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MaEIN2 links ethylene signaling to chlorogenic acid accumulation in mulberry leaves in response to silkworm-feeding

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Research Article

Keywords: Morus alba L., Bombyx mori, ethylene, MaEIN2, virus-induced gene silencing, chlorogenic acid

Posted Date: July 16th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10321544/v1

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Additional Declarations: No competing interests reported.

Abstract

Mulberry (*Morus alba* L) leaves are the sole food source of the domesticated silkworm (*Bombyx mori*) and are rich in chlorogenic acid (ChA), a phenolic metabolite implicated in plant defense and silkworm performance. However, how herbivory-associated cues are integrated with ethylene (ET) signaling to regulate ChA accumulation remains elusive. Here, we found that silkworm feeding markedly enhanced ChA levels in mulberry leaves, accompanied by notable cascading changes in genes associated with the ethylene (ET) signaling pathway, particularly *Morus alba L. ETHYLENE INSENSITIVE 2* (*MaEIN2*), indicating that ET is involved in the stress response elicited by silkworm feeding. Virus-induced gene silencing (VIGS) of *MaEIN2* significantly reduced ChA accumulation and downregulated downstream genes implicated in ChA biosynthesis. Moreover, *MaEIN2*-silenced seedlings exhibited compromised resistance to *A. tumefaciens* harboring tobacco rattle virus (TRV)-based plasmids, supporting a role for *MaEIN2* in biotic stress defense. Collectively, these results indicate that *MaEIN2* mediates ET-dependent defense responses by modulating ChA accumulation in mulberry. Furthermore, herbivory-induced stress appears to operate through *MaEIN2*-dependent ET signaling, which elevates ChA content potentially to accommodate silkworm developmental needs. This study provides new insights into the role of *MaEIN2* in linking silkworm feeding with ChA accumulation in mulberry.

Key message

MaEIN2 acts as an ethylene-signaling hub that promotes chlorogenic acid accumulation and supports mulberry responses to pathogen-associated and silkworm-feeding stresses.

Introduction

Mulberry (*Morus alba* L.) is a perennial tree or shrub of major economic value in sericulture and the sole food source of the domesticated silkworm (*Bombyx mori*). Mulberry leaves are rich in phenolic compounds and flavonoids that contribute to plant defense against pathogens and herbivores. Chlorogenic acid (ChA), the most abundant phenolic compound in mulberry leaves (Jarienė et al. 2019; Can et al. 2021), is widely distributed in plants and has antibiotic and anti-herbivore activities (Jiao et al. 2018; Kundu and Vadassery 2019; Zhang et al. 2020a; Kai et al. 2021; Cheng et al. 2022; Liu et al. 2026). Intriguingly, ChA in mulberry leaves can promote silkworm feeding and larvae growth, correlates positively with growth-related traits, and affects cocoon formation and silk quality (Yamagishi et al. 2018; Šelih et al. 2020). These observations suggest that the domesticated silkworm, which has evolved from wild silkworms over thousands of years of artificial selection, has formed a co-evolutionary relationship with mulberry trees. During growth, mulberry trees are exposed to diverse biotic stresses, including pathogen infection and feeding by wild insects. Silkworm feeding is itself a herbivory-like stress that may remodel secondary metabolism in mulberry leaves. However, how silkworm feeding influences ChA accumulation and how ChA biosynthesis is regulated in mulberry remains poorly understood.

The endogenous phytohormone ethylene (ET) is essential for developmental processes, including fruit ripening in plants. As the sole gaseous hormone in plant cells, ET is extensively involved in regulating secondary metabolites (Bleecker and Kende 2000; Guo and Ecker 2004; Dugardeyn et al. 2008; Hao et al. 2025). It also serves as an important mediator in response to biotic and abiotic stresses in myriad physiological processes of plants (Cao et al. 2007; Peng et al. 2014; Ju and Chang 2015). However, whether ET affects the biosynthesis of ChA in mulberry and its possible regulatory mechanisms remain poorly understood.

ET biosynthesis and signaling are organized through a conserved regulatory cascade. 1-Aminocyclopropane-1-carboxylate synthase (ACS), encoded by a small gene family, catalyzes a rate-limiting step in ET biosynthesis (Alonso and Ecker 2001; Tsuchisaka and Theologis 2004).

ETHYLENE INSENSITIVE2 (EIN2) is an essential positive regulator of ET signaling and is located in the endoplasmic reticulum (ER) membrane (Guo and Ji 2013). In *Arabidopsis*, the N-terminus of EIN2 contains 12 transmembrane domains and shows similarity to the Nramp family of metal-ion transporters (Alonso et al. 1999). Loss-of-function *ein2* mutants are completely insensitive to ET, demonstrating that EIN2 is required for transmitting ET signals from the ER to the nucleus (Roman et al. 1995; Sonia and Peter 2003; Wen et al. 2012). Expression of the C-terminal end of EIN2 (EIN2-CEND; amino acids 454–1294) in *ein2* mutants constitutively activates adult ET-response phenotypes, indicating that EIN2-CEND is sufficient to activate downstream ET responses (Alonso et al. 1999; Qiao et al. 2012; Zheng and Zhu 2016).

The upstream factor CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) is the primary regulator responsible for the dephosphorylation and cleavage of EIN2, resulting in the formation of EIN2-CEND (Ju et al. 2012; Qiao et al. 2012; Wen et al. 2012). CTR1 acts as a Raf-like protein kinase and serves as a negative regulatory factor in ET signaling (Kieber et al. 1993). In the absence of ET, CTR1 forms a complex with ET receptors, leading to phosphorylation of the EIN2-CEND, preventing its cleavage and subsequent nuclear translocation (Kieber et al. 1993; Bisson and Groth 2010; Ju et al. 2012; Guo and Ji 2013; Angulo et al. 2021).In the presence of ET, the binding of ET to its receptors inactivates CTR1, leading to the reduced phosphorylation of EIN2, which subsequently allows the cleavage of EIN2-CEND at the S645/ F646 or S645/S924 sites, generating the increased accumulation of EIN2-CEND fragments and its subsequent nuclear translocation (Ju et al. 2012; Qiao et al. 2012; Guo and Ji 2013; Zhang et al. 2020b). The cleaved EIN2-CEND fragments in the cytosol can also translocate to the nucleus. Additionally, EIN2-CEND localizes to processing bodies (P-bodies), where it suppresses the translation of EIN3-BINDING F-BOX 1/2 (EBF1/EBF2) mRNAs. This prevents EBF1/EBF2-mediated ubiquitin-proteasome degradation of ETHYLENE INSENSITIVE 3/ETHYLENE INSENSITIVE 3-LIKE 1 (EIN3/EIL1), allowing EIN3/EIL1 to accumulate and activate a transcriptional cascade that includes ETHYLENE RESPONSE FACTOR (ERF) genes (Guo and Ecker 2003; An et al. 2010; Park et al. 2023; Hao et al. 2025). Thus, EIN2 is a pivotal transducer that connects ET perception at the ER to nuclear transcriptional reprogramming.

Genetic disruption of EIN2 alters salt sensitivity and disease tolerance, indicating that EIN2-mediated signaling contributes to plant adaptation to abiotic and biotic stresses (Bent et al. 1991; Geraats et al. 2002; Cao et al. 2007; Lei et al. 2011; Low et al. 2022). EIN2 is generally thought to promote stress tolerance by stabilizing EIN3/EIL1 transcription factors and activating downstream defense-related genes such as ERFs (Guo and Ecker, 2003; An et al. 2010; Wang et al. 2013; Hao et al. 2025).

Although the molecular basis of EIN2-regulated ET signaling is well established in *Arabidopsis thaliana*, the contribution of ET signaling to stress responses in woody plants remains much less clear. In mulberry, the potential function of EIN2 has been particularly difficult to assess because efficient and stable transformation systems are still limited.

Virus-induced gene silencing (VIGS) is a reverse-genetic approach for rapid gene-function analysis in plants (Ramegowda et al. 2014). In VIGS, viral vectors carrying host-derived sequences trigger the degradation of homologous endogenous mRNAs, resulting in a transient loss-of-function phenotype; its transformation is commonly mediated by *Agrobacterium* (Lu et al. 2003; Ramegowda et al. 2014). *Tobacco rattle virus* (TRV)-based VIGS vectors are among the most widely used systems because they infect a broad range of plant hosts (Ramegowda et al. 2014; Zhang et al. 2025). VIGS therefore provides an effective strategy for studying gene function in species that are recalcitrant to stable transformation, including mulberry.

In an effort to investigate the relationships among the silkworms, endogenous ET and ChA in mulberry, a *Morus alba* L. *ETHYLENE INSENSITIVE 2* (*MaEIN2*) gene was isolated. Subsequently, *MaEIN2*-VIGS plants were successfully generated using the TRV-based VIGS technique. By comparing the transcriptional changes of *MaEIN2* and its key upstream regulators and downstream targets in both silkworm feeding and *MaEIN2*-VIGS mulberry leaves, along with ChA content measurements, this study further elucidated the role of *MaEIN2* in ET-mediated biotic stress responses in a ChA accumulation manner in mulberry.

We further propose a model describing how biotic stress induced by *Agrobacterium* infection or silkworm feeding on mulberry leaves regulates ET signaling via *MaEIN2* translation, ultimately controlling ChA accumulation.

Materials and methods

Plant materials and growth conditions

The mulberry cultivar 'Jisang 3', a white mulberry (*Morus alba* L.) variety, was used in this study. Mulberry seedlings and fruits were obtained from the National Mulberry Germplasm of Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang city, Jiangsu province. Female flowers and the subsequent fruits of 3-year-old 'Jisang 3' mulberry plants were collected at six spring developmental stages: initial budding (S1, pistil just appeared), full flowering (S2, stigma unpollinated), end of flowering (S3, stigma pollinated), early fruit set (S4, fruit turn from green to red), mid-fruit stage

(S5, fruit turn to deep red), and full ripening (S6, fruit maturity). All samples were immediately flash-frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

Seeds were rinsed from mature S6-stage fruits of 'Jisang 3' and sown in growth medium. Seedlings were transplanted into pots containing vermiculite and loamy soil (pH 6.5) and maintained at 28°C under a 12 h light/12 h dark photoperiod. Seedlings 15–20 cm in height with uniform growth status were used for subsequent experiments.

Cloning and sequence analysis of MaEIN2.

Primers were designed based on the *Morus notabilis* EIN2 gene (*MnEIN2*, Morus024376) to amplify the full-length coding sequence of its homolog from the 'Jisang 3' cultivar (*Morus alba L.*) (Table S1). The obtained sequence was designated *MaEIN2* (*Morus alba L.* ETHYLENE INSENSITIVE 2; GenBank accession no. PP975779). The protein structure and transmembrane domains of *MaEIN2* were predicted using the SMART online tool (http://smart.embl-heidelberg.de/). A three-dimensional (3D) model of *MaEIN2* was generated using the SWISS-MODEL server (http://swissmodel.expasy.org/interactive). Phylogenetic analysis was performed in MEGA 7.0 using the neighbor-joining (NJ) method, and tree reliability was assessed by bootstrap analysis with 2,000 replicates.

Quantification of gene Expression by qRT-PCR

qRT-PCR was used to quantify expression levels of *MaEIN2* and selected upstream and downstream ET-signaling genes. Total RNA was isolated from different tissues of 'Jisang 3', including leaves, buds, stems, roots and fruits. RNA quality was evaluated using a UV spectrophotometer. cDNA was synthesized according to the M-MLV Reverse Transcriptase manufacturer's protocol (TaKaRa-Bio, China). Gene expression was detected using the ABI StepOnePlus Real-Time PCR System (Foster City, CA, USA). qRT-PCR primers for *MaEIN2* were designed to target the *MaEIN2*-CEND domain, specifically the region encompassing the putative S645/F646 cleavage/phosphorylation sites. Primers used for gene cloning, expression analysis, and plasmid construction were designed according to mulberry cDNA sequences deposited in GenBank (Table S1). The relative expression level of *MaEIN2* was calculated using the $2^{-\triangle\triangle Ct}$ method, with *MaActin* serving as the internal reference gene.

