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AtGIS, a C2H2 zinc-finger transcription factor from *Arabidopsis* regulates glandular trichome development through GA signaling in tobacco

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ABSTRACT

Glandular trichome is specialized multicellular structures that have capability to synthesize and secrete secondary metabolites and protect plants from biotic and abiotic stresses. Our previous results revealed that the C2H2 zinc-finger transcription factors (GIS) acts upstream of GL3/EGL3-GL1-TTG1transcriptional activator complex to regulate trichome initiation through GA signal in *Arabidopsis*. In the present study, we are reporting that ectopic expression of *AtGIS* could regulate glandular trichome development through GA signaling in tobacco. X-gluc staining of various organs from transgenic plants showed that *AtGIS* expressed mainly in the glandular trichomes. Statistical analysis demonstrated that over expression of *GIS* increased significantly glandular trichome production on the leaf, stem, branch, and sepal in tobacco. After PAC treatment, reduction of glandular trichome production in transgenic plants was more severe with compared to wild type plants. Furthermore, GA treatment could induce expression of AtGIS. More importantly, our results also demonstrated that overexpressed *AtGIS* significantly affect the main components of trichome exudates, such as significantly increase the content of nicotine, Cembratriene-4, 6-diol. Taken together, these results suggest that ectopic expression of *AtGIS* regulates glandular trichome development and may play a key role in compounds secretion in tobacco.

1. Introduction

Trichomes are easily accessible appendages originating and projecting from the epidermal cell layer of leaves, stems, and floral organs in most terrestrial plants. They may be unicellular, multicellular, branched or non-branched, glandular or non-glandular depending on their morphological classification [1–5]. Trichomes are rightly considered as plants first line of defense due to its protective roles from UV irradiation, high temperature and heat stress and to some extent can hinder herbivores [2,3,6–11]. Trichomes are not essential to plant viability and could provide an excellent model system for studying molecular mechanism of cell-fate determination and interaction of different developmental processes in plants [1,12].

In *Arabidopsis*, trichomes are unicellular, non-glandular, regularly spaced, and have two or more successive branching [8,13]. During trichome development, progenitor cells are involved in the stages of cell fate determination and cellular specification and undergoing four endoreduplication cycles resulting in a DNA content of 32C before turning into mature trichomes [4,12,14,15]. A transcriptional regulatory network of trichomes initiation and development has been determined by using molecular genetics methods. It consist of several groups of transcription factors (TFs), including the R2R3MYB, basic helix-loop-helix (bHLH), and WD40 repeat (WDR) protein, and C2H2 zinc finger proteins [16]. At the core of these networks, GL1, TRANSPARENT TESTA GLABRA1 (TTG1), GLABRA3 (GL3), and ENHANCER OF GLABRA3 (EGL3) form a MYB/bHLH/WD-repeat complex controlling trichome formation and development [4,7,16–19]. Previous studies have identified that the C2H2 zinc finger protein coding transcription factor INFLORESCENCE STEMS (GIS), GIS2, GIS3, ZINC FINGER PROTEIN 8 (ZFP8), ZFP5, and ZFP6, plays a key role in trichome initiation and

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formation in *Arabidopsis* [5,20–24].

Tobacco plant is covered with a high density of glandular trichomes, that secret and store huge amounts of various secondary metabolites [3,25]. Metabolic exudates from glandular trichomes in tobacco are made of terpenes, alkanes, acyl sugars, nicotine, fatty acid derivatives and flavonoids [26]. In addition to its basic function to protect plants, these exudates are serving as an important industrial raw material in production of aromas, flavors and pharmaceuticals [27,28]. However, the molecular mechanism of glandular trichome differentiation in tobacco largely remains unknown, although instrumental characters correlated with pest resistance has been studied in tobacco [9,11].

GLABROUS INFLORESCENCE STEMS (GIS), which encodes the C2H2 zinc-finger transcription factors, act upstream of GL3/EGL3-GL1-TTG1transcriptional activator complex in regulation of trichome initiation in *Arabidopsis* [20]. Overexpression of *GIS* resulted in high density of trichome initiation on inflorescence organs and the formation of ectopic trichomes on carpels, petals, and even stamens during inflorescence development while loss of function mutants of *GIS* displayed a steep decline in trichome production on successive inflorescence leaves, successive stem internodes, branches, and flowers [20]. Furthermore, *GIS* control trichome initiation and trichome branching through GA signaling [13,21]. Here our novel results demonstrated that ectopic expression of *AtGIS* also could regulate glandular trichome development in response to GA signaling in tobacco.

