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An HD-Zip IV transcription factor protein NbGL3 regulates glandular trichome initiation in tobacco

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Abstract

Glandular trichomes, a specialized multicellular structure, are considered as biofactories due to their capability to synthesize and secrete a large amount of secondary metabolites. Tobacco leaves have a high density of glandular trichomes that produce huge amounts of secondary metabolites, which can be used as important industrial raw materials. However, molecular mechanism controlling glandular trichome development in tobacco still remains largely unknown. In the present study, we found that *NbGL3*, an HD-Zip IV family gene from *Nicotiana benthamiana*, was highly expressed in mature leaves, and ethylene or auxin application could increase the expression of *NbGL3*. Virus induced gene silencing *NbGL3* resulted in a decreased glandular trichome density on leaves. Putative downstream genes, such as *micro trichome*, *cyclin B2*, and *wax inducer 1 like* were down regulated in *NbGL3* silenced plants. Our results demonstrate that *NbGL3* could positively regulate initiation of glandular trichomes in tobacco.

Additional key words: auxin, ethylene, *Nicotiana benthamiana*, secondary metabolites, virus induced gene silencing.

Introduction

Plant trichomes, the epidermal outgrowths covering most aerial plant tissue surfaces, are found in a very large number of plant species. According to their morphology, trichomes could be classified into glandular or non-glandular, unicellular or multicellular, branched or non-branched (Werker 2000, Pattanaik *et al.* 2014). Trichomes play various roles in plant growth and development, including protection of plant from UV radiation, arthropod herbivores, and pathogens (Kang *et al.* 2016), and from excess transpiration (Kang *et al.* 2014). Glandular trichomes are characterized by the ability of head cells to secrete or store large quantities of secondary metabolites (Huchelmann *et al.* 2017).

Tobacco shoots are covered with a high density of glandular trichomes. Tobacco glandular trichome cells secrete and store huge amounts of terpenes, alkanes, acyl sugars, nicotine, fatty acid derivatives, and flavonoids (Roda *et al.* 2003), together representing up to 16 % of the dry mass of the leaf (George 1991, Wagner *et al.* 2004). These exudates are important for aromas, flavours, and

pharmaceuticals (Schilmiller *et al.* 2010, Huchelmann *et al.* 2017). Molecular mechanisms controlling trichome development in model plant *Arabidopsis* have been extensively studied and reviewed (Serna and Cathie 2006, Pesch and Hülskamp 2009, Pattanaik *et al.* 2014, Yan *et al.* 2014). However, previous study indicated that transcription networks controlling trichome development are different between tobacco and *Arabidopsis*. Overexpression of *myeloblastosis related transcription factor (MIXTA)* from *Antirrhinum majus* and *Wo^v* gene from tomato could promote glandular trichome initiation in tobacco (Payne *et al.* 1999, Yang *et al.* 2015). Most recent reports demonstrated that C2H2 type zinc finger *NbGIS*, *AtGIS* from *Arabidopsis*, and *JcZFP8* from *Jatropha curcas* can also promote glandular trichome development in tobacco through gibberellic acid (GA) signaling (Liu *et al.* 2017, 2018, Shi *et al.* 2018). However, the molecular mechanism of glandular trichomes initiation and development in tobacco still remains largely unknown.

The homeodomain-leucine zipper (HD-Zip) transcription factor family is unique to the plant kingdom, and characterized by two indispensable conserved domains: the

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Abbreviations: DAI - days after infiltration; GA - gibberellic acid; MIXTA - myeloblastosis related transcription factor; qPCR - quantitative PCR; SEM - scanning electron microscopy; SGT - short glandular trichome; TGT - tall glandular trichome; VIGS - virus induced gene silencing.

