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RESEARCH PAPER

Promoter of a cotton fibre MYB gene functional in trichomes of *Arabidopsis* and glandular trichomes of tobacco

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Abstract

Cotton fibres are unicellular seed trichomes. Our previous study suggested that the cotton R2R3 MYB transcript factor GaMYB2 is a functional homologue of the *Arabidopsis* trichome regulator GLABRA1 (GL1). Here, the *GaMYB2* promoter activity is reported in cotton (*Gossypium hirsutum*), tobacco (*Nicotiana tabacum*), and *Arabidopsis* plants. A 2062 bp promoter of *GaMYB2* was isolated from *G. arboreum*, and fused to a β-glucuronidase (*GUS*) reporter gene. In cotton, the *GaMYB2* promoter exhibited activities in developing fibre cells and trichomes of other aerial organs, including leaves, stems and bracts. In *Arabidopsis* the promoter was specific to trichomes. Different from *Arabidopsis* and cotton that have unicellular non-glandular simple trichomes, tobacco plants contain more than one type of trichome, including multicellular simple and glandular secreting trichomes (GSTs). Interestingly, in tobacco plants the *GaMYB2* promoter directed *GUS* expression exclusively in glandular cells of GSTs. A series of 5′-deletions revealed that a 360 bp fragment upstream to the translation initiation codon was sufficient to drive gene expression. A putative *cis*-element of the T/G-box was located at -233 to -214; a yeast one-hybrid assay showed that *Arabidopsis* bHLH protein GLABRA3 (GL3), also a trichome regulator, and GhDEL65, a GL3-like cotton protein, had high binding activities to the T/G-box motif. Overexpression of *GL3* or *GhDEL65* enhanced the *GaMYB2* promoter activity in transgenic *Arabidopsis* plants. A comparison of *GaMYB2* promoter specificities in trichomes of different plant species with different types of trichomes provides a tool for further dissection of plant trichome structure and development.

Key words: Cotton fibre, glandular, MYB, promoter, tobacco, trichome.

Introduction

Trichomes are specialized epidermal appendages found in the surface of aerial organs of most land plants. There are several types of trichomes: unicellular or multicellular, branched or unbranched, and glandular or non-glandular. Trichomes contribute to many aspects of plant adaptation to biotic and abiotic stresses, such as to fence off insect herbivores, regulate surface temperature, decrease water loss through transpiration, increase tolerance to freezing, assist seed dispersal, and protect plant tissues from UV light (Eisner *et al*., 1998; Werker, 2000; Wagner *et al*., 2004). Glandular secreting trichomes (GSTs) often secrete plant secondary metabolites to constitute natural product-based resistance to herbivores and pathogens (Werker, 2000; Ranger and Hower, 2001; Wagner *et al*., 2004; Medeiros and Tingey, 2006). Many trichome-produced or trichome-stored compounds are of commercial value, such as those used in spice principal and pharmaceuticals production (Krings and Berger, 1998; McCaskill and Croteau, 1999; Wagner *et al*., 2004). For example, artemisinin, a sesquiterpene lactone that is widely used for the treatment of malaria, accumulates in glandular trichomes of *Artemisia annua* (Lommen *et al*., 2006; C Liu *et al*., 2006).

Different plant species may have different types of trichomes, and one plant may bear more than one type of

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Abbreviations: GUS, β-glucuronidase; 4-MU, 4-methylumbelliferone; 3-AT, 3-amino-1, 2, 4-triazole; DPA, days post-anthesis; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; X-Gluc, 5-bromo-4-chloro-3-indolylglucuronide; RT-PCR, reverse transcription-PCR; GST, glandular secreting trichome; ONPG, *O*-nitrophenyl-β-D-galactopyranoside.

trichomes. The annual weed *Arabidopsis thaliana* produces unicellular non-glandular trichomes, which are either branched or unbranched (Szymanski *et al*., 2000). Tobacco plants usually contain multicellular trichomes, including tall glandular secreting trichomes (GSTs) and simple glandless trichomes (Wagner *et al*., 2004). Recently, small procumbent glandular secreting trichomes, which accumulate antimicrobial proteins, were found in the aerial surfaces of tobacco (Shepherd *et al*., 2005), and sunflower (Kroumova *et al*., 2007). Cotton fibres are single-celled and extensively elongated seed trichomes, which provide the most important natural fibre for the textile industry (Kim and Triplett, 2001).

