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ETHYLENE REGULATES TRICHOME INITIATION IN *ARABIDOPSIS*

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Abstract

Plant trichome is the first protective barrier of plants, which is regulated by various genes and plant hormones. Ethylene is closely related to plant growth and development, but the specific mechanism of its regulation on plant trichomes is still unclear. In this study, *Arabidopsis* ethylene-signaling defective mutants (*ein2-1*, *etr1-3*) and wild type (WT) were used to investigate the initiation of plant trichomes via the ethylene signaling pathway. It was found that the trichome number of *ein2-1* and *etr1-3* was significantly less than that of WT on the sepal, stem and rosette leaf of main stem inflorescence. Ethylene synthesis precursor (ACC) solution treatment could promote trichome formation on the main stem inflorescence, main stem and rosette leaves in *Arabidopsis*. Through expression analysis of ACC-treated WT, it was found that ethylene induces the expression of key trichome genes in *Arabidopsis*. At the same time, the expression of key trichome-key genes of the *ein2-1* and *etr1-3* was significantly different from that of the WT. In summary, this study demonstrated that ethylene regulates the trichome initiation by regulating the expression of key trichome genes in *Arabidopsis*. This study offers a significant theory for enhancing the network of plant hormones that regulate trichome initiation.

Key words: Ethylene; Trichome; *Arabidopsis*; *ein2-1*; *etr1-3*

Introduction

Plant trichome is a special structure e which formed by epidermal cells and distributed on the surface of most plants (Hernandez & Park, 2022; Song *et al.*, 2022). They can be divided into single-cell or multi-cell trichomes, glandular or non-glandular trichomes, branched or non-branched trichomes, etc., (Han *et al.*, 2022; Hulskamp, 2019). Trichomes serve as an excellent model for studying plant cell determination and initiation, which benefits from their simple structure and diverse morphology. Trichomes serve as a crucial natural barrier against environmental stress, protecting plants from ultraviolet radiation, pathogens, herbivores, and excessive water loss during growth and development (Andama *et al.*, 2020; Wang *et al.*, 2020; Waseem *et al.*, 2021; Yuan *et al.*, 2021). Glandular tissues synthesize, store, and secrete essential substances, working in conjunction with stomata, cutin, and epidermal wax to perform protective functions. In addition, plant trichomes have high economic, application, and research value (Feng *et al.*, 2021; Livingston *et al.*, 2022).

With the continuous progress in molecular regulatory study of trichomes, many genes related to trichome initiation have been confirmed, most of which are transcription factor genes. Transcription factor genes involved in trichome initiation are categorized as either positive or negative regulators (Cox & Smith, 2019; Han *et al.*, 2022; Li *et al.*, 2021; Song *et al.*, 2022). Among them, the positive regulators include *GLABRA1* (*GL1*), MYB23, *TRANSPARENT TESTA GLABRA1* (*TTG1*), *TTG2*, *GLABRA2* (*GL2*), *ENHANCER OF GLABRA3* (*EGL3*), *GLABRA3* (*GL3*), *GIS* family genes, AP2/ERF family genes and so on (Han *et al.*, 2022). The *Arabidopsis* trichome initiation complex, known as the MYB-bHLH-WD40 complex, consists of the R2R3-MYB transcription factor *GL1*, the bHLH transcription factors *GL3*/*EGL3*, and the WD40 repeat protein *TTG1* (Bernhardt *et al.*, 2003; Payne *et al.*, 2000; Zhao *et al.*, 2008). *GL1* is the earliest cloned positive gene related to trichome initiation (Walker *et al.*, 1999). *GL3* and *EGL3* are homologous genes on chromosome 5 in *Arabidopsis*. *GL3* and *EGL3* exhibit overlapping roles in trichome formation and development (Bernhardt *et al.*, 2003). WD40 protein *TTG1* binds to *GL3* and acts directly on the upstream of *GL2* and *TTG2* to regulate trichome initiation (Pesch *et al.*, 2014; Heydarian *et al.*, 2024; Liu *et al.*, 2024). *GL2*, which encodes an HD-ZIP transcription factor, plays a role in multiple stages of trichome development (Chen & Wang, 2019; Rerie *et al.*, 1994). All *GIS* family genes act as positive regulators in trichome initiation on the main stem and inflorescence (An *et al.*, 2012; Sun *et al.*, 2015; Zhang *et al.*, 2020; Zhou *et al.*, 2013). *GIS* family genes upregulate MYB-bHLH-WD40 complex, and promote trichome initiation via gibberellin and cytokinin signal pathways (Sun *et al.*, 2015; Zhang *et al.*, 2020). *TOE1*, a member of the conserved AP2/ERF gene family, interacts with *GIS* gene family proteins and regulates *GL3* and *GL1* expression to control trichome initiation in *Arabidopsis* (Liu *et al.*, 2023).