1-Methylcyclopropene (1-MCP) application

The method for treating mulberry seedlings with 1-MCP was adapted from Hussain et al. (2018, 2019) (Hussain et al. 2018; Hussain et al. 2019) with some modifications. Seedlings were treated with 1-MCP at 0.8 g/L (0.025 g per pot), and seedlings treated with clean water served as controls. Both the 1-MCP-treated and control seedlings were placed in plastic boxes, sealed with plastic wrap, and maintained at 4°C for 24 h. After removal of the wrap, the seedlings were transferred to a light incubator. Phenotypic changes were recorded, and samples were collected for subsequent analysis.

Virus-induced gene silencing (VIGS)

construction of MaEIN2-VIGS plasmid

The *MaEIN2*-CEND domain was selected as the primary target for gene silencing based on the analysis of the full-length sequence of *MaEIN2*. The optimal VIGS fragment was predicted using the SGN VIGS Tool online SGN-VIGS platform (https://vigs.solgenomics.net/), and the target sequence encompassed nucleotides encoding S645/ F646 within *MaEIN2*-CEND. A 297 bp encoding 99 amino acids was amplified using the *MaEIN2*-VIGS-F/R primers (Table S1). RT-PCR products were analyzed in 1% agarose gels and purified using the Takara Agarose Gel DNA Purification Kit according to the manufacturer's protocol. The purified fragment of the expected size was cloned into the pMD18-T plasmid (Takara) and sequenced to confirm target-sequence identity.

The empty plasmid pNC-TRV2-eGFP, constructed in our laboratory, was used as the control, as described by Shi et al. (2022) (Shi et al. 2022). The *MaEIN2*-VIGS fragment amplified with *MaEIN2*-VIGS-F/R was inserted into pNC-TRV2-eGFP to generate pNC-TRV2-eGFP-*MaEIN2*-CEND. The pNC-TRV2-eGFP and pNC-TRV2-eGFP-*MaEIN2*-CEND plasmids were then transformed into *Agrobacterium tumefaciens* GV3101 using the freeze-thaw method. Recombinant *A. tumefaciens* cultures were incubated at 28°C with shaking and used for infection when the OD600 of freshly grown cells reached 0.6.

Infection and co-cultivation of the mulberry seedlings

Seedlings approximately 20 cm in height were selected, and infection and co-cultivation were performed as described by Shi et al. (2022). (Shi et al. 2022). *A. tumefaciens* strain GV3101 suspensions harboring either the *MaEIN2*-VIGS plasmid or the empty vector (EV, pNC-TRV2-eGFP) were used as infection media, with transformation buffer alone serving as the blank control (WT) (Shi et al. 2022; Mardini et al. 2023). Each *A. tumefaciens* suspension was injected into mulberry seedlings to generate VIGS plants.

Fluorescence detection

Phenotypic changes in mulberry seedlings infected with recombinant *A. tumefaciens* were monitored over a two-week period. Leaf fluorescence was examined daily using a handheld fluorescence detector (Shanghai Luyang Instruments Co., Ltd.). Leaves with detectable fluorescence were collected dynamically, and *MaEIN2* transcript levels were analyzed in parallel to evaluate VIGS efficiency.

Stomatal aperture measurement

Seedling leaves showing distinct fluorescence and the lowest *MaEIN2* transcript level were selected for stomatal observation and aperture measurement by field-emission scanning electron microscopy (FESEM). Reagents for stomatal aperture measurement were purchased from Sangon Biotech (Shanghai, China).

The detection procedure was as follows. Collected mulberry leaves were cut into 0.5 mm x 0.5 mm squares and rinsed two to three times with phosphate buffer. Samples were fixed in 3% glutaraldehyde for 8 h and washed two to three times with phosphate buffer for 10–15 min per wash. Gradient dehydration was performed sequentially with 50%, 70%, 80% and 90% ethanol for 15 min each, followed by dehydration with 100% ethanol three times for 30 min each. Ethanol was replaced with tert-butanol three times for 30 min each. Samples were dried using a freeze vacuum dryer and coated with 10 nm gold using an ion sputter coater. Stomatal morphology was observed and analyzed with a field-emission scanning electron microscope (Zhenjiang Zhuanbo Testing Technology Co., Ltd., China).

Transcriptome Analysis

MaEIN2-VIGS leaves that exhibited distinct fluorescence and the lowest *MaEIN2* transcript level were selected and submitted to BGI Genomics Co., Ltd. (Wuhan, China) for transcriptome sequencing. Leaves from the EV group collected at the corresponding time point were used as controls.

ChA Determination

Chemicals

Acetonitrile and acetic acid (HPLC grade, purity ≥ 98%) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Deionized water was prepared using a Milli-Q water purification system (Millipore, USA). ChA (purity ≥ 98%) was purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China).

plant Samples

Mulberry leaf samples were processed following Zhang et al. (2018) (Zhang et al. 2018), with three biological replicates per group. Seedling leaves showing distinct fluorescence after inoculation with *A. tumefaciens* and the lowest *MaEIN2* transcript level, corresponding to samples used for transcriptome sequencing, were dried in the dark at room temperature, ground into powder and stored in a dry, dark place. EV leaves were used as controls.

Mulberry seedlings approximately 20 cm in height were selected for the feeding experiment. Third-instar silkworm larvae were placed on seedling leaves (10 larvae per seedling) for 2 h. Silkworm-gnawed leaves were then collected, dried in the dark at room temperature, ground into powder and stored in a dry, dark place for subsequent analyses. Unfed leaves (WT) served as controls, with three biological replicates per group.

Extraction of phenolic compounds from mulberry leaves

Dried mulberry leaf powder (0.5 g) was suspended in 50 mL of 70% (v/v) aqueous ethanol. The mixture was vortexed for 1 min and soaked for 30 min to ensure complete hydration. Ultrasound-assisted extraction was then performed twice using an ultrasonic homogenizer (KQ-2200DB, Kunshan, China; 100 W) at 30°C for 20 min each. Combined extracts were centrifuged at 12,000 rpm for 5 min and filtered

through a 0.22-μm organic filter membrane. The filtrate was diluted to 50 mL with 70% (v/v) ethanol to obtain the test solution and protected from light before phenolic-compound analysis.

ChA HPLC analysis

Phenolic compounds, including ChA, were quantified by high-performance liquid chromatography (HPLC). Analyses were performed according to Sugiyama et al. (2017) and Zhou et al. (2018) (Sugiyama et al. 2017; Zhou et al. 2018) with modifications. Samples were filtered through a 0.22-μm PES membrane and analyzed using an HPLC-VWD system (Agilent 1260 Infinity II, Victoria, TX, USA) equipped with a C18 column (4.6 mm x 250 mm, 5.0 μm; Waters, CA, USA). The mobile phase consisted of 1% (v/v) acetic acid in water (eluent A) and HPLC-grade acetonitrile (eluent B). The gradient program was as follows: 0–30 min, 24–48% B; 30–31 min, 48% B; 31–33 min, 48–75% B; and 33–35 min, 24% B. The column temperature was 28°C, the flow rate was 0.8 mL min-1, and the injection volume was 10 μL. Phenolic compounds, including ChA, were monitored at 290 nm. ChA was identified and quantified according to its characteristic peak and corresponding standard curve. A standard curve was generated using ChA standards at 20, 40, 60, 80 and 100 ng μL-1. ChA content in dried mulberry leaves was calculated using the standard-curve equation y (ChA, ng/μL) = 24.64x − 205.7 (R^2 = 0.9988).

Results

Characterization of MaEIN2

The sequence of the *MaEIN2* gene isolated from the mulberry cultivar ‘Jisang 3’was 3924 bp in length and encoded a predicted EIN2 protein (Fig.S1). Phylogenetic analysis showed that MaEIN2 was most closely related to MnEIN2 from *Morus notabilis* and was also relatively close to EIN2 homologs from other Moraceae species, such as *Ficus carica*. By contrast, *MaEIN2* was more distantly related to homologs from Poaceae species, including *Zea mays*, *Triticum aestivum* and *Oryza sativa*, and to the model dicot *Arabidopsis thaliana* (Brassicaceae) (Fig.S2). Protein-domain prediction using the SMART online tool (http://smart.embl-heidelberg.de/) indicated that mulberry MaEIN2 is a typical transmembrane protein. The N-terminal region of MaEIN2 from amino acid 13 to 457 contains 11 transmembrane (TM) domains, located at residues 13–32, 52–74, 94–116, 126–148, 153–175, 195–217, 237–259, 326–348, 355–377, 392–414, and 435–457, respectively. The region spanning amino acids 38–391 corresponded to an Nramp-like metal-ion transporter domain. *MaEIN2* and maize EIN2 both contain 11 predicted TM domains (Mei et al. 2019), whereas *Arabidopsis* EIN2 contains 12 TM domains (Alonso et al. 1999). Downstream of the TM region, two low-complexity regions were identified, NSSNNAFSSSS (residues 549–559) and GAGSLSRLAGLGRAARRQLAA (residues 659–679), suggesting possible roles in protein interaction, transport and stress responses (Coletta et al. 2010) (Fig.S3). Within *MaEIN2*-CEND, putative CTR1-associated phosphorylation/cleavage sites were predicted at S645/F646, consistent with conserved sites in Arabidopsis EIN2 (Guo and Ji 2013). A 3D-model of *MaEIN2* generated by SWISS-MODEL (http://swissmodel.expasy.org/interactive) indicated that S645/F646 are positioned on the cytosolic side of the ER membrane (Fig.S4).

MaEIN2 expression is positively correlated with ET accumulation in mulberry

MaEIN2 expression in mature leaves is significantly suppressed by the ET inhibitor 1-MCP

To examine the relationship between *MaEIN2* and ET signaling in mulberry, we first assessed *MaEIN2* expression under ET-perception inhibition. The ET-binding inhibitor 1-methylcyclopropene (1-MCP) is widely used to block ET perception in plants. *MaEIN2* expression was analyzed in roots, buds, mature leaves and stems after 1-MCP treatment.

In untreated seedlings (CK, air treatment), *MaEIN2* transcript abundance varied among tissues. Expression was highest in mature leaves, followed by stems, and lowest in buds, where cell division is vigorous. This pattern indicates tissue-specific expression and differs from a previous report in which *MnEIN2* was most highly expressed in roots of *Morus notabilis* (Liu et al. 2015). Whether this difference reflects species, genotype, or experimental conditions requires further investigation.

No obvious phenotypic changes were observed within 24 h of 1-MCP treatment (Fig.S5). However, *MaEIN2* transcriptional responses to 1-MCP differed among tissues. In roots and young buds, *MaEIN2* expression was only weakly suppressed, consistent with earlier observations that *MnEIN2* expression in mulberry fruit was not markedly affected by 1-MCP (Liu et al. 2015). In contrast, *MaEIN2* expression was strongly repressed in mature leaves and stems (Fig. 1a). These results suggest that *MaEIN2* basal expression and responsiveness to ET-perception inhibition are tissue dependent, with the highest constitutive expression and strongest repression occurring in mature leaves.

