or *CsTCP11* in tea plants.

Taken together, our results data suggested that CsNAC30 and CsTCP11 acted as positive regulators of tea plant resistance against *E. obliqua* through enhanced JA-induced 3-HAC biosynthesis.

Discussion

Plants have evolved phytochemical defense strategies that protect them from herbivore attacks. Volatile organic compounds are main phytochemicals synthesized by plants as a chemical defense. To date, many reports have shown that 3-HAC plays an important defensive role in crop resistance to herbivores. It protects cotton from the damage of male *Helicoverpa armigera* (Kvedaras *et al.*, 2007) and green ash plantations from *Agrilus planipennis* (Grant *et al.*, 2010). A previous investigation of transcriptional mechanisms reported that 3-HAC promotes the expression of candidate genes potentially associated with the resistance of maize to insects (Wang *et al.*, 2023). Besides, the emission of 3-HAC was associated with the susceptible or resistant phenotype of tea varieties (Xin *et al.*, 2017), which served as the attractants for herbivores in tea gardens (Sun *et al.*, 2010, 2014, 2016; Bian *et al.*, 2018). A strikingly enhanced 3-HAC emission from tea plants attacked by *M. aurolineatus* (Sun *et al.*, 2010), as well as *E. obliqua* (Jing *et al.*, 2021a). In our study, *E. obliqua* induced the accumulation of 3-HAC in tea plants (Fig. 1a,b), and further exogenous 3-HAC supplement enhanced the resistance of tea plants to *E. obliqua* (Fig. 2a–c). In accordance with the HIPV-induced activation of JA signaling in tea plants, which of β-Ocimene, DMNT, (Z)-3-hexenol emission enhanced herbivore resistance through upregulated expression of JA biosynthetic genes (Liao *et al.*, 2021; Jing *et al.*, 2021a,b), the 3-HAC facilitated the mRNA accumulation of *CsLOX1* and *CsLOX3* and JA content (Fig. 2d,e,i,j). Conversely, the JA also accelerated the emission of the 3-HAC in tea plant (Fig. 2k). These results collectively suggested that there was the regulatory crosstalk between 3-HAC biosynthesis and JA signaling in tea plant in response to *E. obliqua* feeding.

Although 3-HAC has gained attention for its involvement in the plant defense, its biosynthesis in different plants remains to be characterized. To date, members of the BAHD acyltransferase family have been investigated to gain insight into their roles in the biosynthesis of various defensive volatile compounds associated with plant resistance to biotic and abiotic stresses, such as pathogen infection, herbivore attack, drought and salt stress (Yu *et al.*, 2008; Gou *et al.*, 2009). Drought, salt and MeJA treatments increased the accumulation of linalyl acetate, and lavandulyl acetate and induced the expression of *LaBAHDs* (W. Y. Zhang *et al.*, 2024). Here, *CsCHAT1* encoded a BAHD acyltransferase, was significantly induced and converted (Z)-3-hexenol into 3-HAC in response to feeding by *E. obliqua* (Figs 3, 4). Suppression of *CsCHAT1* not only led to the deficiency on 3-HAC biosynthesis but also impaired the resistance of tea plant against *E. obliqua* feeding (Fig. 5a–d). Notably, the exogenous 3-HAC and JA significantly rescued the hypersensitive response to the *E. obliqua* feeding (Fig. 5e–h). These results implied that the *CsCHAT1* may acted as the critical intermediator, which was responsible for the 3-HAC biosynthesis and further amplified JA signal to improve the herbivore resistance of tea plant. This finding inspired us to characterize the natural variation in *CsCHAT1* and manipulated its transcriptional expression or protein structure to accelerate the screen and breeding of resistant tea cultivars.

The utilization of 3-HAC biosynthesis for potential tea plant defense strategies necessitates a comprehensive understanding of the mechanisms through which tea plants regulate *CsCHAT1* expression. It is known that TFs (such as bHLH, WRKY, NAC, and TCP) and phytohormones (such as JA) play fundamental roles in increasing plant resistance against herbivores through regulated the biosynthesis of secondary metabolites (Malook *et al.*, 2019; Pecher *et al.*, 2019; Fan *et al.*, 2020; Gong *et al.*, 2023). A study reported that *M. separata* herbivory attack increased JA/JA–Ile and activated benzoxazinoids (Bxs)