Cotton fibre development is a complicated and ordered process under the regulation of a vast number of genes, many of which are up-regulated or highly expressed in developing fibre cells (CH Li *et al*., 2002; Ruan *et al*., 2003; S Wang *et al*., 2004; Li *et al*., 2005; Luo *et al*., 2007). In recent years, comprehensive analyses of gene expression profiles have provided valuable clues to understanding cotton fibre formation (Arpat *et al*., 2004; Yang *et al*., 2006; Shi *et al*., 2006; Gou *et al*., 2007; Lee *et al*., 2007). To explore the molecular mechanisms regulating cotton fibre development, promoters of several cotton fibre genes have been identified. *E6* was the first of such genes to be reported, and the *E6* promoter has been used for engineering cotton fibre quality (John and Keller, 1996). *GhRDL1*, a gene highly expressed in cotton fibre cells at the elongation stage, encodes a BURP domain-containing protein (CH Li *et al*., 2002), and the *GaRDL1* promoter exhibited a trichome-specific activity in transgenic *Arabidopsis* plants (S Wang *et al*., 2004). *GhTUB1* transcripts preferentially accumulate at high levels in fibre, accordingly, the *pGhTUB1*::*GUS* fusion gene was expressed at a high level in fibre but at much lower levels in other tissues (XB Li *et al*., 2002). Promoters of three cotton lipid transfer protein genes, *LTP3*, *LTP6*, and *FSltp4*, were able to direct *GUS* gene expression in leaf and stem GSTs in transgenic tobacco plants (Hsu *et al*., 1999; Liu *et al*., 2000; Delaney *et al*., 2007), however, they did not exhibit a clear tissue-specificity. For example, in *pFSltp4::GUS* transgenic tobacco plants, strong GUS activity could be detected in all types of trichomes; in addition, GUS expression was also visible at the leaf margin, vascular tissue, ovules, and root tips (Delaney *et al*., 2007).

Previously it was reported that the cotton R2R3 MYB transcription factor GaMYB2 is a functional homologue of *Arabidopsis* GLABRA1 (GL1), a key regulator of *Arabidopsis* trichome formation. Northern blot and *in situ* RNA hybridization showed that *GaMYB2* is expressed in cotton fibre cells at the early developmental stages (S Wang *et al*., 2004). In order to dissect the regulation of *GaMYB2* gene expression further, the *GaMYB2* promoter was isolated and its activity in cotton, *Arabidopsis*, and tobacco plants was analysed. It is shown that, while highly active in developing cotton fibre cells, this promoter is trichome-specific in *Arabidopsis* and GST head-specific in tobacco. It is further shown that a *cis*-element of the T/G-box, which can be recognized by bHLH transcription factors, such as *Arabidopsis* GL3 and cotton GhDEL65, contributes to the promoter activity in transgenic *Arabidopsis*.

Materials and methods

Plant materials and growth

Plants of cotton (*Gossypium hirsutum* cv. R15 and *G. arboreum* cv. Qingyangxiaozi) and tobacco (*Nicotiana tabacum*) were grown in a greenhouse at 28±2 °C with a natural photoperiod. Transgenic cotton and tobacco plants were at first cultured under 26 °C in a tissue culture room. Plants of *Arabidopsis thaliana* (Columbia-0, Col-0 ecotype) were grown indoors at 22 °C under a 16 h light period.