MaEIN2 transcript levels during mulberry fruit development

To further understand the relationship between *MaEIN2* and ET levels in mulberry trees, we aimed to investigate the expression pattern of *MaEIN2* under conditions of increased ET levels. The role of ET in mulberry has been successfully elucidated using mulberry fruit as a model. It has been reported that the ET content gradually increases as pistillate flowers develop into mature fruits and then declines rapidly after full ripening (Liu et al. 2015). To further elucidate the expression pattern of *MaEIN2* in ET-regulated mulberry fruit ripening, we analyzed the transcript levels of *MaEIN2*, its upstream negative regulator *Morus alba* L. *CONSTITUTIVE TRIPLE RESPONSE1* (*MaCTR1*), and the ET biosynthesis key gene *Morus alba* L. *ACC Synthase 1* (*MaACS1*) across six representative developmental stages of pistillate flowers and fruits.

MaACS1, *MaCTR1* and *MaEIN2* all exhibited an overall increase followed by a decline during pistillate flower development and fruit ripening, although their expression peaks occurred at different stages. Among these, *MaCTR1*, an upstream negative regulator of *MaEIN2*, peaked at early fruit set (S4), when fruits begin turning from green to red, and then decreased significantly during further maturation. In

contrast, *MaACS1* and *MaEIN2* reached their highest transcript levels at the mid-fruit stage (S5), when drupelets change from red to dark purple, followed by a significant decline at full ripening (S6) (Fig. 1b). This pattern is largely consistent with the reported variation in ET content in mulberry fruit (Liu et al. 2015). These results suggest that *MaACS1* and *MaEIN2* expression are positively associated with ET accumulation during mulberry fruit development.

Together, the 1-MCP and fruit-development analyses indicate that *MaEIN2* expression is positively associated with ET signaling and/or ET accumulation in mulberry.

Induction and validation of MaEIN2-VIGS seedlings

To investigate *MaEIN2* function in mulberry, we used a TRV-based VIGS approach and constructed the pNC-TRV2-eGFP-*MaEIN2*-CEND plasmid (*MaEIN2*-VIGS) (Figs. S6 and S7). The *MaEIN2*-CEND region containing the putative S645/F646 sites was selected as the silencing target. Seedlings expressing eGFP were visually monitored to assess VIGS efficiency, as infected tissues exhibited green fluorescence. From day 4 to day 14 post-infection, clear fluorescence was observed in leaves of both EV and *MaEIN2*-VIGS seedlings (Fig. 2a), indicating successful expression of the eGFP marker and suggesting that the VIGS system became active approximately 4 days after infection.

VIGS of MaEIN2 compromises mulberry seedling resistance to recombinant A. tumefaciens carrying TRV-based plasmids

Phenotypic observation of mulberry seedlings after Agroinfiltration

Phenotypic changes in mulberry seedlings treated with transformation buffer (WT) or infected with recombinant *A. tumefaciens* were monitored for 14 days. WT seedlings injected with transformation buffer showed no obvious phenotypic changes and grew normally. In contrast, both EV and *MaEIN2*-VIGS seedlings showed damage symptoms after injection with recombinant *A. tumefaciens*. Compared with EV seedlings, *MaEIN2*-VIGS seedlings exhibited enhanced susceptibility to *A. tumefaciens* carrying TRV-based plasmids.

From day 8 post-infection, middle and upper leaves of *MaEIN2*-VIGS seedlings displayed visible damage symptoms, including water-soaked spots and chlorosis. By day 12, apical growth had ceased, and several axillary buds began to sprout (Fig. 2b). By day 14, plant growth was markedly retarded, and some leaves turned yellow, curled and abscised. EV seedlings treated with the same concentration of *A. tumefaciens* suspension exhibited milder leaf damage, with less severe water-soaked spots and chlorosis and no obvious wilting or leaf drop by day 14 (Fig. 2c).

Virus infection itself can interfere with plant stress responses (Ramegowda et al. 2014). During VIGS, mulberry seedlings are exposed to two biotic stress components: *A. tumefaciens* inoculation and subsequent TRV infection. Each factor can function as a pathogen-associated stress. Symptoms such

as water-soaked spots, growth retardation, leaf curling, chlorosis and wilting may therefore arise from mechanical damage during agroinfiltration, *A. tumefaciens* infection and TRV-induced stress.

Under infection with recombinant *A. tumefaciens* suspensions of identical concentration but carrying different plasmids, *MaEIN2*-VIGS seedlings exhibited lower tolerance to these combined pathogen-associated stresses than EV seedlings. Thus, silencing *MaEIN2* increased the sensitivity of mulberry seedlings to recombinant *A. tumefaciens* carrying TRV-based plasmids.

Stomatal morphology

Stomata are an important first line of defense against pathogens, and plants can regulate stomatal opening and morphology in response to environmental cues. Stomatal closure can reduce water loss and delay leaf wilting, representing an active adaptation to biotic stress (Peng et al., 2022; Meddya et al. 2023). To assess physiological differences among WT, EV and *MaEIN2*-VIGS seedlings after *A. tumefaciens* infection, leaves were collected at day 12 post-infection, when fluorescence was most pronounced, and stomatal morphology was examined by FESEM (Fig. 2d).

Quantitative analysis of stomatal aperture, calculated as the ratio of stomatal width to length, showed no significant difference between WT and EV seedlings. In contrast, stomatal aperture in *MaEIN2*-VIGS leaves was significantly lower than in WT and EV leaves, with average reductions of 38.33% and 35.23%, respectively (Fig.S8). Several stomata in *MaEIN2*-VIGS leaves appeared nearly closed. Because stomatal closure is closely associated with plant metabolism and pathogen-induced stress, *MaEIN2*-CEND knockdown may trigger a complex physiological response that alters stomatal morphology and function. Although reduced stomatal aperture can contribute to stress adaptation (Meddya et al. 2023), this response did not prevent wilting and premature leaf drop in *MaEIN2*-VIGS seedlings.

ET can restrict pathogen spread by promoting leaf abscission when disease symptoms intensify (Geraats et al. 2002; van Loon et al. 2006). In *MaEIN2*-VIGS seedlings, axillary bud sprouting accompanied leaf damage, suggesting that altered ET signaling under *MaEIN2* knockdown may activate leaf-abscission and regrowth responses.

In summary, *MaEIN2* silencing reduced mulberry seedling resistance to infection by *A. tumefaciens* carrying TRV plasmids. This phenotype was associated with reduced stomatal aperture, wilting, premature leaf drop and activation of axillary bud growth.

MaEIN2 silencing alters the expression of ET-signaling genes in mulberry

To determine whether the reduced pathogen-stress tolerance of *MaEIN2*-VIGS seedlings was associated with *MaEIN2* knockdown, *MaEIN2* transcript levels were monitored dynamically in leaves of WT, EV and *MaEIN2*-VIGS seedlings at different post-infection time points.

In WT and EV seedlings, *MaEIN2* expression fluctuated only slightly from day 4 to day 14. In the EV group, *MaEIN2* expression at days 6, 8, 10 and 12 was higher than in WT seedlings at the corresponding

time points. In *MaEIN2*-VIGS seedlings, *MaEIN2* expression decreased continuously from day 6 post-infection, reached its minimum at day 12, and partially recovered by day 14. Thus, *MaEIN2* silencing was most effective at approximately 12 days after infection (Fig. 3a).

These results suggest that altered *MaEIN2* transcription, and the accompanying changes in ET signaling, are likely to contribute to the phenotypic differences among WT, EV and *MaEIN2*-VIGS seedlings under pathogen-associated stress. *MaEIN2* silencing may weaken mulberry defense responses against pathogens such as *A. tumefaciens*.

Because EV and *MaEIN2*-VIGS seedlings showed distinct phenotypic responses to *A. tumefaciens* infection, we next focused on the expression patterns of *MaEIN2* and its upstream and downstream ET-signaling genes in these two groups.

Leaves were collected at day 12 post-infection, coinciding with the lowest *MaEIN2* expression level. We analyzed the transcript abundance of *MaEIN2*, the upstream genes *MaACS1* and *MaCTR1*, and the downstream effectors *Morus alba L. Ethylene Insensitive 3* (*MaEIN3*) and *Morus alba* L. *ethylene response factor 1* (*MaERF1*).

Under the same concentration of *A. tumefaciens* infection, *MaEIN2* silencing at day 12 was accompanied by significant upregulation of the upstream genes *MaACS1* and *MaCTR1*, whereas the downstream genes *MaEIN3* and *MaERF1* were markedly downregulated compared with the EV group (Fig. 3b). *MaACS1* is closely associated with ET biosynthesis in mulberry (Liu et al. 2014; Liu et al. 2015), its upregulation under *MaEIN2*-VIGS conditions resembles the increase in *OsACS1* transcription observed in rice plants carrying *OsEIN2* antisense constructs (Jun et al. 2004). This similarity suggests that, as in rice, the knockdown of *MaEIN2* in mulberry may trigger a certain feedback regulatory effect on ET synthesis. However, this feedback was insufficient to restore *MaEIN2* transcript levels or rescue the reduced resistance of *MaEIN2*-VIGS seedlings to TRV-based *A. tumefaciens* infection.

CTR1 is widely recognized as a negative regulator of ET signaling. However, Park et al. (2023) showed that CTR1 can dissociate from ET receptors and relocate to the nucleus, where nuclear-localized CTR1 stabilizes EIN3 through an unknown mechanism and enhances ET responses. This finding highlights the complexity of intracellular ET signaling (Park et al. 2023). Whether the strong increase in *MaCTR1* mRNA under *MaEIN2*-CEND knockdown results in enhanced *MaCTR1* nuclear accumulation remains unknown.

EIN3/EIL1 and ERFs participate in plant growth, development, environmental adaptation and stress resistance (Guo and Ecker 2003; Dietz et al. 2010; Liu et al. 2019; Zhang et al. 2026), and ERF1 contributes to defense responses against pathogen attack (Fang et al. 2019). In mulberry, AP2/ERF family members associated with stress responses, including *MaERF1* and *MnEIL3* (a homolog of *MaEIN3*), have been identified and characterized (Shang et al. 2014, Liu et al. 2019; Dou et al. 2024). The downregulation of *MaEIN2*, together with upregulation of its upstream negative regulator *MaCTR1*, may therefore contribute to the reduced expression of *MaEIN3* and *MaERF1*. Downregulation of these stress-

related downstream genes may ultimately underlie the poor tolerance of *MaEIN2*-VIGS seedlings to TRV-based *A. tumefaciens* infection.

MaEIN2 silencing reduces ChA accumulation in mulberry

Transcriptome sequencing of MaEIN2-VIGS seedlings

To further explore *MaEIN2* function, we examined transcriptomic changes associated with *MaEIN2* silencing. Because EV and *MaEIN2*-VIGS seedlings differed in phenotype and stomatal morphology under the same concentration of recombinant *A. tumefaciens* infection, we compared the transcriptomes of these two groups. Leaf samples from EV and *MaEIN2*-VIGS seedlings at day 12 post-infection were submitted to BGI Genomics Co., Ltd. for transcriptome sequencing.

After transcriptome sequencing and alignment to the *Morus notabilis* reference genome, 1,272 differentially expressed genes (DEGs) were identified between EV and *MaEIN2*-VIGS seedlings. KEGG pathway enrichment analysis showed that DEGs were primarily enriched in metabolic pathways and biosynthesis of secondary metabolites, with 294 and 222 DEGs in these categories, respectively (Fig. 3c). A total of 52 DEGs were associated with plant-pathogen interaction and were also annotated under environmental adaptation. Notably, nine DEGs were involved in phenylpropanoid biosynthesis, indicating that *MaEIN2*-CEND silencing may alter phenylpropanoid-derived secondary metabolism in mulberry cells.