2. Materials and methods

2.1. Plant materials and treatments

Arabidopsis thaliana ecotype (Columbia Col-0) and *Nicotiana tabacum* (Xanthi) were used as plant materials in this study. For uniform germination, seeds were cold treated in the dark for 3 days. Cold treated seeds were germinated on Murashige and Skoog medium in petri plates in the growth chamber under a 16 h light/8 h dark photoperiod at 22 °C. 8 days old uniform seedlings were selected and transferred to soil and grown under same light/dark regime in phytotron [20,22,24].

2.2. RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted from leaves with TrIzol Reagent (Invitrogen). Quality and quantity of RNA were assessed by Nanodrop 2000. About 2 μg of total RNA were reverse-transcribed using the M-MLV transcriptase (Promega) and oligo dT18 primers in a 20 μL reaction according to the manufacturer's instructions. Quantitative real-time reverse transcription PCR were carried out using SYBR Premix Ex TaqII (TaKaRa) in a 20 μL volume and a CFX96™ Real-time Detection System. According to the manufacturer's instructions, the cDNAs were diluted to 80 μL, and 2 μL was added to 10 μL of SYBR-green PCR mix (Takara, Tokyo, Japan) and 1 μl of each primer (500 nM final concentration) in 20 μL reactions. The expression of actin was detected and used as an internal reference to normalize expression of the other genes. Relative expression levels were calculated by subtracting the Ct (threshold cycle) values for actin from those of the target gene (to give ΔCt) and then calculating $2^{-\Delta Ct}$ [29,30].

2.3. Gene cloning and vector construction

The full-length cDNA and putative promoter region of GIS was amplified by PCR using Prime STAR HS DNA polymerase (TaKaRa) and inserted into the NotI and SalI sites of pENTR1A. The ends of the insert from pENTR1A were sequenced and subsequently cloned into an appropriate destination vector using the Gateway LR reaction (Invitrogen). All destination vectors were obtained from VIB (Flanders Inter university Institute for Biotechnology) [20,24]. The destination vectors pH2GW7 containing 35S CaMV promoter was used for generating the *35S:GIS* construct, and pHGWFS7 containing a GUS fusion was employed to create the *pGIS:GUS* fusion construct.

Agrobacterium tumefaciens strain EHA105 was used to transform tobacco plants by the leaf disk method using binary vectors. Transformed plants were identified and selected by growing on MS solid medium supplemented with 15 mg/l of hygromycin, followed by PCR confirmation. Transgenic plants expressing *35S:GIS*, were verified by using RT-PCR.

2.4. GUS histochemical analysis

The histochemical assay for GUS was carried out with the progeny of the self-pollinated T0 plants (T1 plants) according to the procedure of Jefferson et al. [31]. Plantlets and different plant organs to be analyzed were submersed in GUS substrate containing 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) solution overnight at 37 °C in the dark.

2.5. Glandular trichomes number counts

Tobacco glandular trichomes were examined with Stereo Microscope and images were analyzed using software Image-Pro Plus. Glandular trichomes from wild type and transgenic plants were selected and counted at the same developmental stage. Fully expanded leaves of 40d old plants were selected for counting leaf glandular trichomes. Leaf sections were made by cutting the leaves at similar position. A 1 cm^{-2} area of Leaf sections was observed and photographed using Stereo Microscope, followed by trichomes counting using Image-Pro Plus software. Glandular trichomes on the stems, branches, flowers and sepals were measured from 60 d old plants. At least 3 fields of vision were photographed for every section in every plant. About 16 plants were used for glandular density analysis for each of the treatment × genotype.

2.6. GA treatments and gene expression analysis

Plants were treated with GA3 and paclobutrazol (the GA biosynthesis inhibitor) by spraying with 100 μM corresponding solutions. For measuring the effect of GA applications on gene expressions, the plants were grown to 20 days on soil. The plants were then sprayed with either 10 μM GA3, 100 μM GA3, or their corresponding mock solutions. After 4 h treatment, the above ground portion was harvested for RNA extraction. For hormone-treated phenotype experiments, 20 days old plants were sprayed with 1 μM PAC, 10 μM PAC, 50 μM PAC or their corresponding mock solution. Treatment was repeated every 7 days, until plants were harvested for analyses [24,32].