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homeodomain (HD) and the leucine zipper (Zip) domain (Schena and Davis 1992, Ariel *et al.* 2007). Based on the distinctive features of DNA-binding specificities, gene structures, additional common motifs, and physiological functions, the members of HD-Zip family can be divided into four groups, HD-Zip I to IV (Henriksson *et al.* 2005, Ariel *et al.* 2007). HD-Zip proteins play a wide variety of roles during plant growth and development, including lateral organ initiation (Otsuga *et al.* 2010), leaf cell expansion and endoreduplication (Hur *et al.* 2015), floral meristem regulation (Williams *et al.* 2005), and responses to biotic or abiotic stresses (Ariel *et al.* 2007, Brandt *et al.* 2014). The HD-Zip IV proteins have been demonstrated to play a pivotal role in trichome initiation in numerous plants. *Glabrous 2* from *Arabidopsis* is the first homeodomain-leucine zipper IV gene identified, and this gene participates in trichome initiation (Cristina *et al.* 1996). Further studies on *Arabidopsis* demonstrate that other HD-Zip IV proteins, including protodermal factor 2 (Abe *et al.* 2003), homeodomain glabrous 2, homeodomain glabrous 11, and homeodomain glabrous 12 (Khosla *et al.* 2014) also participate in trichome formation and epidermal cell differentiation. Woolly (Wo) is a HD-Zip IV protein that affects trichome formation in tomato (Yang *et al.* 2011) and GL3 is a HD-Zip IV protein from cucumber. the GL3 spontaneous mutant cucumber plants, *trichome-less (tril)* and *csgl3*, show a completely glabrous phenotype (Pan *et al.* 2015, Zhao *et al.* 2015, Cui *et al.* 2016). Comparative transcriptomic analysis of the wild type and *gl3* mutant cucumber indicate that numerous transcription factors, which belonging to homeodomain, MADS, myeloblastosis, ethylene-responsive, and zinc finger family, are differently expressed (Zhao *et al.* 2015). In addition, *Gossypium hirsutum homeodomain leucine zipper 1* (Walford *et al.* 2012) and *Gossypium barbadense meristem layer 1* from cotton (Zhang *et al.* 2010), and *outer cell layer 4* from maize (Vernoud *et al.* 2010), also belong to HD-Zip IV protein family and participate in trichome development.

The aim of this study was to investigate the expression pattern of *NbGL3*, a HD-Zip IV family gene, in different tobacco organs and the expression of *NbGL3* in response to plant hormones ethylene and auxin. In order to study the function of *NbGL3*, we employed virus induced gene silencing (VIGS) system to silence the expression of *NbGL3* and observed trichome development with scanning electron microscopy (SEM).

Materials and methods

Plants and growth conditions: Tobacco (*Nicotiana benthamiana* Domin) seedlings were cultured in a greenhouse under a 16-h photoperiod, an irradiance of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$, day/night temperatures of 23/19 °C, and a relative humidity of 80 %. The seeds were germinated, the seedlings were grown until the second leaf could be observed, and then transplanted into the small basins. Then the seedlings with 4 - 5 true leaves (about 20 d after transplantation) were used for *Agrobacterium* infiltration.

The wild type tobacco plants about two months after transplantation which had entered flowering stage were used to collect samples (root, stem, mature leaf, young leaf, petiole, petal, sepal, fruit) for *NbGL3* expression pattern analysis. Systemic leaves 20 days after infiltration (DAI) were used for RNA extraction and VIGS gene verification. The leaves one week after infiltration were collected for RNA extraction and downstream gene expressions.

Vector construction: For VIGS vector construction, a 660 bp fragment from the *NbGL3* coding sequence was amplified with specific primers: 5' - AAGGTTACCGAATTCTCTA GATCTTGTTAATTCAGGCCTCGC - 3' and 5' - TG TCTTCGGGACATGCCCCGGGGTCGAACCATCTGG TAGTATC-3'. Linearized *pTRV2* vector was generated using the *XbaI* and *SmaI* restriction enzymes. The PCR product was ligated with linearized *pTRV2* vector using the *ClonExpress II* one step cloning kit (Vazyme, Nanjing, China). The reconstructed vector was transformed into competent *Escherichia coli* cells and verified by sequencing. The verified *pTRV2:NbGL3*, empty *pTRV2*, and *pTRV1* vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 by heat shock method.