Determination of ChA content in MaEIN2-VIGS seedlings

Plant secondary metabolites, including flavonoids and ChA-related phenolics, are closely associated with resistance to plant pathogens (Kai et al. 2021; Cheng et al. 2022). In mulberry, enhanced resistance to mulberry ring rot disease has been linked to enrichment of phenylpropanoid-biosynthesis pathways (Qian et al. 2024).

Because the phenylpropanoid pathway provides the biosynthetic foundation for many phenolic compounds, including ChA, and contributes to diverse stress responses (Vogt 2010; Yadav et al. 2020), we next measured ChA in the same leaf samples used for transcriptome sequencing (day 12 post-infection). ChA content was determined using methods modified from Sugiyama et al. (2017) and Zhou et al. (2018) (Sugiyama et al. 2017; Zhou et al. 2018) (Fig.S9).

ChA content was significantly lower in *MaEIN2*-VIGS leaves than in either WT or EV leaves. In contrast, EV leaves accumulated significantly more ChA than WT leaves (Fig. 4a). These results demonstrate that *MaEIN2* knockdown decreases ChA accumulation in mulberry leaves.

ChA is a common phenolic compound synthesized through the phenylpropanoid pathway and is widely distributed in plants, where it contributes to pathogen-stress resistance (He et al., 2025). *A. tumefaciens* infection with TRV-based plasmids therefore represents a biotic stress that may stimulate ChA accumulation, as reflected by the higher ChA content in EV leaves than in WT leaves. The markedly reduced ChA content in *MaEIN2*-VIGS leaves suggests that downregulation of *MaEIN2* disrupts ET-

associated regulation of ChA synthesis, thereby contributing to the reduced tolerance of *MaEIN2*-VIGS seedlings to *A. tumefaciens* carrying TRV plasmids.

Together, these findings support the hypothesis that ET signaling contributes to pathogen defense by modulating ChA accumulation. *MaEIN2* knockdown reduced expression of the stress-related genes *MaEIN3* and *MaERF1*, which was accompanied by lower ChA accumulation and weakened defense against recombinant *A. tumefaciens* carrying TRV-based plasmids. Whether ChA is a primary metabolite underlying mulberry resistance to diverse natural biotic stresses, including pathogens, viruses and insect feeding, remains to be determined.

Silkworm feeding promotes MaEIN2 expression and ChA accumulation in mulberry leaves

ChA accumulation in mulberry after silkworm feeding

ChA is a well-studied phenolic compound with direct and multifaceted roles in chemical defense against insect herbivores (Kundu and Vadassery 2019). The domesticated silkworm, which has been selected for thousands of years from wild silkworms, is a specialist herbivore whose exclusive food source is mulberry leaves. We therefore hypothesized that ChA content in mulberry leaves changes after silkworm feeding.

To further test the relationship among *MaEIN2* transcript abundance, ChA accumulation and mulberry resistance to biotic stress, we simulated herbivory by allowing silkworm larvae to crawl on and feed from mulberry leaves (Fig. 4b). We then quantified transcript levels of *MaEIN2*, the upstream genes *MaACS1* and *MaCTR1*, and the downstream genes *MaEIN3* and *MaERF1*. ChA content was also measured in leaves after 2 h of silkworm feeding.

ChA content in mulberry leaves gnawed by silkworms for 2 h was significantly higher than in unfed WT leaves, demonstrating that silkworm feeding strongly enhances ChA accumulation in mulberry leaves (Fig. 4a).

MaEIN2 expression in response to silkworm feeding

Expression analysis showed that the 2 h silkworm-feeding treatment produced a pattern opposite to that observed under *MaEIN2*-VIGS conditions. *MaEIN2* and the downstream genes *MaEIN3* and *MaERF1* were significantly upregulated, whereas the upstream genes *MaACS1* and *MaCTR1* were markedly downregulated (Fig. 4c). These results suggest that upregulation of *MaEIN2* downstream regulators, particularly the stress- and metabolism-associated transcription factor *MaERF1*, may contribute to enhanced ChA synthesis. Because these genes are central components of ET signaling, ET signaling is likely involved in ChA biosynthesis in mulberry leaves, with *MaEIN2* acting as a critical hub in ET-mediated ChA accumulation.

Discussion

MaEIN2 is essential for ET regulation in mulberry

Despite being domesticated for thousands of years on mulberry leaves, the effects of silkworm feeding behavior on these leaves remain largely unexplored. ET has been widely demonstrated to be involved in stress responses and the synthesis of secondary metabolites in plants, including ChA. Thus, we attempted to establish the molecular mechanism by which the *MaEIN2* gene of interest might influence ChA accumulation in mulberry leaves resulting from the feeding behavior of silkworms.

As an ER-localized protein, EIN2 plays a central role in relaying the ET signal from ER perception to the nucleus in *Arabidopsis thaliana* (Guo and Ji 2013). Translocation from the ER to the nucleus of the EIN2-CEND is sufficient to constitutively activate ET response (Alonso et al. 1999; Qiao et al. 2012; Zheng and Zhu 2016).

In this study, the isolated *MaEIN2*-CEND region contained putative *MaCTR1*-associated S645/F646 sites corresponding to conserved sites in *Arabidopsis* EIN2. *MaEIN2* expression in mature mulberry leaves was significantly suppressed by the ET-perception inhibitor 1-MCP, and *MaEIN2* expression during fruit development was positively associated with ET accumulation.

Silencing *MaEIN2*-CEND by VIGS significantly altered the expression of the upstream regulators *MaACS1* and *MaCTR1* and the downstream effectors *MaEIN3* and *MaERF1*. These changes indicate that *MaEIN2* silencing can trigger feedback regulation of ET biosynthesis and reprogram ET-signaling gene expression in mulberry.

Collectively, these results indicate that *MaEIN2* is an essential component of ET-signaling regulation in mulberry and likely functions as a central node within the ET signal-transduction pathway. The *MaEIN2*-CEND region appears to have an important regulatory role in this process.

MaEIN2 contributes to pathogen-stress resistance through ChA accumulation

TRV-derived plasmids are frequently used in dicots because they generally cause mild symptoms in host plants (Valentine et al. 2004). However, TRV-based VIGS also has limitations. Because VIGS depends on pathogen-host interactions, inoculating plants with a virus can alter plant development, including plant height and leaf morphology. Although TRV efficiently infects meristematic tissues and is broadly used in dicots, TRV-infected plants may still display host-damage symptoms such as moderate stunting (Lu et al. 2003, Burch-Smith et al. 2004; Unver and Budak 2009; Ramegowda et al. 2014; Ma et al. 2015).

In dicotyledonous mulberry, the TRV-based plasmid induced symptoms such as leaf curling during VIGS, similar to previous reports (Ma et al. 2015). In addition, the TRV-based VIGS system relies on wound-inoculated *Agrobacterium* to deliver viral vectors into plant cells. *A. tumefaciens* is a pathogen of many dicotyledonous species; thus, agroinfiltration represents a transient pathogen attack that can elicit chlorosis and cell necrosis in infected host leaves (Du et al. 2014). Consequently, mulberry plants subjected to VIGS may experience dual biotic stress from both TRV-based plasmids and *A. tumefaciens*.

In our study, *MaEIN2*-VIGS seedlings exhibited greater susceptibility to *A. tumefaciens* carrying TRV-based plasmids than EV seedlings, with more severe leaf curling, yellowing, stomatal closure and induction of axillary bud growth. These phenotypes suggest that *MaEIN2* silencing disrupts ET-mediated defense responses and weakens mulberry resistance to pathogen-associated stress.

In rice, *ein2* mutants exhibit reduced ChA content and lower ROS-scavenging capacity than WT plants (Chen et al. 2022). Consistently, our data showed that *MaEIN2*-VIGS significantly reduced ChA accumulation in mulberry leaves and impaired seedling tolerance to pathogen-associated stress. ChA is known for its antibiotic and anti-stress properties (Jiao et al. 2018; Kundu and Vadassery 2019; Zhang et al. 2020a; Kai et al. 2021; Cheng et al. 2022). We therefore propose that *MaEIN2* is a potential target for enhancing pathogen-stress resistance in mulberry and that the reduced stress tolerance of *MaEIN2*-VIGS seedlings results, at least in part, from *MaEIN2* transcript knockdown and the associated decline in ChA accumulation.

Nevertheless, current evidence does not indicate that EIN2 or ET directly regulates ChA biosynthesis. Under *MaEIN2*-VIGS conditions, the downstream genes *MaEIN3* and *MaERF1*, which are associated with stress responses and secondary-metabolite regulation, were significantly downregulated, and ChA accumulation decreased markedly. These findings suggest that *MaEIN2* does not directly catalyze or control ChA synthesis but instead acts through an indirect ET-signaling cascade.

Taken together, our results suggest that *MaEIN2* contributes to pathogen-induced defense responses in a ChA-dependent manner in mulberry.

MaEIN2 confers silkworm-feeding-induced ChA accumulation.

Silkworm feeding enhances MaEIN2 expression and ChA accumulation in mulberry leaves

ChA is the dominant phenolic constituent in mulberry leaves (Jarienė et al. 2019; Can et al. 2021). High ChA concentrations in leaves promote feeding in silkworm larvae and correlate positively with growth parameters, particularly during early larval stages, and can influence cocoon formation and silk quality (Yamagishi et al. 2018; Šelih et al. 2020). Although ChA has anti-herbivore activity against many insects, the domesticated silkworm appears to have adapted to this compound during long-term domestication and may even exploit it for growth and development.

Here, silkworm feeding significantly increased ChA accumulation in mulberry leaves. This result suggests a dual interpretation: mulberry leaves may respond to silkworm feeding by enhancing ChA synthesis as part of an anti-herbivore response, while silkworms may benefit from the increased ChA that feeding induces. These findings further support a close co-evolutionary relationship between mulberry and the domesticated silkworm.

Silkworm-feeding-induced stress may be mediated through MaEIN2-regulated ET signaling, leading to increased ChA production in mulberry leaves.

Our experiments showed that silkworm feeding significantly enhanced *MaEIN2* expression, accompanied by marked upregulation of *MaEIN3* and *MaERF1*, suggesting that ET signaling may indirectly regulate ChA synthesis.

Damage caused by silkworm larval feeding is a form of biotic stress to mulberry leaves. As discussed above, both ChA and ET participate in plant biotic-stress responses. In plants, the pathway from ET biosynthesis to perception and downstream stress responses depends on the transcriptional regulation of numerous genes and forms a complex signaling network (Li and Guo, 2007; Wang et al. 2013).

ET is not directly involved in the biosynthesis of ChA. However, ChA treatment can reduce ET synthesis in apple, suggesting potential competition or feedback between ET and ChA metabolism (Xi et al. 2016). In other contexts, ET can induce ChA-derivative accumulation in chamomile, indicating that ET may regulate ChA metabolism indirectly (Petrulova et al. 2020). Under biotic stress, ET and ChA may also act synergistically, because strengthened ET signaling often promotes phenolic or ChA-related accumulation (Li et al. 2022; Xu et al. 2017; Tosetti et al. 2020). Whether ET and ChA act cooperatively or competitively in mulberry remains unresolved.

ET is generally thought to regulate plant defense through EIN3/EIL1-controlled ERFs, key transcription factors in the ET-signaling pathway (Thirugnanasambantham et al. 2015). ERFs are also important integrators that control defense-related metabolites, including ChA (Xu et al. 2022; Ma et al. 2024). Although ET and ChA arise from distinct biosynthetic pathways and phenylalanine is not a major precursor in ET biosynthesis, EIN2-regulated ERFs may form a key regulatory node that coordinates ET signaling with ChA-mediated defense. The phenylpropanoid pathway provides the biosynthetic foundation for phenolic compounds, including ChA. In tobacco, ET signaling activates ERFs that bind to promoters of phenylpropanoid-pathway genes and thereby promote ChA biosynthesis. ET signaling may also target key biosynthetic enzymes such as phenylalanine ammonia-lyase (PAL) to increase ChA production and improve biotic-stress resistance (Xu et al. 2022; He et al. 2024; Zhong et al. 2025).