Genome walking

Genomic DNA was isolated from *G. arboreum* leaf tissue as described (XB Li *et al*., 2002), and genome walking was performed to isolate the *GaMYB2* upstream fragment according to the Genome Walker kit (Clontech, Palo Alto, CA). The DNA was completely digested with selected restriction enzymes, and ligated to the corresponding adaptors to generate several DNA fragment libraries. The corresponding library was subjected to a first round of PCR amplification with the outer adaptor primer (AP1) and an outer gene-specific primer (GSP1), while the inner adaptor primer (AP2) and inner gene-specific primer (GSP2) were used for the second round of PCR. After two rounds of PCR, DNA fragments amplified were cloned into the pMD18-T vector (TakaRa, Japan) for sequencing.

All the primers used in this investigation are shown in Supplementary Table S1 at *JXB* online.

Vector construction

A 2062 bp promoter fragment of *GaMYB2* was re-amplified with a pair of primers carrying an *Xba*I and a *Bam*HI restriction site, respectively. Shorter promoter fragments with different lengths of 5′-terminal deletions were similarly amplified with each primer pairs. After digestion, these DNA fragments were inserted into pBI101.1 vector (Clontech), upstream of *GUS* gene coding region, resulting in a series of *pGaMYB2::GUS* binary vectors, namely P-2000 (-2062/-1, 2062 bp), P-1000 (–1000/-1, 1000 bp), P-750 (-750/-1, 750 bp), P-440 (-440/-1, 440 bp), P-360 (-360/-1, 360 bp), and P-220 (-220/-1, 220 bp). To construct the P-AB1 (-440/-1, the 20 bp fragment of -233 to -214 was deleted) and P-AB2 (-440/-1, the 87 bp fragment of -317 to -231 deleted) vectors, a PCR-based two-step DNA synthesis method was used as described (Wang and Malcolm, 2002).

The 35*S* promoter and *NOS* terminator were inserted into the *Sac*I/*Eco*RI and *Hin*dIII/*Pst*I sites of pCAMBIA1300, respectively, forming a p1300–35S-NOS intermediate vector. The *Arabidopsis GLABRA3* (*GL3*) cDNA and the genomic sequence of *GhDEL65* were amplified by PCR and were inserted into the *Bam*HI/*Pst*I site of the p1300–35S-NOS, respectively, generating 35S*::GL3* and *35S::GhDEL65* fusion gene constructs. The vectors were transferred into *Agrobacterium tumefaciens* strain LBA4404 or GV3101 and used for plant transformation.

Plant transformation and GUS assay

Agrobacterium-mediated cotton transformation was performed as described (XB Li *et al*., 2002). The hypocotyl segments were used as explants for transformation. After the stages of callus induction, proliferation, embryogenic callus induction, embryo differentiation, and finally plantlet regeneration, the plantlets were transferred to pots in greenhouse for further growth. For tobacco transformation, a leaf disc transformation method (Horsch *et al*., 1985) was employed. Transformants were selected on MS medium containing 100 mg l^{-1} of kanamycin and 500 mg l^{-1} of cefotaxime. Transgenic *Arabidopsis* plants were generated by a floral dip method (Clough and Bent, 1998), and screened on half-strength MS agar medium containing 50 mg l^{-1} of kanamycin or hygromycin. Histochemical localization and fluorometric quantification of GUS activities were performed as described (Jefferson *et al*., 1987).

RNA analysis

Total RNAs were isolated from plant materials using a Trizol reagent (Invitrogen, Carlsbad, CA). For RT-PCR, the first strand cDNA was prepared, followed by a standard PCR protocol: 95 °C for 5 min, 27–34 cycles (according to the gene expression level) of denaturation at 95 °C for 20 s and annealing/extension at 56 °C for 30 s.

Yeast one-hybrid assay

Yeast one-hybrid assay was performed using the MATCHMAKER one-hybrid system (Clontech). The DNA fragment of four tandem copies of T/G-box [4× T/G-box (CTGCCACGTTGACAA)] was synthesized and inserted directly into the multiple cloning sites of reporter plasmids of pLacZi and pHISi-1, respectively. These two bait constructs were linearized and integrated into the genome of yeast strain YM4271, the dual reporter strain was selected and maintained on synthetic dextrose (SD)/-His/-Ura medium. For construction of the pGAD-GL3 fusion, GL3 cDNA was ligated with GAL4 activation domain in pGAD424 plasmid, and then was introduced into yeast strain with dual reporter genes, with the blank pGAD424 plasmid as control. Yeast transformants were tested on SD/-Leu/-His/-Ura medium containing different concentrations of 3-amino-1,2,4-triazole (3-AT) and 80 mg l^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 1× BU salt.