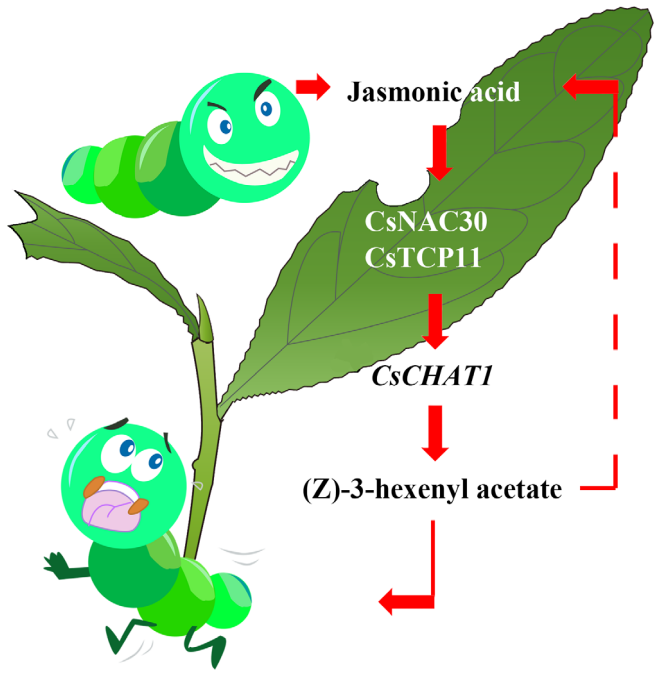

Fig. 8 A scheme model showing how *CsNAC30*, *CsTCP11*, *CsCHAT1*, and 3-HAC interact to protect tea plants from the herbivore *Ectropis obliqua*. When *E. obliqua* consumes tea leaves, wounding induces the accumulation of JA. Increased JA production activates the expression of *CsNAC30* and *CsTCP11*. The two TFs bind to the *CsCHAT1* promoter and activate its expression. In turn, CsCHAT1 catalyzes the formation of 3-HAC, which not only drives away *E. obliqua*, but regulates the JA biosynthesis in a feedback loop to amplify the defense signal. Standard arrows indicated the results supported by experiments in this study. Dashed arrow indicated potential regulatory pathways.

biosynthesis to enhance resistance against *M. separata*, while bHLH57 and WRKY34 were predicted to positively regulate Bxs biosynthetic genes (Malook *et al.*, 2019). TCPs also involved in defense responses to leafhopper feeding in *Arabidopsis*, but not in maize (Pecher *et al.*, 2019). In our study, the herbivore-induced CsNAC30 and CsTCP11 bound to the *CsCHAT1* promoter to stimulate its expression (Fig. 6), and positively regulated 3-HAC biosynthesis to enhance resistance against *E. obliqua* (Fig. 7a–e). Further silencing either one considerably impaired JA-induced 3-HAC biosynthesis in tea plants (Fig. 7h,i). In contrast to the limited effect of individual TF on expression of downstream resistance genes, the potential synergetic function of TF complex had been raised in interaction between plant and environment. Recently, CsNAC17-CsbHLH62 TF complexes was found that synergistically reduced susceptibility of tea plants to pathogens (Han *et al.*, 2024). Although we determined the positive role of *CsNAC30* and *CsTCP11* on *E. obliqua* resistance, biochemical and genetic analyses were necessary to confirm whether these two TFs could assemble into a regulatory complex that fine-tuned the *CsCHAT1* expression.

Based on experimental data, a model was proposed to describe this defensive pathway (Fig. 8). When tea plants are consumed by the larvae of *E. obliqua*, leaf wounding induces the accumulation of JA, which activates or enhances the expression of *CsNAC30* and *CsTCP11*. The TFs then bind to the *CsCHAT1* promoter and activate its transcription, whereafter the encoded enzyme catalyzes the formation of 3-HAC to repel *E. obliqua*. This pathway supports the production high-quality tea leaf products without the use of anti-herbivore chemicals.

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Competing interests

None declared.

Author contributions

JZ, CW, DQ and HG have made substantial contributions to the conception or design of the work; DQ, KH, SL, D-YX and HG revised it critically for important intellectual content; JL, ML, YY, HX and HG performed experiments and acquired, analyzed, or interpreted working data; JZ, CW and HG drafted the work; HG, JL and DQ contributed equally to this work. All authors discussed the results, reviewed the article, and approved the final article.

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Data availability

Sequence data from this study can be found in the National Center for Biotechnology Information (NCBI) under the following accession nos.: *CsCHAT1* promoter (OR058582), *CsCHAT1* (OR058579), *CsNAC30* (OR058580), *CsTCP11* (OR058581), and raw data of transcriptome from leaves consumed by *Ectropis Obliqua* (PRJNA901518).

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Supporting Information

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

Fig. S1 Identification of the *CsCHAT* candidates.

Fig. S2 Enzyme activity analysis of *CsCHAT1 in vitro*.

Fig. S3 The herbivore damage assay in *CsCHAT1*-silenced tea plants.

Fig. S4 The expression level of transcription factors CsNAC30 and CsTCP11 in response to *Ectropis obliqua* feeding and JA treatment.

Fig. S5 The expression levels and scheme illustrating CsNAC30-AsODNs and CsTCP11-AsODNs.

Fig. S6 The contents of eight main volatiles emitted from leaves of *CsNAC30*/*CsTCP11*-silenced tea plants.

Fig. S7 The level of JA and JA–Ile in leaves of *CsNAC30*/*CsTCP11*-silenced tea plants.

Fig. S8 The herbivore damage assay in *CsNAC30*/*CsTCP11*-silenced tea plants.

Methods S1 RNA-Seq and bioinformatics analysis.

Methods S2 Volatile extraction and measurement.

Methods S3 Phytohormone content determination.

Methods S4 Sequence amplification.

Methods S5 Quantitative reverse transcription polymerase chain reaction analysis.

Methods S6 Heterologous expression and purification of protein.

Methods S7 Subcellular localization of CsCHAT1 in *Nicotiana benthamiana*.

Methods S8 Electrophoresis mobility shift assay.

Methods S9 Dual-luciferase (dual-LUC) assay.

Methods S10 Suppression of *CsCHAT1*, *CsNAC30*, *and CsTCP11* expression in tea plants under 3-HAC and JA treatment.

Table S1 Primers used in this study.

Table S2 CHAT amino acid sequence used in this study.

Table S3 3659 differentially expressed genes associated with the *CsCHAT1* expression in transcriptome results of *Ectropis obliqua* damaged tea plants at 3, 6, 12, and 24 h.

Table S4 The original FPKM values of 309 significantly differentially expressed TFs used for Pearson correlation analysis.

Table S5 The Pearson correlation between *CsCHAT1* and 12 TFs.

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