On the basis of our results, ChA biosynthesis in mulberry may be regulated by a cascade within the ET-signaling pathway. Under biotic stresses such as *Agrobacterium* infection or silkworm feeding, mulberry adjusts the expression of the core transducer *MaEIN2*, which in turn affects the downstream transcription factor *MaERF1*. *MaERF1* may then directly or indirectly modulate key enzyme genes in ChA biosynthesis, ultimately influencing ChA accumulation.

Although silkworm feeding and *Agrobacterium* infection represent distinct forms of biotic stress, both induced responses that converge on the ET-signaling cascade, in which *MaEIN2* likely serves as a central regulatory node. Overall, *MaEIN2* is a pivotal target gene contributing to ET-regulated ChA accumulation.

Future work should determine how *MaEIN2* regulates *MaERF1* and whether *MaERF1* modulates enzymes such as PAL to control ChA synthesis. Such studies will clarify how silkworm feeding enhances ChA levels in mulberry leaves.

Based on these findings, we propose a model for *MaEIN2*-mediated ChA accumulation in mulberry in response to TRV-based *A. tumefaciens* infection and silkworm feeding (Fig. 5).

In EV seedlings, infection with *A. tumefaciens* harboring the pNC-TRV2-eGFP plasmid may promote translocation of cleaved *MaEIN2*-CEND fragments from the cytoplasm to the nucleus under pathogen-associated stress. This translocation would suppress *MaEBF1/2*-mediated ubiquitin-proteasome degradation of *MaEIN3*, promoting *MaEIN3* accumulation and transcriptional reprogramming in the nucleus, including activation of downstream *MaERF1* expression. Increased *MaERF1* expression may activate downstream effectors associated with ChA biosynthesis, thereby enhancing ChA accumulation. Elevated ChA may then contribute to increased mulberry leaf resistance to *A. tumefaciens* infection.

In *MaEIN2*-VIGS seedlings, infection with *A. tumefaciens* carrying pNC-TRV2-eGFP-*MaEIN2*-CEND induces degradation of *MaEIN2* mRNA. Consequently, only limited amounts of cleaved *MaEIN2*-CEND are expected to translocate to the nucleus. Under this condition, *MaEBF1/2*-mediated ubiquitin-proteasome degradation of *MaEIN3* may be enhanced, leaving insufficient MaEIN3 to activate downstream *MaERF1*. Reduced *MaERF1* expression may restrict downstream effectors involved in ChA biosynthesis, resulting in low ChA production and enhanced susceptibility to *A. tumefaciens*.

Under silkworm feeding, herbivory stress alters ET signaling and significantly upregulates *MaEIN2* expression. This may increase nuclear translocation of cleaved *MaEIN2*-CEND fragments, promote EIN3/EIL1 accumulation and activate downstream *MaERF1* expression. Elevated *MaERF1* may then induce ChA biosynthesis, leading to ChA enrichment that may benefit silkworm development while also reflecting a mulberry defense response.

Correlation analyses showed a positive relationship between *MaEIN2* expression and ChA accumulation under biotic stress. Because stable transgenic technology for mulberry remains underdeveloped, the present study examined *MaEIN2* function mainly at the transcriptional level. In addition, VIGS allows *MaEIN2* knockdown rather than the generation of stable *MaEIN2* mutants. Future studies should prioritize efficient mulberry transformation and genome-editing systems to define *MaEIN2* function more precisely.

Nevertheless, *MaEIN2* silencing and silkworm-feeding assays revealed a positive role for *MaEIN2* in ET-associated ChA accumulation in mulberry leaves. This work provides new insight into the relationships among ET signaling, ChA metabolism and silkworm feeding, and offers preliminary evidence that *MaEIN2* contributes to biotic-stress tolerance through ET-dependent regulation of ChA accumulation in mulberry.

Conclusions

In this study, silkworm feeding triggered a marked increase in ChA levels in mulberry leaves and was associated with strong upregulation of *MaEIN2*, a core ET-signaling gene. Using a VIGS approach, we showed that *MaEIN2* silencing substantially reduced ChA accumulation, weakened pathogen-associated stress resistance and downregulated downstream genes potentially involved in ChA biosynthesis. Our results suggest that silkworm-feeding-induced stress is linked to *MaEIN2*-mediated ET signaling and enhanced ChA accumulation in mulberry leaves, a response that may contribute to both mulberry defense and silkworm performance. This study provides preliminary evidence that *MaEIN2* participates in the molecular dialogue between mulberry and silkworm. Future studies using stable *MaEIN2* mutants and mechanistic analysis of ET-signaling components and ChA-biosynthetic enzymes will further clarify the role of *MaEIN2* in silkworm-feeding-induced ChA biosynthesis.

Abbreviations

1-MCP :1-Methylcyclopropene

ChA: chlorogenic acid

ET: ethylene

ER: endoplasmic reticulum

MaEIN2: *Morus alba* L. *ETHYLENE INSENSITIVE 2*

MaCTR1: *Morus alba* L. *CONSTITUTIVE TRIPLE RESPONSE1*

MaACS1: *Morus alba* L. *ACC Synthase 1*

MaEIN3: *Morus alba* L. *ETHYLENE INSENSITIVE 3*

MaEBF1: *Morus alba* L. *EIN3- EIN3-BINDING F-BOX 1*

MaERF1: *Morus alba* L. *ETHYLENE RESPONSE FACTOR 1*

TRV: *Tobacco rattle virus*

VIGS: virus-induced gene silencing

Declarations

Acknowledgements

The authors would like to thank the Silkworm Germplasm Resource Group of the Sericulture Research Center, Chinese Academy of Agricultural Sciences, for providing the silkworms.

Author contributions

R.F. and L.L. designed research and revised manuscript; P. L. and Q. Y. performed research and drafted manuscript; G.Y. and H. L. detected and analyzed ChA; N. C. and M. L analyzed data. P. L. and Q. Y. contributed equally to this work. All authors have read and approved the final version of the manuscript.

Funding

This work was supported by the China Agriculture Research System of the Ministry of Finance and Ministry of Agriculture and Rural Affairs (CARS-18), Crop Germplasm Resources Protection Project of the Ministry of Agriculture and Rural Affairs of the People's Republic of China (22250312), and National Crop Germplasm Resources Center(NCGRC-2025-041).

Data availability

All data generated and analyzed during this study are included in this published article and its supplementary files.

Conflict of interest: The authors declare no competing interests.

References

1. Alonso J.M, Ecker J.R (2001) The Ethylene Pathway: a Paradigm for Plant Hormone Signaling and Interaction. Sci. STKE 70**:**1-10. https://doi.org/10.1126/stke.2001.70.re1
2. Alonso J.M, Hirayama T, Roman G, Nourizadeh S, Ecker JR (1999) EIN2, a Bifunctional Transducer of Ethylene and Stress Responses in *Arabidopsis*. Science 284**:**2148-2152. https://doi.org/10.1126/science.284.5423.2148
3. An F, Zhao Q, Ji Y, Li W, Jiang Z, Yu X, Zhang C, Han Y, He W, Liu Y, Zhang S, Ecker JR, Guo H (2010) Ethylene-Induced Stabilization of ETHYLENE INSENSITIVE3 and EIN3LIKE1 Is Mediated by Proteasomal Degradation of EIN3 Binding F-Box 1 and 2 That Requires EIN2 in *Arabidopsis*. Plant Cell 22**:**2384-2401. https://doi.org/10.1105/tpc.110.076588
4. Angulo M, García MJ, Alcántara E, Pérez-Vicente R, Romera FJ (2021) Comparative Study of Several Fe Deficiency Responses in the *Arabidopsis thaliana* Ethylene Insensitive Mutants *ein2-1* and *ein2-5*. Plants 10**:**1-17. https://doi.org/10.3390/plants10020262
5. Bent AF, Innes RW, Ecker JR, Staskawiz BJ (1991) Disease Development in Ethylene-Insensitive *Arabidopsis thaliana* Infected with Virulent and Avirulent *Pseudomonas* and *Xanthomonas* Pathogens. Mol. Plant-Microbe Interact 5**:**372-378. https://doi.org/10.1094/MPMI-5-372
6. Bisson MMA, Groth G (2010) New Insight in Ethylene Signaling: Autokinase Activity of ETR1 Modulates the Interaction of Receptors and EIN2. Mol Plant 3**:**882-889. https://doi.org/10.1093/mp/ssq036
7. Bleecker AB, Kende H (2000) Ethylene: a gaseous signal molecule in plants. Annu. Rev. Cell Dev. Biol. 16**:**1-18. https://doi.org/10.1146/annurev.cellbio.16.1.1

8. Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP (2004) Applications and advantages of virus-induced gene silencing for gene function studies in plants. Plant J. 39**:**734-746. https://doi.org/10.1111/j.1365-313X.2004.02158.x
9. Can A, Kazankaya A, Orman E, Gundogdu M, Ercisli S, Choudhary R, Karunakaran R (2021) Sustainable mulberry (*Morus nigra* L., *Morus alba* L. and *Morus rubra* L.) production in Eastern Turkey. Sustainability 13**:**1-13. https://doi.org/10.3390/su132413507
10. Cao WH, Liu J, He XJ, Mu RL, Zhou HL, Chen SY, Zhang JS (2007) Modulation of Ethylene Responses Affects Plant Salt-Stress Responses. Plant Physiol. 143**:**707-719. https://doi.org/10.1104/pp.106.094292
11. Chen H, Zhang Q, Lv W, Yu X, Zhang Z (2022) Ethylene positively regulates Cd tolerance via reactive oxygen species scavenging and apoplastic transport barrier formation in rice. Environ. Pollut. 302**:**1-12. https://doi.org/10.1016/j.envpol.2022.119063
12. Cheng Z., Li R, Jiang Z, Tang Y, Li W, Shao Y (2022) Combined effect of *Bacillus siamensis* and chlorogenic acid on maintenance of quality and control of disease in stored wax apple fruit. Food Qual. Saf. 6**:**1-12. https://doi.org/10.1093/fqsafe/fyac026
13. Coletta A, Pinney JW., Solís DYW, Marsh J, Pettifer SR, Attwood TK (2010) Low-complexity regions within protein sequences have position-dependent roles. BMC Syst Biol. 4**:**1-13. https://doi.org/10.1186/1752-0509-4-43
14. Dietz KJ, Vogel MO, Viehhauser A (2010) AP2/EREBP transcription factors are part of gene regulatory networks and integrate metabolic, hormonal and environmental signals in stress acclimation and retrograde signalling. Protoplasma 245**:**3-14. https://doi.org/10.1007/s00709-010-0142-8
15. Dou H, Wang T, Zhou X, Feng X, Tang W, Quan JE, Bi H (2024) Genome-Wide Identification and Expression of the *AP2/ERF* Gene Family in *Morus notabilis*. Forests 15**:**1-18. https://doi.org/10.3390/f15040697
16. Du J, Rietman H, Vleeshouwers VGaA (2014) Agroinfiltration and PVX Agroinfection in Potato and *Nicotiana benthamiana*. J. Vis. Ex.p. 83:1-7. https://doi.org/10.3791/50971
17. Dugardeyn J, Straeten DVD (2008) Ethylene: Fine-tuning plant growth and development by stimulation and inhibition of elongation. Plant Sci. 175:59-70. https://doi.org/10.1016/j.plantsci.2008.02.003
18. Fang RJ, Li AQ, Tian RN, Zhang WJ, Zou AL, Wu FY, Liao YH, Wang XM., Pang YJ, Yang RW (2019) Heterologous overexpression of *Lithospermum erythrorhizon LeERF-1* gene increases drought and pathogen resistance in *Arabidopsis*. Acta Physiol. Plant 41:1-9. https://doi.org/10.1007/s11738-019-2807-z
19. Geraats BP, Bakker PA, Van Loon L (2002) Ethylene insensitivity impairs resistance to soilborne pathogens in tobacco and *Arabidopsis thaliana*. Mol. Plant-Microbe Interact 15**:**1078-1085. https://doi.org/10.1094/MPMI.2002.15.10.1078