2.7. GC-MS analysis

Tobacco glandular exudates were extracted from fresh leaves by dipping the leaves in methylene chloride for 2 s (1 mL for 100 mg fresh weight), followed by drying. The whole process was repeated 7 times. The extract was dehydrated by anhydrous sodium sulfate and filtered into Erlenmeyer flask through filter paper. The filtered extract was concentrated to 1 ml by using rotator evaporators at 40 °C before GC-MS analysis. Extracted samples were injected immediately into a 7890A gas chromatograph coupled to a 5975C mass spectrometer (Agilent Technologies, http://www.agilent.com).

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3. Results

3.1. Expression pattern of the AtGIS promoter–GUS constructs in tobacco

To probe the tissue-specific expression patterns of *AtGIS* in the tobacco plant, 1.5 kb sequence upstream of the *AtGIS* coding region was fused upstream of the GUS gene in pHGWFS7 to create *pGIS:-GUS*. Strong expression was displayed by *pGIS:GUS* in various organs of 60 days old tobacco plants compared with wild type control (Fig. 1). In the mature transgenic tobacco (60days), GUS expression is visible in aerial parts, including stems, leaves, and sepals (Fig. 1A, C, E). In particular, GUS expression was apparently observed in glandular trichomes of transgenic tobacco. As shown in Fig. 1G, GUS staining activities were present in the glandular head, stalk, and basal portion of glandular trichomes. Apart from glandular trichomes, *pGIS:GUS* also showed strong expressions in other tissue and organ, such as epidermal and vein.

3.2. Over expression of AtGIS increases glandular trichome productions in tobacco

GLABROUS INFLORESCENCE STEMS (GIS) was reported to plays a key role in the control of trichome initiation and development in *Arabidopsis* [20]. In order to evaluate the role of *AtGIS* in tobacco, we generated *AtGIS* overexpression transgenic lines. Several independent *35S:GIS* overexpression lines were randomly chosen, and three representative T2 lines were selected for investigation in this study. Transgenic lines were identified by genomic PCR and expression level PCR. Compared to WT tobacco plant, significant increase in glandular trichomes was recorded in transgenic plants (Fig. 2). As shown in Fig. 2A, abundant glandular trichomes were presented on the epidermis of leaf, stem, branch, sepal in both WT and transgenic lines. However, barely noticeable non-glandular trichomes can be viewed in the tobacco plants either WT or transgenic lines. It is particularly worth mentioning here that both lines exhibited noticeable high density of glandular trichomes than wild-type plants. In order to better evaluate the influence of overexpression of *AtGIS* on glandular trichome production, we counted glandular trichome and had a statistical analysis on these data. Through comparative analysis, the quantity of glandular trichomes on all the transgenic lines was remarkable high than WT. At 40 days, significantly higher numbers of glandular trichomes were also observed on the leaf in the overexpressors compared to wild-type control, especially transgenic line *35S:GIS-1* exhibited 45% increase in glandular trichomes (Fig. 2B). Such an increase in glandular trichome numbers also were exhibited on the epidermis of other organ in 60 days old overexpression lines, most significant on the stem, with 22.3%–36.4% higher than WT (Fig. 2C, D, E).

3.3. AtGIS controls trichome initiation through GA signaling in tobacco

In *Arabidopsis*, *GIS* controls trichomes initiation on leaves, stems and inflorescence through gibberellic acid responsive pathway [21]. However, the role of GA signaling in regulation of glandular trichomes has not been fully investigated previously. In order to investigate implication of GA signaling on glandular trichome production in tobacco, we sprayed different concentrations of paclobutrazol (PAC), a gibberellic acid biosynthesis inhibitor on tobacco plants. The effectiveness of PAC treatment was evident from the significant decreased plants height (Fig. 3C). For PAC treatment, tobacco glandular trichome number was counted and analyzed at the mature stage. Our results showed that there was a decreasing tendency with the increasing concentration of PAC in both wild type and transgenic plants and overexpressed *AtGIS* transgenic plants were more sensitive to the PAC treatment (Fig. 3A). These results suggested that glandular trichome initiation in tobacco also requires gibberellic acid signaling. Interestingly, transgenic plants showed more distinct and greater fluctuation in the quantity of glandular trichome on the leaf compared to wild type plants with different concentrations of PAC treatment (Fig. 3A). The sensitivity of transgenic lines to PAC application with respect to glandular trichome initiation coupled with up-regulation of GIS expression by GA, supported the hypothesis that *AtGIS* controls tobacco trichome initiation through GA signaling (Fig. 3B).