Plant hormones can trigger downstream gene expression via signal transduction, contributing to trichome formation (Li *et al.*, 2021; Matias-Hernandez *et al.*, 2016a). Gibberellin (GA) is the most studied hormone in the regulatory network of trichome formation (Perazza *et al.*, 1998). GA is involved in controlling the growth, development and morphogenesis of *Arabidopsis* trichomes, which is partially mediated by the transcription factor *GIS* and acts on the MYB-bHLH-WD40 transcription complex (Gan *et al.*, 2006). SA negatively impacts trichome initiation

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and formation, diminishing the positive influence of JA (Yan *et al.*, 2017; Zhou *et al.*, 2022). CTK and GA can coordinate the growth and development of trichomes by integrating *GIS*, *ZFP8* and *GIS2* in *Arabidopsis* (Matías-Hernández *et al.*, 2016b; Zeng *et al.*, 2023). Ethylene is a simple but important gaseous plant hormone, which can regulate fruit ripening, leaf senescence, material metabolism, plant resistance, plant growth and development (Gan *et al.*, 2007; Yoshida *et al.*, 2009; Zhou *et al.*, 2011; Qiao *et al.*, 2024). Ethylene (ET) signaling is mediated by two-component kinase-type receptors located on the endoplasmic reticulum, including ethylene resistance 1 (*ETR1*), *ETR2*, ethylene-responsive sensor 1 (*ERS1*), *ERS2*, and ethylene-insensitive 4 (*EIN4*) (Binder, 2020). Ethylene has been shown to enhance the initiation and elongation of cotton fibers *In vitro* (Kim *et al.*, 2015). In *Arabidopsis*, a loss-of-function mutation in *etr2* impacts trichome branch formation in rosette leaves (Plett *et al.*, 2009; Yu *et al.*, 2021). However, the specific mechanism by which ethylene regulates plant trichome initiation remains unclear.

In this study, the mutants *ein2-1*, *etr1-3*(*Arabidopsis* ethylene-related mutants) and WT were used to explore the function of ethylene on trichome initiation in *Arabidopsis*. We found that ethylene can promote the initiation of the *Arabidopsis* trichome by upregulating the expression of *GIS* family genes and MYB-bHLH-WD40 complex genes. This study significantly enhances the understanding of ethylene's role in regulating plant trichome initiation mechanisms.

Material and Methods

Plant materials: Seeds of *Arabidopsis* wild type (WT), *ein2-1*, and *etr1-3* were disinfected, vernalized at 4°C for one week, and then sown in MS medium for cultivation in a light chamber. About one week later, the seedlings with consistent growth and strong growth were transplanted into the well-configured nutrient soil (nutrient soil: vermiculite=1:1). Following with cultured in the chamber under 16 h light and 8 h dark cycle for subsequent experiments.

Ethylene treatment: When *Arabidopsis* seedlings developed 2-3 true leaves, they were treated with four concentrations of ACC (1-aminocyclopropyl-1-carboxylic acid) solution: 0, 10, 50, and 100 μmol/L. ACC treatment experiments were repeated every week until plants were harvested at the maturity stage, and the trichome number of stem inflorescence sepals was analyzed. 20-day-old plants were treated with 100 μmol/L ACC or negative control, harvested after 4 h, and subjected to RNA extraction and real-time quantitative fluorescence PCR analysis.

Trichome count: The trichome number of main stems and cauline leaves was counted and analyzed when *Arabidopsis* ethylene mutants *ein2-1*, *etr1-3* and WT came into the mature period. The sepal trichome number was calculated when the first flower of the main stem inflorescence just opened. The trichome number of the main stem was counted 2-4 cm above the stem base of the main stem when plants are mature. The 8th rosette leaf was used for trichome number analysis of rosette leaves. Total trichomes were observed by stereomicroscope. At least 16 plants were calculated for trichome number for each genotype (Liu *et al.*, 2023). The significance analysis was carried out by IBM SPSS Statistics 27 software.