20. Guo H, Ecker J (2004) The ethylene signaling pathway: new insights. Curr. Opin. Plant Biol. 7:40-49. https://doi.org/10.1016/j.pbi.2003.11.011
21. Guo H, Ecker JR (2003) Plant Responses to Ethylene Gas Are Mediated by $SCF^{EBF1/EBF2}$-Dependent Proteolysis of EIN3 Transcription Factor. Cell 115:667-677. https://doi.org/10.1016/S0092-8674(03)00969-3
22. Guo H, Ji Y (2013) From Endoplasmic Reticulum (ER) to Nucleus: EIN2 Bridges the Gap in Ethylene Signaling. Mol Plant 6**:**11-14. https://doi.org/10.1093/mp/sss150
23. Hao D, Li W, Guo H (2025) Ethylene signaling in *Arabidopsis*: a journey from historical discoveries to modern insights. Plant Horm. 1**:**1-15. https://doi.org/10.48130/ph-0025-0015
24. He S, Xu X, Gao Q, Huang C, Luo Z, Liu P, Wu M, Huang H, Yang J, Zeng J, Wang Z (2024) *NtERF4* promotes the biosynthesis of chlorogenic acid and flavonoids by targeting *PAL* genes in *Nicotiana tabacum*. Planta 259**:**1-16. https://doi.org/10.1007/s00425-023-04301-1
25. He Y, Mao S, Zhao Y, Yang J (2025) Research Advances in the Synthesis, Metabolism, and Function of Chlorogenic Acid. Foods 14**:**1-21. https://doi.org/10.3390/foods14111914
26. Hussain S, Bai Z, Huang J, Cao X, Zhu L, Zhu C, Khaskheli MA, Zhong C, Jin Q, Zhang J (2019) 1-Methylcyclopropene Modulates Physiological, Biochemical, and Antioxidant Responses of Rice to Different Salt Stress Levels. Front Plant Sci. 10**:**1-18. https://doi:10.3389/fpls.2019.00124
27. Hussain S, Zhong C, Bai Z, Cao X, Zhu L, Hussain A, Zhu C, Fahad S, James AB, Zhang J (2018) Effects of 1-Methylcyclopropene on Rice Growth Characteristics and Superior and Inferior Spikelet Development Under Salt Stress. J. Plant Growth Regul. 37:1368-1384. https://doi.org/10.1007/s00344-018-9800-4
28. Jarienė E, LevickienėD., Danilčenko H, Vaitkevičienė N, Kulaitienė J, Jakštas V, Ivanauskas L, Gajewski M (2019) Effects of biodynamic preparations on concentration of phenolic compounds in the leaves of two white mulberry cultivars. Biol. Agric. Hortic. 35**:**132-142. https://doi.org/10.1080/01448765.2018.1535329
29. Jiao W, Li X, Wang X, Cao J, Jiang W (2018) Chlorogenic acid induces resistance against *Penicillium expansum* in peach fruit by activating the salicylic acid signaling pathway. Food Chem. 260**:**274-282. https://doi.org/10.1016/j.foodchem.2018.04.010
30. Ju C, Chang C (2015) Mechanistic Insights in Ethylene Perception and Signal Transduction. Plant physiol. 169**:**85-95. https://doi.org/10.1104/pp.15.00845
31. Ju C, Yoon GM, Shemansky JM, Lin DY, Ying ZI, Chang J, Garrett WM, Kessenbrock M, Groth G, Tucker ML, Coopere B, Kieberb JJ, Chang C (2012) CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in *Arabidopsis*. Proc. Natl. Acad. Sci. USA 109**:**19486-19491. https://doi.org/10.1073/pnas.1214848109
32. Jun S.H, Han M.J, Lee S, Seo Y.S, Kim WT, An G (2004) OsEIN2 is a positive component in ethylene signaling in rice. Plant Cell Physiol. 45**:**281-289. https://doi.org/10.1093/pcp/pch033
33. Kai K, Wang R, Bi W, Ma Z, Shi W, Ye Y, Zhang D (2021) Chlorogenic acid induces ROS-dependent apoptosis in Fusarium fujikuroi and decreases the postharvest rot of cherry tomato. World

J.Microbiol. Biotechnol. 37:1-9. https://doi.org/10.1007/s11274-021-03062-x

34. Kieber J, Rothenberg M, Roman G, Feldmann K, Ecker J (1993) *CTR1*, a Negative Regulator of the Ethylene Response Pathway in *Arabidopsis*, Encodes a Member of the Raf Family of Protein Kinases. Cell 72:427-441. https://doi.org/10.1016/0092-8674(93)90119-B
35. Kundu A, Vadassery J (2019) Chlorogenic acid-mediated chemical defense of plants against insect herbivores. Plant Biol. 21:185-189. https://doi.org/10.1111/plb.12947
36. Lei G, Shen M, Li ZG, Zhang B, Duan KX, Wang N, Cao YR, Zhang WK, Ma B, Ling HQ (2011) EIN2 regulates salt stress response and interacts with a MA3 domain-containing protein ECIP1 in *Arabidopsis*. Plant Cell Environt. 34:1678-1692. https://doi.org/10.1111/j.1365-3040.2011.02363.x
37. Li H, Guo H (2007) Molecular basis of the ethylene signaling and response pathway in *Arabidopsis*. J. Plant Growth Regul. 26:106-117. https://doi.org/10.1007/s00344-007-0015-3
38. Li X, Li B, Li M, Fu X, Zhao XX, Min D, Li F, Zhang X (2022) Ethylene pretreatment induces phenolic biosynthesis of fresh-cut pitaya fruit by regulating ethylene signaling pathway. *Postharvest Biol. Technol.* 192:1-9. https://doi.org/10.1016/j.postharvbio.2022.112028
39. Liu CY, LÜ RH, LI J, Zhao AC, Wang XL, Diane U, Wang XH., Wang CH., Yu YS, Han SM, Lu C, Yu MD. (2014) Characterization and expression profiles of *MaACS* and *MaACO* genes from mulberry (*Morus alba* L.). J. Zhejiang Univ. Sci. B 15:611-623. https://doi.org/10.1631/jzus.B1300320
40. Liu C, Li J, Zhu P, Yu J, Hou J, Wang C, Long D, Yu M, Zhao A. (2019) Mulberry EIL3 confers salt and drought tolerances and modulates ethylene biosynthetic gene expression. *PeerJ.* 7:1-17. https://doi.org/10.7717/peerj.6391
41. Liu C, Zhao A, Zhu P, Li J, Han L, Wang X, Fan W, LüR, Wang C, Li Z (2015) Characterization and Expression of Genes Involved in the Ethylene Biosynthesis and Signal Transduction during Ripening of Mulberry Fruit. Plos One 10:e0122081. https://doi.org/10.1371/journal.pone.0122081
42. Liu X, Wang H, Yue Z, Wang J, Wang J, Dou T, Zheng L, Liu X, Dai H, Yu J (2026) Exogenous hydrogen sulfide enhances the resistance of cabbage seedlings to black rot by improving phenolic synthesis and reducing reactive oxygen species. Plant Mol. Biol. 116:1-14. https://doi.org/10.1007/s11103-026-01683-0
43. Low YC, Lawton MA, Di R (2022) *Ethylene insensitive 2* (*EIN2*) as a potential target gene to enhance *Fusarium* head blight disease resistance. Plant Sci. 322:1-10. https://doi.org/10.1016/j.plantsci.2022.111361
44. Lu R, Martin-Hernandez AM, Peart JR, Malcuit I, Baulcombe DC (2003) Virus-induced gene silencing in plants. Methods 30:296-303. https://doi.org/10.1016/S1046-2023(03)00037-9
45. Ma N, Sun P, Li ZY, Zhang FJ, Wang XF, You CX, Zhang CL, Zhang Z (2024) Plant disease resistance outputs regulated by AP2/ERF transcription factor family. Stress Biol. 4:1-12. https://doi.org/10.1007/s44154-023-00140-y
46. Ma X, Nicole M-C, Meteignier L-V, Hong N, Wang G, Moffett P (2015) Different roles for RNA silencing and RNA processing components in virus recovery and virus-induced gene silencing in plants. J. Exp. Bot. 66:919-932. https://doi.org/10.1093/jxb/eru447

47. Mardini M., Kazantsev M, Ivoilova E, Utkina V, Vlasova A, Kirov I (2023) Simple Seed-vacuum Protocol for *Agrobacterium*-mediated Virus Induced Gene Silencing (VIGS) in Sunflower *Helianthus annuus* L. . protocols.io 1-9. https://doi.org/10.17504/protocols.io.261ged56dv47/v1
48. Meddya S., Meshram S, Sarkar D, Rakesh S, Datta R, Singh S, Avinash G, Kumar Kondeti A, Savani AK, Thulasinathan T (2023) Plant stomata: An unrealized possibility in plant defense against invading pathogens and stress tolerance. Plants 12**:**1-20. https://doi.org/10.3390/plants12193380
49. Mei X, Li P, Wang L, Liu C, Zhou L, Li C, Cai Y (2019) Imprinting, methylation, and expression characterization of the maize ETHYLENEINSENSITIVE 2-like gene. Crop J. 7**:**49-57. https://doi.org/10.1016/j.cj.2018.08.001
50. Park HL, Seo DH, Lee HY, Bakshi A, Park C, Chien YC, Kieber JJ, Binder BM, Yoon GM (2023) Ethylene-triggered subcellular trafficking of CTR1 enhances the response to ethylene gas. Nat. Commun. 14**:**1-15. https://doi.org/10.1038/s41467-023-35975-6
51. Peng J, Li Z, Wen X, Li W, Shi H, Yang L, Zhu H, Guo H, Yu H (2014) Salt-Induced Stabilization of EIN3/EIL1 Confers Salinity Tolerance by Deterring ROS Accumulation in *Arabidopsis*. Plos Genet. 10**:**e1004664. https://doi.org/10.1371/journal.pgen.1004664
52. Peng P, Li R, Chen ZH, Wang Y (2022) Stomata at the crossroad of molecular interaction between biotic and abiotic stress responses in plants. Front. Plant Sci. 13**:**1-14. https://doi.org/10.3389/fpls.2022.1031891
53. Petrulova V, Vilkova M, Kovalikova Z, Sajko M, Repcak M (2020) Ethylene Induction of non-enzymatic metabolic antioxidants in *Matricaria chamomilla*. Molecules 25**:**1-16. https://doi.org/10.3390/molecules25235720
54. Qian Q, Deng X, Mureed S, Gan Y, Xu D, Wang X, Ali H (2024) Integrating transcriptomics and metabolomics to analyze the defense response of *Morus notabilis* to mulberry ring rot disease. Front. Microbiol. 15**:**1-13. https://doi.org/10.3389/fmicb.2024.1373827
55. Qiao H, Shen Z, Huang S-SC, Schmitz RJ, Urich MA, Briggs SP, Ecker JR (2012) Processing and subcellular trafficking of ER-tethered EIN2 control response to ethylene gas. Science 338**:**390-393. https://doi.org/10.1126/science.1225974
56. Ramegowda V, Mysore KS, Senthil-Kumar M (2014) Virus-induced gene silencing is a versatile tool for unraveling the functional relevance of multiple abiotic-stress-responsive genes in crop plants. Front. Plant Sci. 5**:**1-12. https://doi.org/10.3389/fpls.2014.00323
57. Roman G, Lubarsky B, Kieber J, Rothenberg M, Ecker J (1995) Genetic analysis of ethylene signal transduction in *Arabidopsis* thaliana: five novel mutant loci integrated into a stress response pathway. Genetics 139**:**1393-1409. https://doi.org/10.1093/genetics/139.3.1393
58. Šelih M, Mikulič Petkovšek M, Krajnc D, Berčič RL, Urbanek Krajnc A (2020) Screening of leaf metabolites in historical mulberry trees (*Morus alba* L.) from different eco-geographical regions of Slovenia. Trees 34:971-986. https://doi.org/10.1007/s00468-020-01974-z
59. Shang J, Song P, Ma B, Qi X, Zeng Q, Xiang Z, He N (2014) Identification of the mulberry genes involved in ethylene biosynthesis and signaling pathways and the expression of *MaERF-B2-1* and