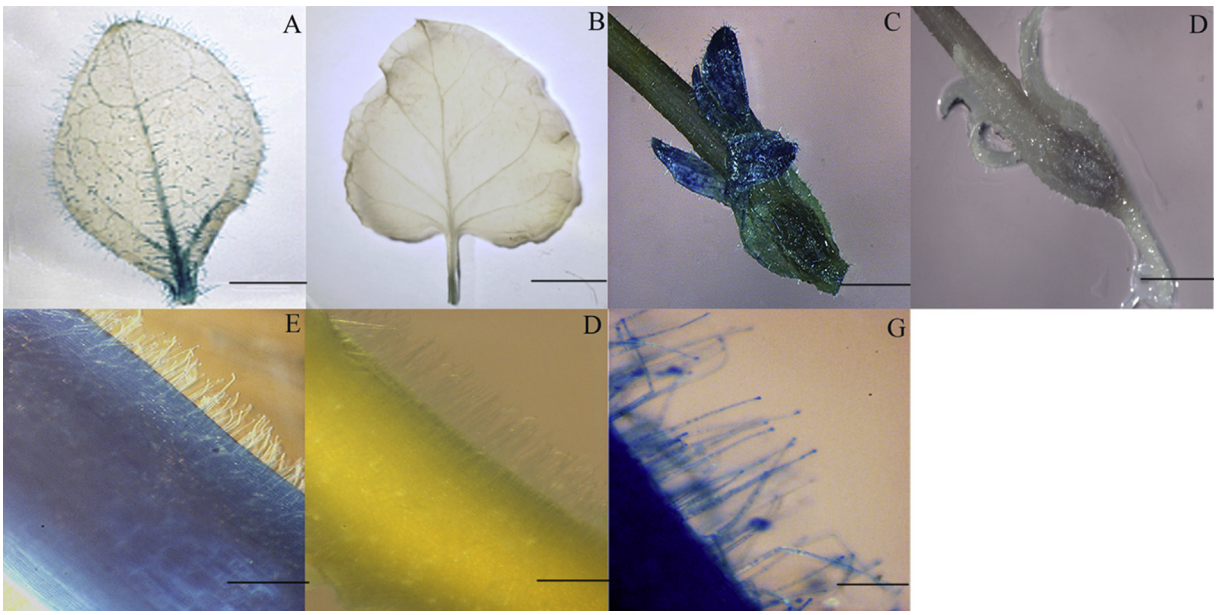

Fig. 1. Histochemical determination of the expression pattern of *AtGIS* in glandular trichomes. Different section was stained for GUS activity in transgenic plants with *pGIS:GUS* (A,C.E,G), and wild-type plants (B, D, F). A, B, leaf; B, C, sepal; E, F, stem; G detail view of glandular trichomes in main stem. Scale bars: A, B 1 cm; C, D 250 μm; E, F 150 μm; G 40 μm.