RNA extraction: Total plant RNA was extracted with Trizol Reagent (TAKARA). The plant tissue sample was ground with 1 mL Trizol in a precooled 2 mL centrifuge tube, then shaken for mixing and cracked for 10 min. After adding 0.2 mL chloroform and shaken for 15 s, the mixture was stood for 5 min before centrifuging for 15 min. The supernatant was transferred and mixed with 500μL isopropanol, then precipitated for 10 min, followed by centrifugation for 10 min. The supernatant was discarded, and 1 mL of 75% ethanol was added, and centrifuged for 5 min. The sample was centrifuged at 5000 g for 1 min for removing the supernatant, and dried at room temperature. Finally, 30μL DEPC H_2O was added, and the precipitate was dissolved completely by vibration and mixed evenly. The purity of total RNAs was detected by Nanodrop.

cDNA synthesis and Quantitative real-time PCR: The first strand of cDNA was synthesized by reverse transcriptase kit (TAKARA) following the manufacturer's introduction. 10μL a final volume was added to a 200μL centrifuge tube: 2μL 5×gDNA digester Buffer, 1 μL gDNA digester, 1μg RNA, followed by DEPC-H_2O to 10μL; incubated using a PCR instrument for removing DNA. Subsequently, 2μL 5 × HifairTM II Buffer, 2 μL HifairTM II Enzyme Mix, 1μL dNTP, 1μL Oligo $(dT)_{18}$ Primer (50μM), and followed by DEPC-H_2O were added to reach a final volume of 20μL. The mixture was centrifuged, and the PCR instrument was programmed according to the manufacturer's protocol.

Quantitative real-time PCR was performed according to the Faststart Essential DNA Green Master (TAKARA) protocol. In 20μL reactions, 5μL cDNA was combined with 10μL Faststart Essential DNA Green Master and 2μL of 10 mM upstream and downstream primers. The relative expression level of each target gene was normalized by a reference gene *Actin* and calculated by $2^{-\Delta\Delta Ct}$. Lightcycle 96 was used for fluorescence quantitative PCR experiments. The program was configured as follows: preheated at 95°C for 300 s; 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s; followed by melting at 95°C for 10 s, 65°C for 60 s, and 97°C for 1 s; and cooled at 37°C for 30 s. Three biological and three technical replicates were performed to ensure the reliability of the quantitative real-time PCR analysis.

Results

The number of trichomes in *Arabidopsis* ethylene mutant was significantly less than that of WT: Ethylene is a crucial plant hormone that significantly influences plant growth and development (Binder, 2020; Feng *et al.*, 2017; Song *et al.*, 2022). To investigate the impact of ethylene on *Arabidopsis* trichome initiation, WT *Arabidopsis* and ethylene-signaling mutants *ein2-1* and *etr1-3* were utilized. Trichomes on the sepals of the main stem inflorescence, main stem, and rosette leaves were observed and photographed. As shown (Fig. 1A), the trichome density on the sepal, stem and rosette leaf of main stem inflorescence in the mutant plants *ein2-1* and *etr1-3* were significantly less than that of WT plants. To quantify this difference, trichome numbers were statistically analyzed using software (Fig. 1B-D). Among them, Fig. 1B showed that the difference of the sepal is the largest between the mutants and WT (21 and 38 trichomes in *ein2-1* and *etr1-3* mutants, respectively, compared to 49 in WT plants).

ACC treatment promoted the initiation of trichomes: In order to further explore the regulation of ethylene on *Arabidopsis* trichome initiation, *Arabidopsis* WT plants were used as experimental materials. Ethylene biosynthesis involves the conversion of S-adenosylmethionine to ACC by ACC synthase, followed by the transformation of ACC to ethylene by ACC oxidase (Binder, 2020). Because ethylene is a gaseous substance that cannot be directly used to spray plants. It has been reported that ACC solution has a similar efficacy to ethylene in plants (Li *et al.*, 2022). Therefore, ACC solution is used to spray plants instead of ethylene. *Arabidopsis* WT seedlings were treated weekly with varying concentrations of ACC solution until the first flower on the main stem inflorescence opened. Then, the number of sepals trichomes on the main stem inflorescence was observed and counted. As shown (Fig. 2) with the increase of ACC solution concentration, the number of sepals trichomes on the main stem inflorescence showed an increasing trend in *Arabidopsis*. Especially, the number of the sepal trichomes with 50/100 μmol/L ACC solution was significantly more than that of 0/10 μmol/L ACC solution. The results showed that ethylene treatment could promote trichome formation on the main stem inflorescence in *Arabidopsis*.