MaERF-B2-2 in the response to flooding stress. Funct. Integr. Genomics 14**:**767-777. https://doi.org/10.1007/s10142-014-0403-2

60. Shi Y, Zhang Q, Wang L, Du Q, Ackah M, Guo P, Zheng D, Wu M, Zhao W (2022) Functional Characterization of *MaZIP4*, a Gene Regulating Copper Stress Tolerance in Mulberry (*Morus atropurpurea* R.). Life 12**:**1-15. https://doi.org/10.3390/life12091311
61. Sonia G, Peter M (2003) Cross-talk in Plant Hormone Signalling: What Arabidopsis Mutants Are Telling Us. Ann. Bot. 91**:**605-612. https://doi.org/10.1093/aob/mcg064
62. Sugiyama M, Katsube T, Koyama A, Itamura H (2017) Seasonal changes in functional component contents in mulberry (*Morus alba* L.) leaves. Hortic. J. 86**:**534-542. https://doi.org/10.2503/hortj.OKD-053
63. Thirugnanasambantham K, Durairaj S, Saravanan S, Karikalan K, Muralidaran S, Islam VIH (2015) Role of ethylene response transcription factor (ERF) and its regulation in response to stress encountered by plants. Plant Mol. Biol. Rep. 33**:**347-357. https://doi.org/10.1007/s11105-014-0799-9
64. Tosetti R, Elmi F, Pradas I, Cools K, Terry LA (2020) Continuous exposure to ethylene differentially affects senescence in receptacle and achene tissues in strawberry fruit. Front. Plant Sci. 11**:**1-9. https://doi.org/10.3389/fpls.2020.00174
65. Tsuchisaka A, Theologis A (2004) Unique and overlapping expression patterns among the *Arabidopsis* 1-amino-cyclopropane-1-carboxylate synthase gene family members. Plant Physiol. 136**:**2982-3000. https://doi.org/10.1104/pp.104.049999
66. Unver T, Budak H (2009) Virus-induced gene silencing, a post transcriptional gene silencing method. Int. J. Plant Genomics 2009**:**1-9. https://doi.org/10.1155/2009/198680
67. Valentine T, Shaw J, Blok VC, Phillips MS, Oparka KJ, Lacomme C (2004) Efficient Virus-Induced Gene Silencing in Roots Using a Modified Tobacco Rattle Virus Vector. Plant Physiol. 136**:**3999-4009. https://doi.org/10.1104/pp.104.051466
68. van Loon LC, Geraats BP, Linthorst HJ (2006) Ethylene as a modulator of disease resistance in plants. Trends Plant Sci. 11**:**184-191. https://doi.org/10.1016/j.tplants.2006.02.005
69. Vogt T (2010) Phenylpropanoid biosynthesis. Mol. Plant 3**:**2-20. https://doi.org/10.1093/mp/ssp106
70. Wang F, Cui X, Sun Y, Dong C-H (2013) Ethylene signaling and regulation in plant growth and stress responses. Plant Cell Rep. 32**:**1099-1109. https://doi.org/10.1007/s00299-013-1421-6
71. Wen X, Zhang C, Ji Y, Zhao Q, He W, An F, Jiang L, Guo H (2012) Activation of ethylene signaling is mediated by nuclear translocation of the cleaved EIN2 carboxyl terminus. Cell Res. 22**:**1613-1616. https://doi.org/10.1038/cr.2012.145
72. Xi Y, Cheng D, Zeng X, Cao J, Jiang W (2016) Evidences for Chlorogenic Acid — A Major Endogenous Polyphenol Involved in Regulation of Ripening and Senescence of Apple Fruit. Plos One 11**:**e0146940. https://doi.org/10.1371/journal.pone.0146940
73. Xu J, Zhu J, Lin Y, Zhu H, Tang L, Wang X, Wang X (2022) Comparative transcriptome and weighted correlation network analyses reveal candidate genes involved in chlorogenic acid biosynthesis in

sweet potato. Sci. Rep. 12:1-11. https://doi.org/10.1038/s41598-022-06794-4

74. Xu L, Yue Q, Bian Fe, Sun H, Zhai H, Yao Y (2017) Melatonin Enhances Phenolics Accumulation Partially via Ethylene Signaling and Resulted in High Antioxidant Capacity in Grape Berries. Front. Plant Sci. 8:1-12. https://doi.org/10.3389/fpls.2017.01426
75. Yadav V, Wang Z, Wei C, Amo A, Ahmed B, Yang X, Zhang X (2020) Phenylpropanoid pathway engineering: An emerging approach towards plant defense. Pathogens 9:1-25. https://doi.org/10.3390/pathogens9040312
76. Yamagishi T, Endo H, Fukumura K, Nagata S, Hayakawa T, Adegawa S, Kasubuchi M, Sato R (2018) Glucose, some amino acids and a plant secondary metabolite, chlorogenic acid induce the secretion of a regulatory hormone, tachykinin-related peptide, from the silkworm midgut. Peptides 106:21-27. https://doi.org/10.1016/j.peptides.2018.06.004
77. Zhang DY, Wan Y, Hao JY, Hu RZ, Chen C, Yao XH, Zhao WG, Liu ZY, Li L (2018) Evaluation of the alkaloid, polyphenols, and antioxidant contents of various mulberry cultivars from different planting areas in eastern China. Ind. Crops Prod. 122:298-307. https://doi.org/10.1016/j.indcrop.2018.05.065
78. Zhang D, Bi W, Kai K, Ye Y, Liu J (2020a) Effect of chlorogenic acid on controlling kiwifruit postharvest decay caused by *Diaporthe* sp. LWT-Food Sci. Technol. 132:1-8. https://doi.org/10.1016/j.lwt.2020.109805
79. Zhang J, Chen Y, Lu J, Zhang Y, Wen CK (2020b) Uncertainty of EIN2$^{Ser645/Ser924}$ Inactivation by CTR1-mediated Phosphorylation Reveals Complexity of Ethylene Signaling. Plant Commun. 1:1-18. https://doi.org/10.1016/j.xplc.2020.100046
80. Zhang J, Yu M, Guo Y, Li J, Gu G, Hussain G, Ma S, Li F, Xue H, Yang Z (2026) Evolutionary Divergence of an Ethylene-Responsive Transcriptional Cascade Governs a Dose-Dependent Balance between Cotton Fiber Length and Strength. Adv. Sci. 13:1-16. https://doi.org/10.1002/advs.202514154
81. Zhang T, Hou J, Chu H, Guo P, Sang Q, Liu Z, Cao L (2025) Establishment of a Virus-Induced Gene Silencing System in *Abelmoschus manihot* L. Plants 14:1-14. https://doi.org/10.3390/plants14020150
82. Zheng Y, Zhu Z (2016) Relaying the Ethylene Signal: New Roles for EIN2. Trends Plant Sci. 21:2-4. https://doi.org/10.1016/j.tplants.2015.11.007
83. Zhong J, Ran Q, Han Y, Gan L, Dong C (2025) Biosynthetic Mechanisms of Plant Chlorogenic Acid from a Microbiological Perspective. Microorganisms 13:1-21. https://doi.org/10.3390/microorganisms13051114
84. Zhou P, Wang X, Liu P, Huang J, Wang C, Pan M, Kuang Z (2018) Enhanced phenolic compounds extraction from *Morus alba* L. leaves by deep eutectic solvents combined with ultrasonic-assisted extraction. Ind. Crops Prod. 120:147-154. https://doi.org/10.1016/j.indcrop.2018.04.071

Figures

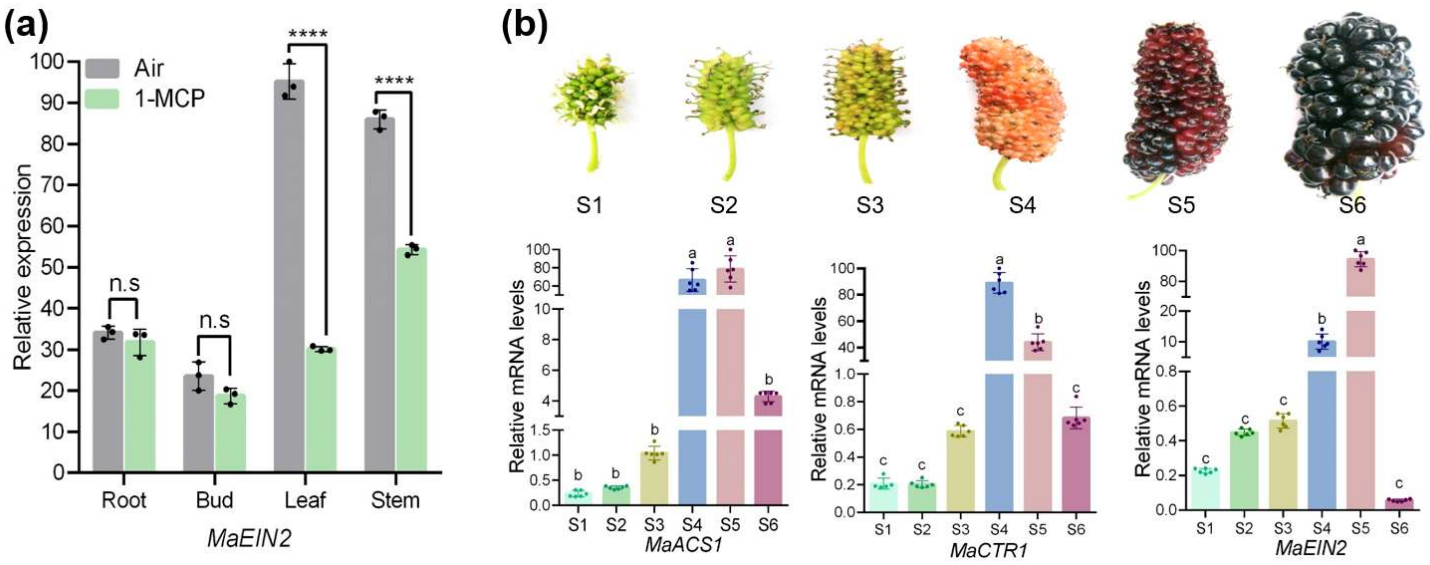

Figure 1

Relationship between *MaEIN2*expression and ET-associated developmental or treatment conditions in mulberry. (**a**) *MaEIN2* expression in roots, buds, leaves and stems under 1-MCP treatment. Significance is indicated by different letters (****$P < 0.0001$). (**b**) Expression profiles of *MaACS1*, *MaCTR1* and *MaEIN2* at different developmental stages of pistillate flowers and fruits: initial flowering (S1), full flowering (S2, unpollinated stigma), end of flowering (S3, pollinated stigma), early fruit set (S4), mid-fruit stage (S5) and full ripening (S6). Significance is indicated by different letters ($P < 0.05$)