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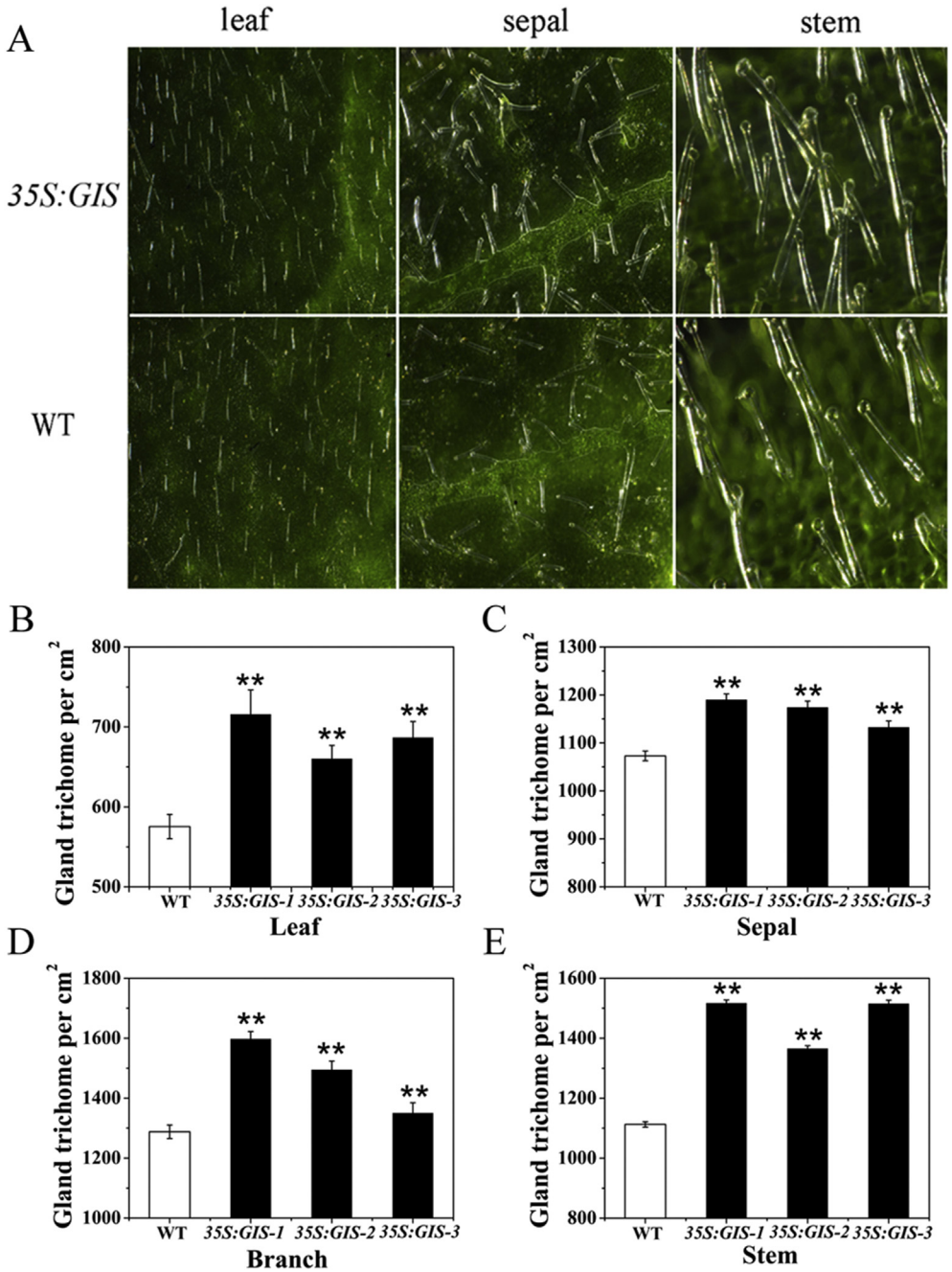

Fig. 2. Glandular trichome population on different organs of wild-type and transgenic tobacco. A Trichomes phenotype of 35S:GIS overexpressor and wild-type in tobacco. B to E, Glandular trichome density on the leaf (B), sepal (C), branch (D) and main stem (E) in wild-type tobacco and transgenic lines. Error bars represent + SE. A Student's t-test was calculated at the probability of 1% (**, $P < 0.01$).

3.4. Analysis of compounds in whole-leaf surfaces and trichome extracts

Whole-leaf exudates from WT and *AtGIS*-over-expressed transgenic tobacco plants were collected by methylene chloride and analyzed by GC-MS. The GC-MS chromatograms revealed the detection of various fatty acids and alkane hydrocarbons and alcohol. The majority of fatty acids and alkane hydrocarbons, which had a retention time ranging from 13 to 20 min, were the prominent compound identified (Fig. 4A and B). Docosane was the most abundant compound in all of detected components. Compared the GC chromatograms between wild type and transgenic lines, it had no significantly difference about the content of alkane hydrocarbons and fatty acids. Nonetheless, nicotine and Cembratriene-4,6-diol, two significant compound in tobacco exudates, were highly increased in all overexpressing lines (Fig. 4). By means of area normalization method, the average contents of nicotine and Cembratriene-4,6-diol for transgenic line were 18.058% and 17.321%, while for wild type plants, the contents of nicotine and Cembratriene-4,6-diol were 7.604% and 4.5% (Fig. 4C).