Agrobacterium infiltration: *A. tumefaciens* strains carrying TRV-mediated vectors were cultured overnight at 28 °C in the appropriate antibiotic selection medium. Then the cultures were spun down and resuspended in infiltration medium (10 mM MES, 10 mM MgCl_2 , 200 mM acetosyringone) to an absorbance of 1 at 600 nm. The resuspended cells were incubated at room temperature for 3 h. Before infiltration, *A. tumefaciens* strain harboring *pTRV1* vector were mixed with that harboring *pTRV2:NbGL3* or *pTRV2* empty vector at a volume ratio of 1:1. The strain harboring *pTRV2:NbPDS* was used as a positive control to assess the efficiency of VIGS system. *N. benthamiana* infiltration was performed as previously reported (Senthilkumar *et al.* 2014).

Plant hormone treatment: To examine the effect of plant hormones on *NbGL3* expression, the tobacco plants (20 DAI) were sprayed with GA (100 μM), 6-benzylaminopurine (6-BA; 50 μM), methyl jasmonate (MeJA; 100 μM), naphthaleneacetic acid (NAA; 100 μM), or 1-amino-1-cyclopropanecarboxylic acid (ACC; 1 μM), respectively. The control plants were sprayed with their corresponding mock solutions. The fourth leaf from the top at least from three plants at 3 and 6 h after treatment were harvested for RNA extraction.

Real-time quantitative PCR (qPCR) analysis: Total RNA was extracted using the plant total RNA isolation kit (Foregene, Chengdu, China). The cDNA was synthesized using *PrimeScript*TM RT reagent kit (Takara, Dalian, China) according to the manufacturer's instructions. The qPCR was performed on a *CFX96*TM real-time PCR system using *SYBR Green Master Mix* (Vazyme). The house-keeping gene *GAPDH* was used as an internal reference. The primers used are listed in Table 1 Suppl.

Scanning electron microscopy: Systemic leaves, stems, and sepals from the plants 20 DAI with *pTRV2:NbGL3* or *pTRV2* empty vector were used for SEM analysis. Samples treatment and SEM images were conducted as previously described (Chen *et al.* 2014).

Results

To determine the *NbGL3* function in glandular trichome development, we first investigated the expressions of *NbGL3* in different organs and developmental stages. Our results showed that *NbGL3* was expressed in all organs, and the highest expression occurred in mature leaves. Sepals and petals also possessed relatively high transcript abundance, while transcript abundance in root was quite low (Fig. 1A). The expression pattern of *NbGL3* was relatively consistent with the distribution of glandular trichomes, which indicated that *NbGL3* probably participated in glandular trichome development in tobacco.

Plant hormone plays an important role in trichome development. In order to study whether *NbGL3* controls the trichome initiation through hormone signaling, we first examined whether the expression of *NbGL3* in mature leaves could be induced by the application of plant hormones. Tobacco plants were sprayed with 100 μ M GA, 50 μ M 6-BA, 100 μ M MeJA, 100 μ M NAA, or 1 μ M ACC and the leaves were harvested at 3 and 6 h after hormone application. The real-time qPCR analysis was performed to examine the *NbGL3* transcript abundance. The expression of *NbGL3* was significantly higher in NAA and ACC treated plants than in their negative controls. However, GA, 6-BA, and MeJA treatment plants had no effect on *NbGL3* expression. These results suggest that *NbGL3* expression could be induced by auxin and ethylene.

In order to explore the role of *NbGL3* in tobacco, we employed VIGS system to silence its expression. The *Nicotiana benthamiana* *phytoene desaturase* gene was used as a positive control to assess the efficiency of VIGS system. At 20 DAI, tobacco plants showed systematical bleaching phenotype, which indicated successful silencing *Nicotiana benthamiana* *phytoene desaturase* (Fig. 1 Suppl.). Real-time qPCR analysis performed on leaves, stems, and sepals collected from the infiltrated plants at 20 DAI further confirmed silencing *Nicotiana benthamiana* *phytoene desaturase* (Fig. 1 Suppl.). The transcription of *NbGL3* gene was significantly decreased in the *pTRV2:NbGL3* infiltrated plants comparing with that infiltrated with the *pTRV2* empty vector (Fig. 1C). These results suggested that *NbGL3* gene was successfully silenced.