Figure 2

Evaluation of mulberry seedlings after VIGS treatment. (**a**) Fluorescence observation at day 4 post-infection. (**b**) Phenotypic changes at day 12 post-infection. (**c**) Leaf phenotypes at day 14 post-infection. Arrows indicate newly sprouted axillary buds or disease spots formed after infection. (**d**) Stomatal morphology in mulberry leaves at day 12 post-infection

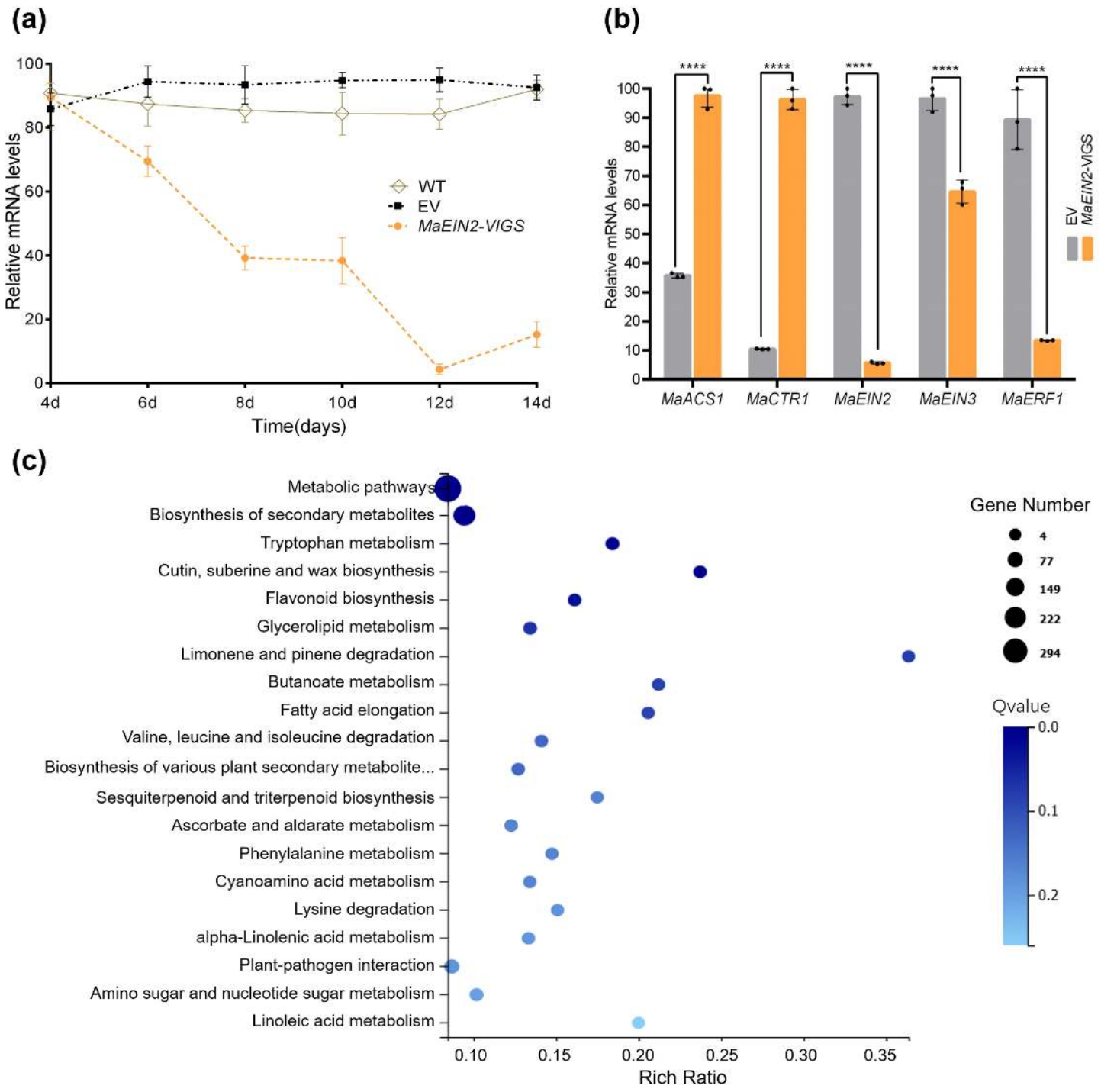

Figure 3

Transcriptional changes in mulberry leaves after *MaEIN2*-VIGS treatment. **(a)** Dynamic expression patterns of *MaEIN2* in mulberry leaves infected with transformation buffer (WT), recombinant *A. tumefaciens* harboring pTRV2-eGFP (EV) or pTRV2-eGFP-*MaEIN2*-CEND (*MaEIN2*-VIGS); **(b)** Expression patterns of *MaEIN2*, *MaACS1*, *MaCTR1*, *MaEIN3* and *MaERF1* in EV and *MaEIN2*-VIGS leaves at day 12 post-infection. Asterisks indicate significant differences between EV and *MaEIN2*-VIGS seedlings (****P <0.0001); **(c)** KEGG pathway enrichment bubble chart of differentially expressed genes between EV and *MaEIN2*-VIGS seedlings at day 12 post-infection

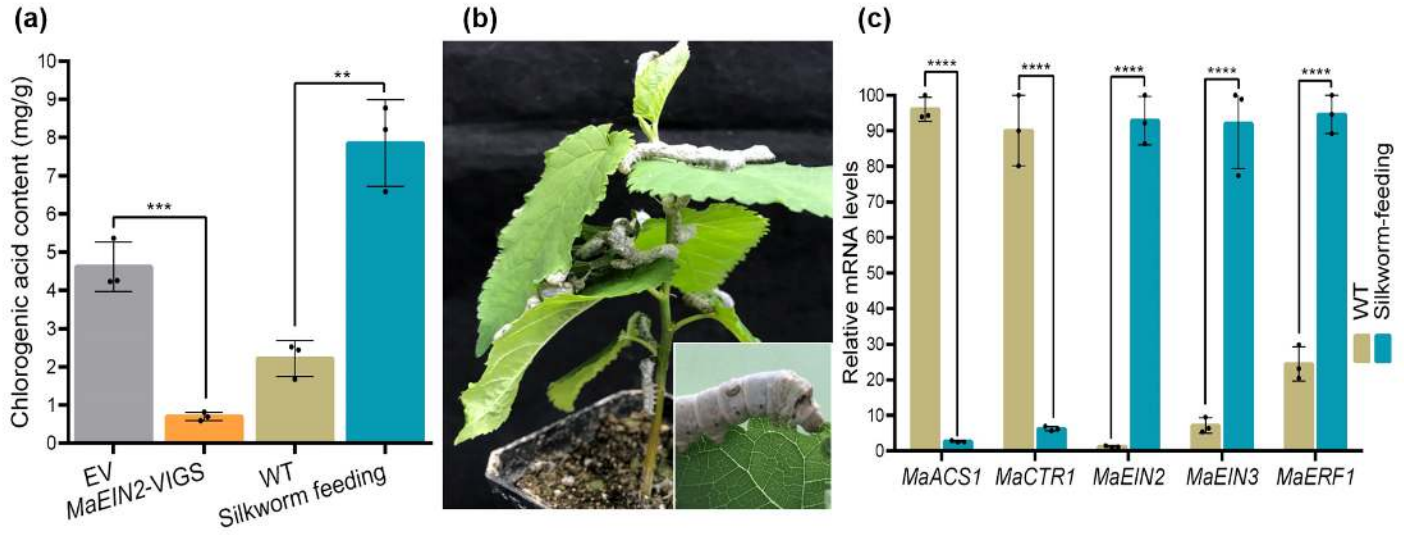

Figure 4

Role of *MaEIN2* in ChA accumulation in mulberry leaves.**(a)** ChA content detection in mulberry leaves (***P* <0.01, ****P* <0.001); **(b)**silkworm-gnawed seedlings; **(c)** Expression patterns of *MaEIN2*, its upstream regulators *MaACS1*, *MaCTR1*, and its downstream targets *MaEIN3* and *MaERF1* in mulberry leaves harvested after 2 h of silkworm feeding. Asterisks indicate significant differences between WT and silkworm-feeding treatments (*****P* <0.0001)

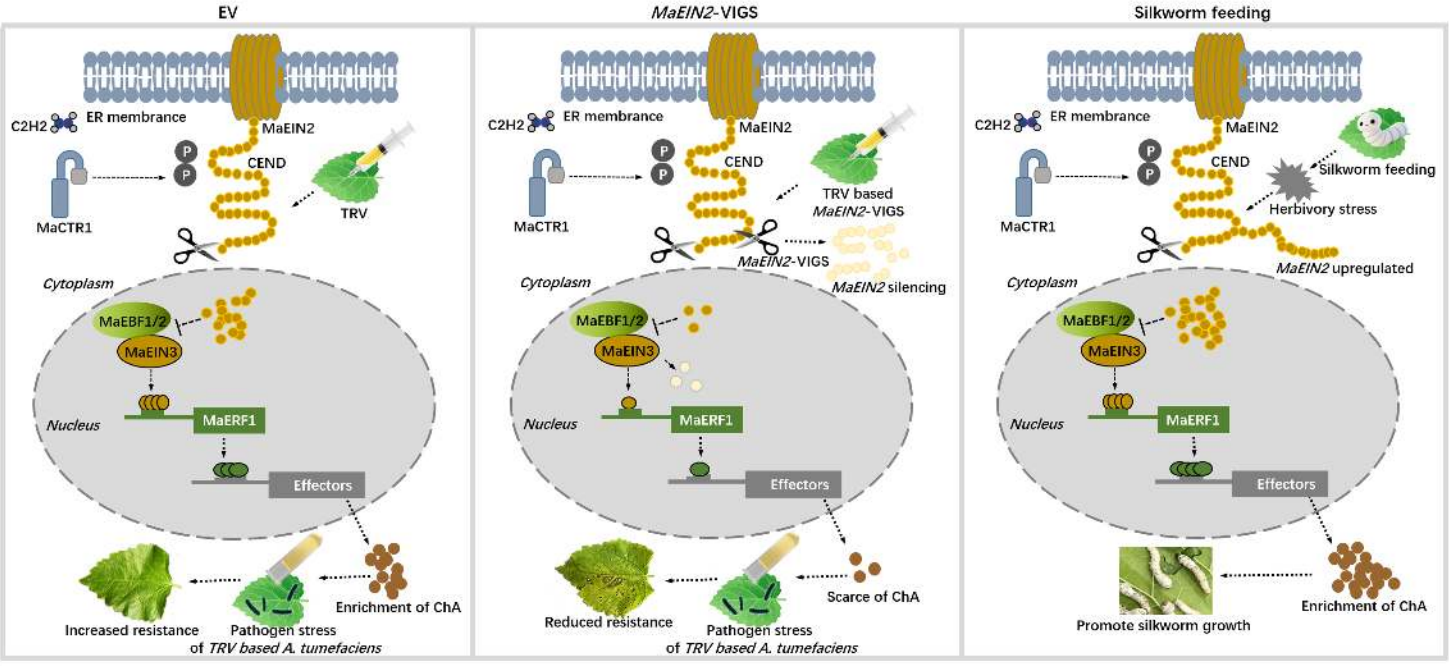

Figure 5

Proposed regulatory model for *MaEIN2*-mediated ChA accumulation in mulberry in response to biotic stress

Supplementary Files

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