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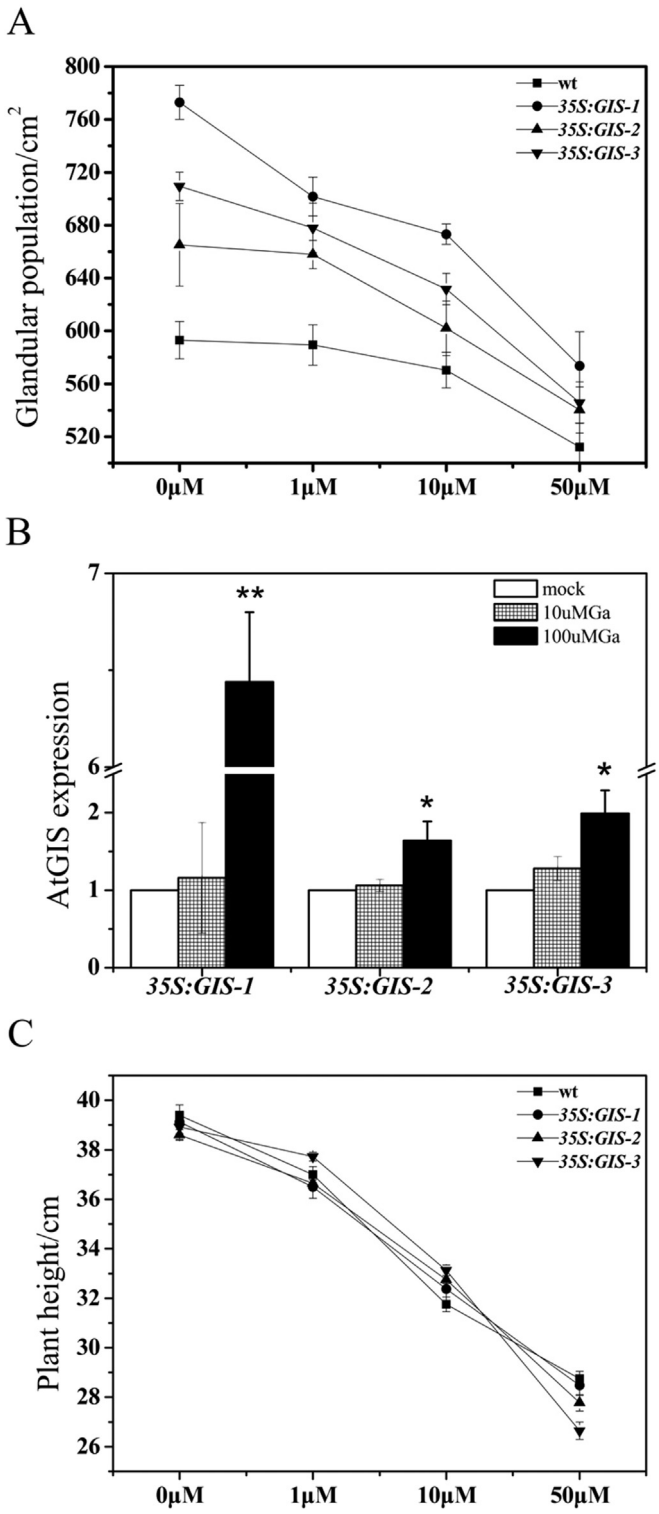

Fig. 3. The effect of GA on glandular trichome initiations and the expression of AtGIS in tobacco. A Glandular trichome population on the leaf of wild-type and transgenic tobacco after treatment with varying concentrations of PAC. B The relative expression levels of AtGIS in leaf of transgenic tobacco after treatment with varying concentrations of GA for 4 h. C Developmental effect of PAC applications on wild-type and transgenic tobacco. Error bars represent + SE. A Student's t-test was calculated at the probability of either 5% (*, $P < 0.05$) or 1% (**, $P < 0.01$).