The expression of *NbGL3* was significantly reduced in *pTRV2:NbGL3* infiltrated plant (Fig. 1C). To assess the effect of *NbGL3* on glandular trichome development, we checked the glandular trichome density on leaves, stems, and flower sepals from *pTRV2:NbGL3* and *pTRV2* empty vector infiltrated plants. Compared to empty vector control plants, a decrease in glandular trichome density was observed in *NbGL3* silenced plants (Figs 2A and 3A). In order to determine whether *NbGL3* regulates glandular

trichome initiation in tobacco, we counted the number of glandular trichomes in *pTRV2:GL3* and *pTRV2* empty vector infiltrated plants. The results showed that trichome number per area was significantly decreased on *NbGL3* gene silenced leaves, stems, and sepals comparing with the negative control plants (Figs 2B and 3B).

There are two kinds of glandular trichomes on *N. benthamiana*: short glandular trichomes (SGTs) and the tall glandular trichomes (TGTs) (Fig. 2 Suppl.). The SGTs

Fig. 1. Real-time quantitative PCR analysis of the *NbGL3* gene. A - The expression pattern of the *NbGL3* gene in different organs. B - Expression of the *NbGL3* gene in response to different plant hormones. C - Expression of the *NbGL3* gene in different organs treated with virus induced gene silencing. Expression in control plants was set to 1. *TRV2* - control plant treated with the *TRV2* empty vector. Means $\pm$ SEs, $n = 3$, * - $P < 0.05$, ** - $P < 0.01$ (the Student's *t*-test).

Fig. 2. Glandular trichomes on different organs. *A* - Trichomes on different organs from *NbGL3* silenced (pTRV2:GL3) and control (pTRV2) plants. *B* - Glandular trichome density on leaves, sepals, and stems in *NbGL3* silenced and control (pTRV2) plants. Three *TRV2:NbGL3* infiltrated plants were used for analysis. Means $\pm$ SEs, $n = 3$, * - $P < 0.05$, ** - $P < 0.01$ (the Student's *t*-test).

have a short unicellular stalk, on the top of which sits a head containing several nonchlorophyllous cells. The TGTs have a multicellular stalk and a head with a single or several chlorophyllous cells (Nielsen *et al.* 1991). *NbGL3* silenced plant showed a reduction of glandular trichome density. To determine which kind of glandular trichome initiation was affected by *NbGL3*, SEM was performed to observe the glandular trichome phenotypes in detail. SEM images showed that the ratio of TGTs and SGTs on different organs was different. Tobacco leaves and sepal edge showed a relatively higher amount of TGT, while sepal and stem had an opposite phenotype (Fig. 3A,B). The TGTs were not observed on *NbGL3* silenced plant leaves, stems, and sepals. The SGTs density showed a mixed result, with an increased density on leaves and a decreased on sepals and stems. These results indicated that *NbGL3* could promote the long glandular trichomes initiation.

Silencing *NbGL3* in tobacco resulted in inhibition of long glandular trichomes; this results indicated that *NbGL3* might function in a similar way as its homologous genes in cucumber and tomato (Yang *et al.* 2011, Cui *et al.* 2016). In order to further confirm the function of *NbGL3* in tobacco, real-time qPCR analysis was conducted to examine the transcripts abundance of the genes whose homologs were differently expressed in *tril* and *csgl3* mutant plant. As shown in Fig. 4, expression of all downstream genes were

decreased in *NbGL3* silenced plants, and our results were consistent with previous reports (Yang *et al.* 2011, Cui *et al.* 2016, Wang *et al.* 2016).

Discussion

A key and unique feature of glandular trichomes is their ability to synthesize and secrete large amounts of specialized metabolites (Huchelmann *et al.* 2017). As such, they can be considered as “chemical factories”, making them interesting targets for metabolic engineering (Schillmiller *et al.* 2010, Huchelmann *et al.* 2017). Elucidating the transcription network regulating glandular trichome development will provide great foundation for metabolic engineering targeting to the chemicals produced by glandular trichomes. Genes controlling trichome development have been extensively studied in *Arabidopsis*. However, according to the present knowledge, mechanisms controlling the multicellular glandular trichome development in tomato and tobacco is partially different from that of unicellular trichomes in *Arabidopsis* and cotton (Serna and Cathie 2006). *GLABROUS1 (GL1)* is a myeloblastosis gene required for trichome development in *Arabidopsis* (Marks and Feldmann 1989). However, overexpression of *GL1* in tobacco could not induced trichome formation (Payne *et al.* 1999). Overexpression

Fig. 3. Glandular trichomes analyzed by scanning electron microscopy. *A* - Glandular trichomes on different organs from *NbGL3* silenced and control (*pTRV2*) plants. Scale bars: 1.00 mm (left and middle) and 500 μ m (right). *B* - Glandular trichome density on different organs from *NbGL3* silenced and control (*pTRV2*) plants. TGT - tall glandular trichome, SGT - short glandular trichome. Means $\pm$ SEs, $n = 3$, * - $P < 0.05$, ** - $P < 0.01$ (the Student's *t*-test).