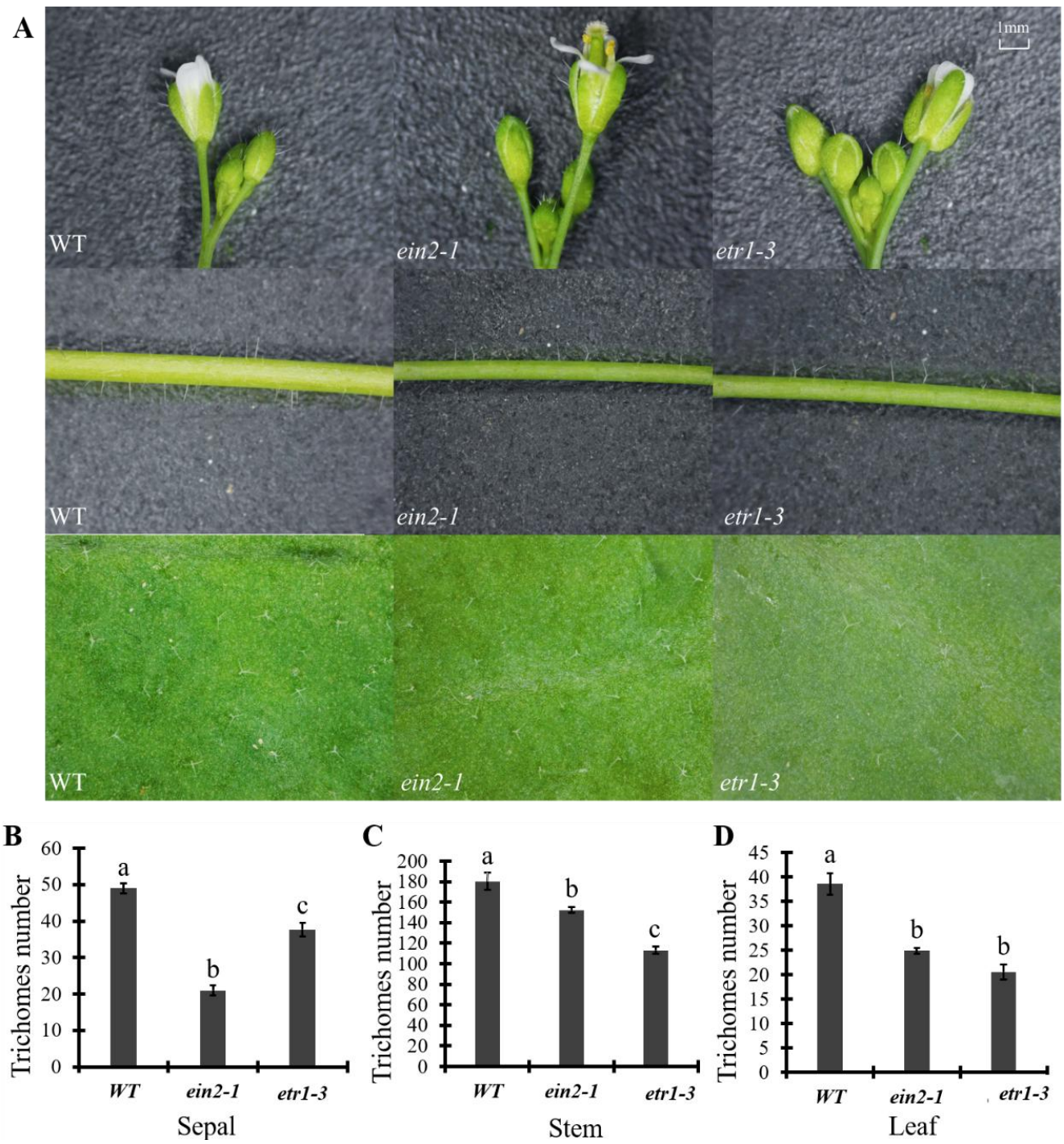

Fig. 1. Trichome phenotype of WT and mutant lines in *Arabidopsis*. A: The trichomes of sepals, stem rosette leaf in WT and mutant *ein2-1*, *etr1-3* plants. Scale: 1 mm. B: Trichome count on sepals in WT and mutant *ein2-1*, *etr1-3* plants. C: Trichome count on the main stems of WT and mutant *ein2-1*, *etr1-3* plants. D: Trichome count on the adaxial side of the rosette leaf in WT and mutant *ein2-1*, *etr1-3* plants. The counting number of every genotype is more than 16. The error bar represents ± SE.