4. Discussion

4.1. AtGIS regulates the trichome initiation through GA signaling in tobacco

Arabidopsis trichomes, as a model of trichome development and regulation, are controlled by the MYB/bHLH/WD-repeat complex and regulated by C2H2 transcriptional factors through integrating GA and Cytokinin signaling [5,13,20–24]. MIXTA-like genes were reported not only promotes unicellular trichome differentiation in Arabidopsis but also promote multicellular trichomes initiation in tobacco [1,4,33]. MIXTA is an R2R3-type MYB transcription factor controlling trichome initiation as a member of the family trichome formation complex. However, ectopic expression of a bHLH factor failed to influence trichomes differentiation in tobacco, whereas it initiates redundant trichomes in *Arabidopsis* [34]. In addition, GL1, whose conserved DNA-binding domain similar to that of R2R3 MYB transcriptional regulators, had no significant effect on glandular trichome formation. Similarly, ectopic expression of MIXTA in *Arabidopsis* does not complement the *gl1-1* mutant phenotype. Furthermore, There is no physical interaction between MIXTA and the R-like bHLH proteins GL1 in tobacco, which signify that the two proteins function independently [4,35]. These results indicated some key similarities and differences in trichome formation between *Arabidopsis* and tobacco. The formation of conical trichomes and glandular trichomes is regulated by distinct developmental pathways, neither homologous nor different.

Our results revealed that *AtGIS* is expressed in glandular trichomes and epidermis cell, and triggers excess trichomes differentiation in tobacco. However, function of *AtGIS* in tobacco is similar but not corresponding to the function of *AtGIS* in *Arabidopsis*. In *Arabidopsis*, *GIS* resulted in stimulation of trichome initiation in the inflorescence, stems and floral organ [20]. In tobacco, however, in addition to *AtGIS* functions in inflorescence, stems and floral organs, it also functions in the leaf. It seems that different regulatory networks in tobacco and *Arabidopsis* could account for this. In addition, our results also confirmed that GA plays a key role in regulates glandular development and induced the expression of *AtGIS* in tobacco. Furthermore, the higher sensitivity of *AtGIS* transgenic plants as a consequence of PAC applications implied that *AtGIS* is involved in GA signaling pathway in the regulation of trichome initiation and development. In summary, *AtGIS* plays an important role in controlling trichome development through GA signaling in tobacco.

4.2. AtGIS may involve in lipid secretion from glandular trichomes

In *Nicotiana tabacum*, the secretion of glandular trichomes accounts for as much as 16% of the dry leaf weight [36]. The prominent CBT-diols of glandular trichome exudate account for ~60% of exudate weight or as much as 8–10% of leaf dry weight [36]. The higher level of CBT-ols in trichome exudate enhances its toxicity to aphids and reduced aphids attack [11]. Similarly, the production and accumulation of the toxic alkaloid nicotine increases herbivore attack on *Nicotiana attenuate* [37]. Our results from GC-MS analyses showed that the most prominent compound was docosane, which is not significantly affected by the expression of *AtGIS*. The level of nicotine and Cembratriene-4,6-diol in transgenic lines was significantly increased in the whole-leaf surface extracts compared to the WT. These result indicates that the over expression of *AtGIS* could affect the main components of trichome exudates (nicotine and Cembratriene-4,6-diol), which could enhance transgenic plants resistance to aphidicidal activity in comparison to WT.

Our results demonstrated that *Arabidopsis* transcription factor gene, *AtGIS*, was up-regulated by GA and expressed in whole plant,