Fig. 4. Real-time quantitative PCR analysis of seven putative downstream genes of *NbGL3*. Leaves from *NbGL3* silenced and control (*pTRV2*) plants were used for analysis. *GL2-like* - *glabrous 2 like*, *CycB2* - *cyclin B2*, *HD-8* - *homeodomain 8*, *MIXTA* - *myeloblastosis related transcription factor*, *WIN1-like* - *wax inducer 1 like*, *MYB76-like* - *myeloblastosis 76-like*, *Mict* - *micro trichome*. Means $\pm$ SEs, $n = 3$, * - $P < 0.05$ (the Student's *t*-test).

of *MIXTA*, a myeloblastosis related transcription factor controlling conical cell formation in snapdragon, could boost trichome initiation in tobacco (Payne *et al.* 1999) and *A. majus* (Glover *et al.* 1998). Whereas ectopic expression of *MIXTA* in *Arabidopsis gl1* mutant plants failed to rescue the glabrous phenotype (Payne *et al.* 1999).

As described above, HD-Zip IV proteins seem to play an important roles in trichome development. In this study, we investigated the role of NbGL3, a HD-Zip IV protein, in *N. benthamiana*. The expression pattern of *NbGL3* showed that *NbGL3* has a relatively higher transcription in leaves and sepals (Fig. 1A), this expression pattern was consistent with the TGTs distribution. In *Arabidopsis*, *glabrous 2* promotes trichome initiation in shoots, but acts as a repressor in root hair development (Qing and Aoyama 2012). The low expression of *NbGL3* in root indicated that *NbGL3* may not play an important role in root hair development in tobacco.

Plant hormones play an important role in trichome development in most plant. However, hormone regulation pathways are not conserved among different plants. In addition, different hormones may regulate trichome development through different transcription networks in the same species (Pattanaik *et al.* 2014). Previous reports showed that *AtGIS* and *NbGIS* regulated glandular trichomes in tobacco through GA signaling pathway. In our study, expression of *NbGL3* only showed response to ethylene and auxin, and external gibberellin, cytokinin, and jasmonic acid methyl ester had no effect on *NbGL3* expression (Fig. 1B). These results indicated that *NbGL3* might regulate glandular trichome development in tobacco through ethylene and auxin signaling.

In tobacco plant, tall and short glandular trichome types have been identified (Nielsen *et al.* 1991). While the former have been extensively studied and match the classic picture of trichome function, the short trichomes have remained less characterized (Sallets *et al.* 2014). TGTs were completely disappeared on *NbGL3* silenced plant surfaces (Fig. 3A,B). However, the changes of SGTs densities were more complex, with an increase on leaves and a decrease on sepals and stems (Fig. 3B). These results indicated that development of TGTs and SGTs might be controlled by different mechanisms and *NbGL3* mainly functioned in TGTs initiation. Wo is a HD-Zip IV protein and regulates glandular trichomes development in tomato. Wo could induce the expression of a cell cycle protein coding gene, *cyclin B2*, and further physically interacted with cyclin B2 protein to regulate glandular trichome development (Yang *et al.* 2011, 2015). Transcriptomic analysis in cucumber *csgl3* and *tril* mutant (Zhao *et al.* 2015, Cui *et al.* 2016, Wang *et al.* 2016), tomato *wolly* plant (Yang *et al.* 2011), and tobacco plant transformed with *Wo* from tomato (Yang *et al.* 2015) identified a number of putative downstream genes. Several putative downstream genes were analyzed by real-time qPCR in the present study and our results were consistent with previous reports (Fig. 4). However, further studies have to be conducted to confirm if the NbGL3 protein could directly bind to the promoter region of the downstream genes to regulate their expression.

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