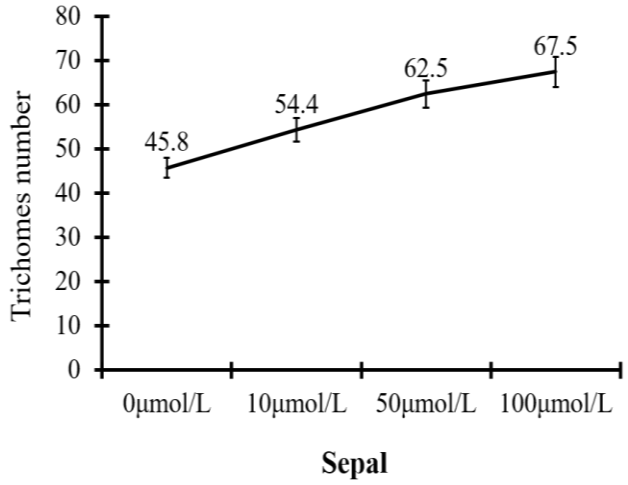

Fig. 2. The sepal trichome count in WT *Arabidopsis* plants treated with 0, 10, 50, and 100 μmol/L ACC solution. The error bar represents ± SE.

ACC treatment promoted the expression of trichome key genes: In order to further explore the molecular mechanism of ethylene affecting trichome formation, ACC treatment and quantitative analysis were performed to study whether trichome key genes are involved in regulating trichome via the ethylene signaling pathway in *Arabidopsis*. The above results showed that 100 μmol/L ACC solution increased most effectively the number of the sepals trichomes. We used quantitative real-time PCR to detect the expression of trichome regulatory genes in WT *Arabidopsis* seedlings treated with 100 μmol/L ACC solution. The expression levels of key genes *GL1*, *GL2*, *GL3*, *TTG1*, *TOE1*, *GIS*, *GIS3*, *ZFP5*, *ZFP6*, and *ZFP8* were lower in WT plants with water compared to those treated with 100 μmol/L ACC solution (Fig. 3). *GL1*, *GL2*, *GL3*, *TTG1*, *TOE1*, *GIS*, *GIS2*, *GIS3*, *ZFP5*, *ZFP6*, and *ZFP8* positively influence trichome initiation in *Arabidopsis* (Han *et al.*, 2022). The result also showed that the ethylene signaling pathway is involved in trichome initiation in *Arabidopsis*.

The expression of key trichome genes was significantly lower in the mutants *ein2-1* and *etr1-3* compared to WT: To validate the results, we used quantitative real-time PCR to measure the expression of trichome regulatory genes in WT and ethylene signal-deficient mutants *ein2-1* and *etr1-3* in *Arabidopsis*. The expression levels of *GL1*, *GL2*, *GL3*, *TTG1*, *TOE1*, *GIS*, *GIS2*, *GIS3*, *ZFP5*, *ZFP6*, and *ZFP8* in the *ein2-1* and *etr1-3* mutants were significantly lower compared to the WT in *Arabidopsis* (Fig. 4). The expression level of *GIS2* did not significantly differ from that of the mutant *ein2-1*. Ethylene may enhance trichome formation by influencing the expression of positive trichome regulatory genes (*GL1*, *GL2*, *GL3*, *TTG1*, *TOE1*, *GIS*, *GIS2*, *GIS3*, *ZFP5*, *ZFP6*, and *ZFP8*) in *Arabidopsis*.