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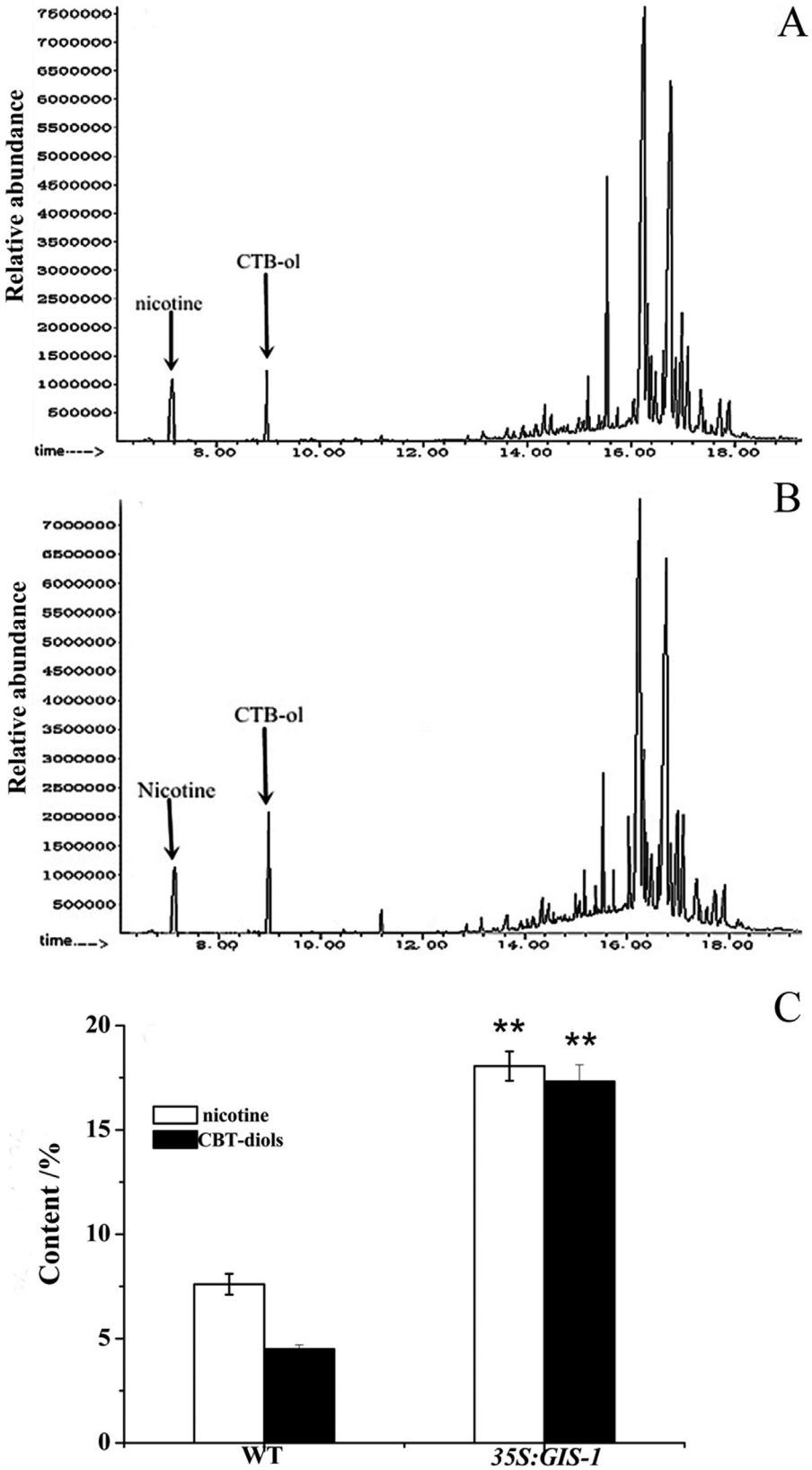

Fig. 4. Gas chromatographic analyses on the leaf surface exudates from tobacco plants. A GC-MS chromatograms of whole-leaf surface exudates from wild-type tobacco. B GC-MS chromatograms of whole-leaf surface exudates from transgenic tobacco. C Levels of nicotine and cembranoid diterpenes in the wild-type tobacco or transgenic lines. Experiments were performed in triplicate. Error bars represent + SE. A Student's t-test was calculated at the probability of 1% (**, $P < 0.01$).

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especially in the glandular trichomes. *AtGIS* controls glandular trichomes development in response to GA signaling in tobacco. This is the first time to report that C2H2 zinc-finger transcription factor gene plays an important role in glandular trichomes development in tobacco. The findings from the present study can provide valuable information in understanding the pathway of multicellular trichomes formation. Although we cannot elaborate whether these phenomena were caused directly or indirectly by overexpressing *AtGIS* in this study, but the alteration in the phenotype were absolutely generated by overexpressing *GIS*. Further studies are necessary to identify the mechanisms of *AtGIS*-mediated glandular development and secretion formation components. Besides, *AtGIS* transgenic plants stimulated the production of nicotine and Cembratriene-4, 6-diol from tobacco exudate could be used by genetic engineering to improve tobacco favor.

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Abbreviations

bHLH	Basic Helix-Loop-Helix
CBT-diols	Cembratriene-4,6-diol
Ct	Cycle Threshold
GA	Gibberellin Acids
GC-MS	Gas Chromatography and Mass Spectrometry
GIS	GLABROUS INFLORESCENCE STEMS
PAC	Paclobutrazol

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.12.164.

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