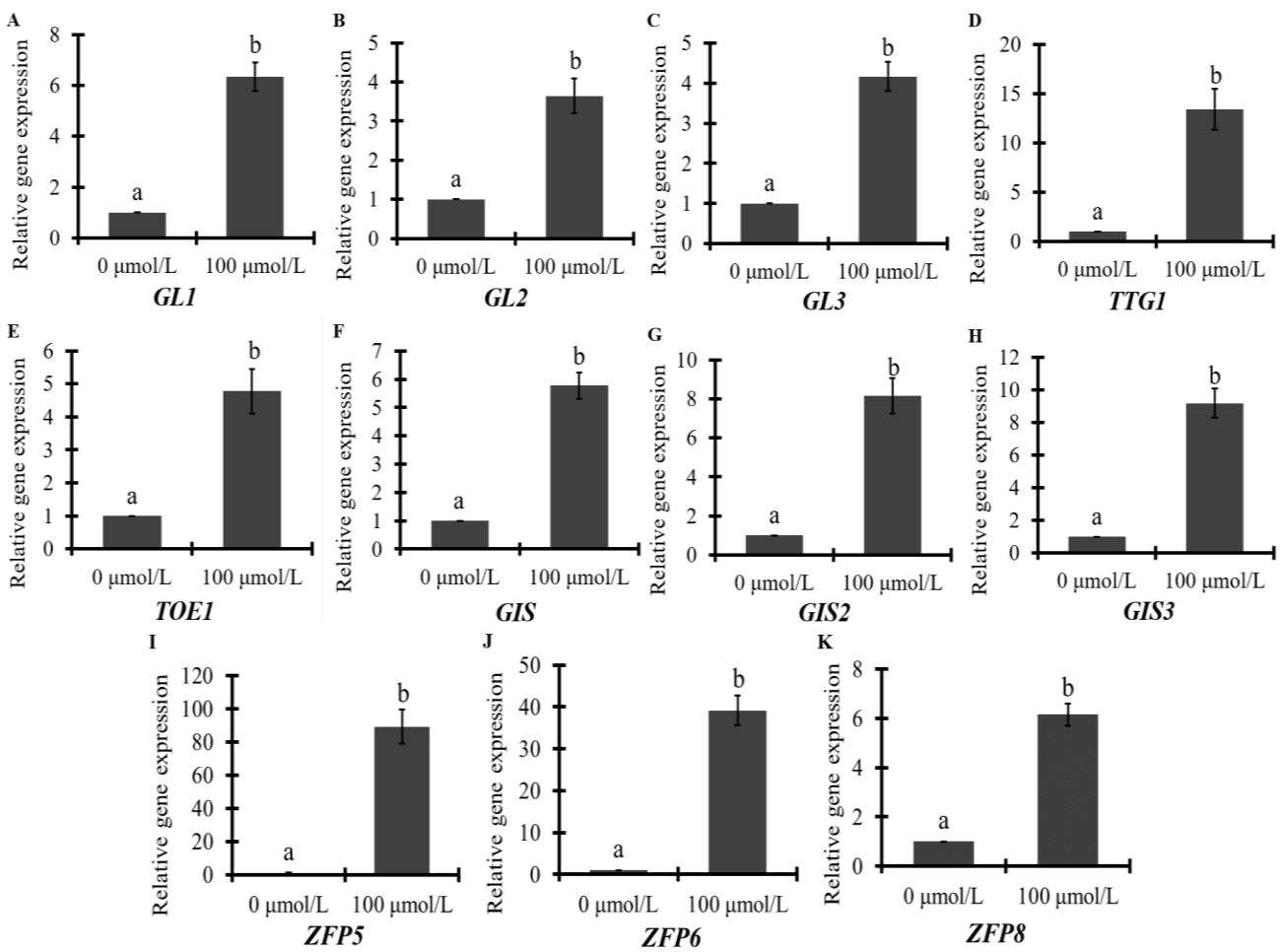

Fig. 3. The relative expression analysis of WT plants with 0 μmol/L and 100 μmol/L AAC solution. A-H represent the relative expression levels of *GL1*, *GL2*, *GL3*, *TTG1*, *TOE1*, *GIS*, *GIS2, GIS3*, *ZFP5*, *ZFP6*, and *ZFP8*, respectively. The error bar represents ± SE.

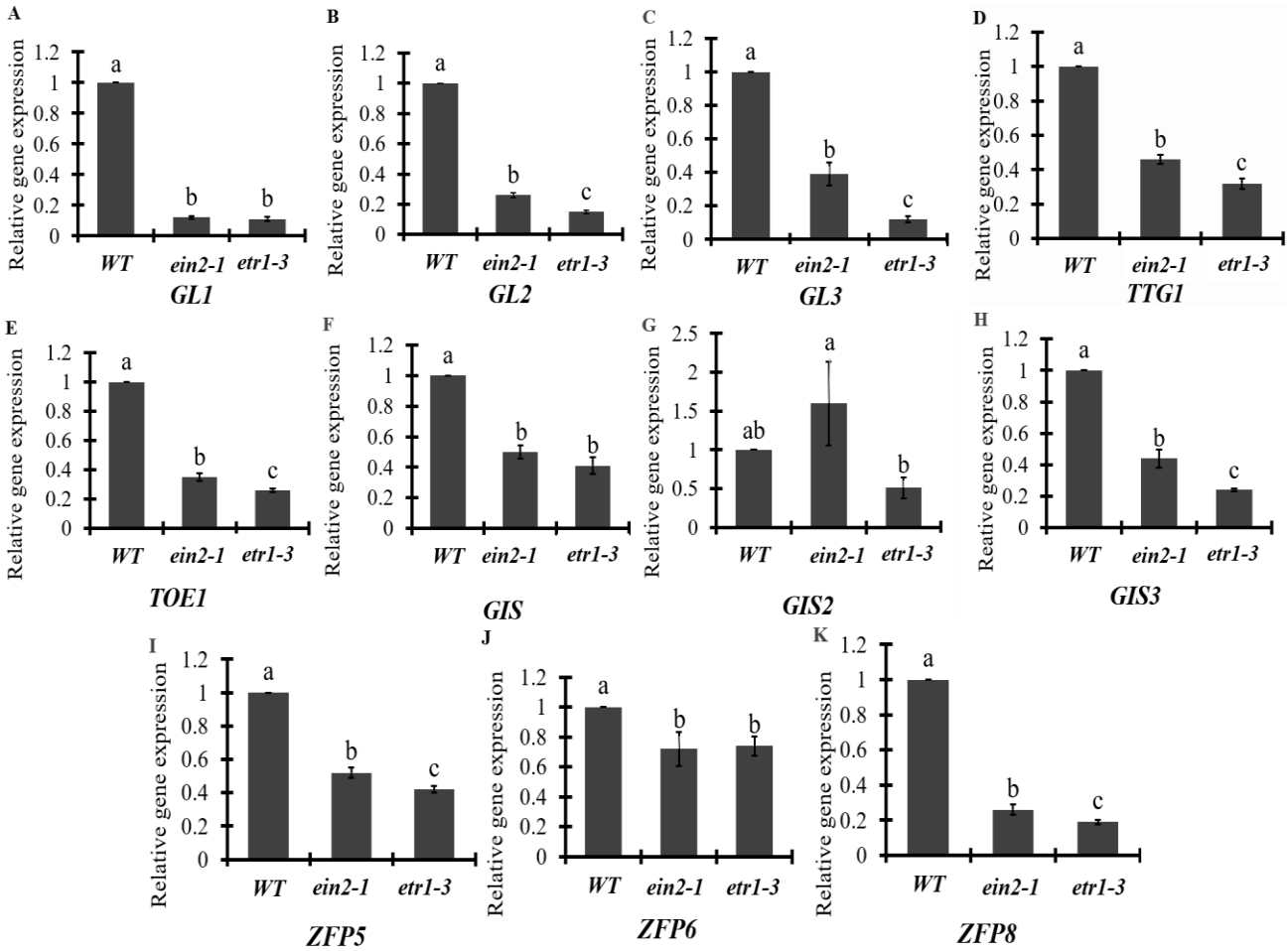

Fig. 4 The relative expression levels of trichome-related genes in WT and mutant *ein2-1*, *etr1-3*. The labels A-H correspond to the relative expression levels of *GL1*, *GL2*, *GL3*, *TTG1*, *TOE1*, *GIS*, *GIS2*, *GIS3*, *ZFP5*, *ZFP6*, and *ZFP8*, respectively. The error bar represents ± SE.

Discussions

Trichomes, which develop on the surfaces of aerial plant organs, function as effective mechanical barriers against environmental stressors including intense ultraviolet radiation, temperature extremes, and excessive transpiration (Hulskamp, 2019). Trichome formation in plants is coordinately regulated by multiple genetic and hormonal pathways. Plant hormones regulate trichome initiation and formation by influencing the expression of trichome-related genes. Among them, the most studied and most famous are GA and CTK signaling regulating trichome pathways (Lies & Alain, 2010; Nosheen *et al.*, 2024). In *Arabidopsis*, *GIS* family genes are involved in the GA and CTK signaling pathways, targeting the MYB-bHLH-WD40 trichome initiation complex to activate *GL2* expression, thereby regulating trichome initiation and formation (An *et al.*, 2012; Gan *et al.*, 2007; Sun *et al.*, 2015). In addition, other plant hormones have also been studied more or less, but the specific mechanism is still unclear. Ethylene is essential for regulating plant growth and development. Previous studies have found that ethylene can regulate the branch of cucumber trichome by regulating the assembly of microtubules (Zhang *et al.*, 2021). *CsTBH* regulates the trichomes morphogenesis of cucumber fruit via the ethylene signaling pathway (Xue *et al.*, 2019). Ethylene synergies with the ROS pathway to play a positive role in fiber elongation (Kim *et al.*, 2015). However, the mechanism of trichome determination and initiation is different from that of trichome morphogenesis. Furthermore, there are similarities and differences in trichome initiation and development in different plants. To date, the mechanism of ethylene regulating trichome determination and initiation in plants is still unclear.

This study found that the trichome number on the sepal, main stem and rosette leaf of the main stem inflorescence in mutant *ein2-1* and *etr1-3* was significantly less than that in WT in *Arabidopsis*. With the increase of ACC solution concentration, the trichome number on the sepal of the main stem inflorescence showed an increasing trend in *Arabidopsis*. It can be seen that ethylene can promote trichome formation in *Arabidopsis*. In *Arabidopsis*, the key regulatory pathway of controlling trichome formation is that *GIS* gene family protein interacts with *TOE1* and acts upstream on the MYB-bHLH-WD40 complex, and in turn directly activates *GL2* expression (Liu *et al.*, 2023). Our findings indicated that the expression levels of key trichome genes (*GL1*, *GL2*, *GL3*, *TTG1*, *TOE1*, *GIS*, *GIS2*, *GIS3*, *ZFP5*, *ZFP6*, and *ZFP8*) were significantly elevated in WT plants treated with ACC solution compared to the control. Moreover, those gene expressions in mutant *ein2-1* and *etr1-3* were significantly lower than those in WT. However, no significant difference in *GIS2* expression was observed between the mutant *ein2-1* and WT. Similarly, *GIS2* does not play a role in the CTK signaling pathway in

regulating trichome initiation and development (Gan *et al.*, 2007). We presumed that the reason may be some functional redundancy among *GIS* family genes, or *GIS2* is not directly regulated by ethylene. In summary, those results indicated that ethylene regulates trichome initiation by regulating the expression of trichome key genes (*GL1*, *GL2*, *GL3*, *TTG1*, *TOE1*, *GIS*, *GIS2*, *GIS3*, *ZFP5*, *ZFP6*, and *ZFP8*) (Fig. 5). However, it is still unclear the molecular mechanism of those trichome key genes controlling trichome initiation via the ethylene signaling pathway. Moreover, *GIS* family genes control trichome formation through the synergistic effect of GA and CTK (Gan *et al.*, 2007). Commonly, interactions between various plant hormones can be synergistic or antagonistic (Liu *et al.*, 2017; Song *et al.*, 2022). Ethylene has different effects (synergistic, antagonistic, or not) with different plant hormones in different life processes. Thus, it is not known whether ethylene has interaction with GA and CTK to participate in trichome initiation. In addition, there are similarities and differences between non-glandular trichomes and glandular trichome formation and development in plants. So, it is not clear whether ethylene can regulate the formation of glandular trichomes in economic crops such as tomato, peppermint and *Artemisia annua*. Further study can analyze the specific mechanism of ethylene regulating plant trichome initiation by biological function analysis, interaction analysis, proteomics, and so on. This study offers a crucial theory for enhancing the network of plant hormones that regulate trichome development and serves as a fundamental basis for examining the impact of ethylene on trichome initiation in key horticultural plants and field crops.

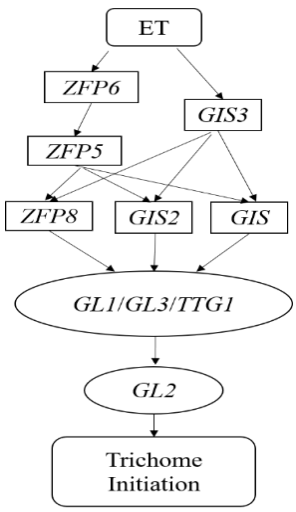

Fig. 5. Molecular regulation mechanism model of *Arabidopsis* trichome.

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