β-galactosidase assay was conducted as described (PT3024–1, Clontech). The ORF of GhDEL65, TT8, AtMYC2, and GL3 were in-frame fused with the GAL4 activation domain of the one-hybrid vector pGAD424, and then transferred into yeast cells containing pLacZi-4×T/G-box plasmids, respectively; the blank pGAD424 plasmid was used as control. The unit of β-Gal activity was determined by the equation of $U=1000\times[OD_{420}]/(\text{time (in min)}\times\text{volume (in ml)}\times[OD_{600}]$. For each transformation sample, multiple independent yeast isolates were tested, each for three times.

Results

Isolation and sequence analysis of GaMYB2 promoter

Based on the cDNA sequence of *GaMYB2* (S Wang *et al*., 2004), a 2062 bp promoter fragment upstream to the coding region was isolated from *G. arboreum* by genome walking (see Supplementary Table S2 at *JXB* online). The A of translation initiation codon (ATG) of *GaMYB2* gene was defined as +1. A putative TATA box (-93 to -86) and a CAAT box (-131 to -128), which serve as basal promoter elements for the transcription of eukaryotic genes, were found in the *GaMYB2* promoter. Sequence analysis using PLACE (http://dna.affrc.go.jp/PLACE) showed that a number of putative tissue-specific or stress-induced regulatory motifs corresponding to known *cis*-elements of plant genes were present, such as MYB recognition site, E-box, and T/G box (see Supplementary Table S3 at *JXB* online), implying that the *GaMYB2* promoter may be under a complex regulation.

GaMYB2 promoter has a high activity in cotton fibre

Our previous investigation showed that *GaMYB2* was preferentially expressed in fibre cells at the early developmental stages, and this R2R3 MYB gene was able to rescue the glabrous phenotype of the *Arabidopsis gl1* mutant (S Wang *et al*., 2004). These experimental data suggest that *GaMYB2* may play a role in controlling cotton fibre development. To dissect the *GaMYB2* gene expression pattern further, its promoter activity was examined in cotton plants. The chimeric gene of *P-2000::GUS*, in which a β-glucuronidase (*GUS*) reporter gene was placed behind the promoter, was transferred into cotton (*G. hirsutum*) plants through *Agrobacterium tumefaciens*-mediated transformation. Nine T_0 transgenic lines were generated, and histochemical staining of each line exhibited a similar pattern of GUS expression. Intensive GUS staining was observed in epidermis of young ovules, such as the 0-DPA ovule from which the fibre initials were emerging, and in developing fibre cells (Fig. 1A, B). To a lesser extent, GUS staining was observed in trichomes of other aerial organs, including leaves, stems, and bracts (Fig. 1C–E). Weak GUS staining was also detected in other tissues, such as roots, stamens, and petals, without a clear tissue-specificity (data not shown). Consistent with histochemical staining, the *in vitro* assay of protein abstracts showed the highest specific activity of GUS in cotton fibres, and a lower activity in the 0-DPA ovule. In the 9-DPA ovule from which the fibres were stripped, GUS specific activity was almost completely lost (Fig. 1F). The differences in specific activities among the organs investigated were probably a reflection of the portions of trichome proteins present in each sample, and in cotton plants the *GaMYB2* promoter has a high activity in developing fibre cells and epidermal trichomes.

GaMYB2 promoter displays trichome-specific activity *in* Arabidopsis

To examine the expression pattern of *GaMYB2* promoter in different plant species, the *P-2000::GUS* gene was introduced into *Arabidopsis*. *Arabidopsis* leaf trichomes are mostly branched, but trichomes on stem and sepals are often unbranched; both branched and unbranched trichomes are unicellular (Szymanski *et al*., 2000).

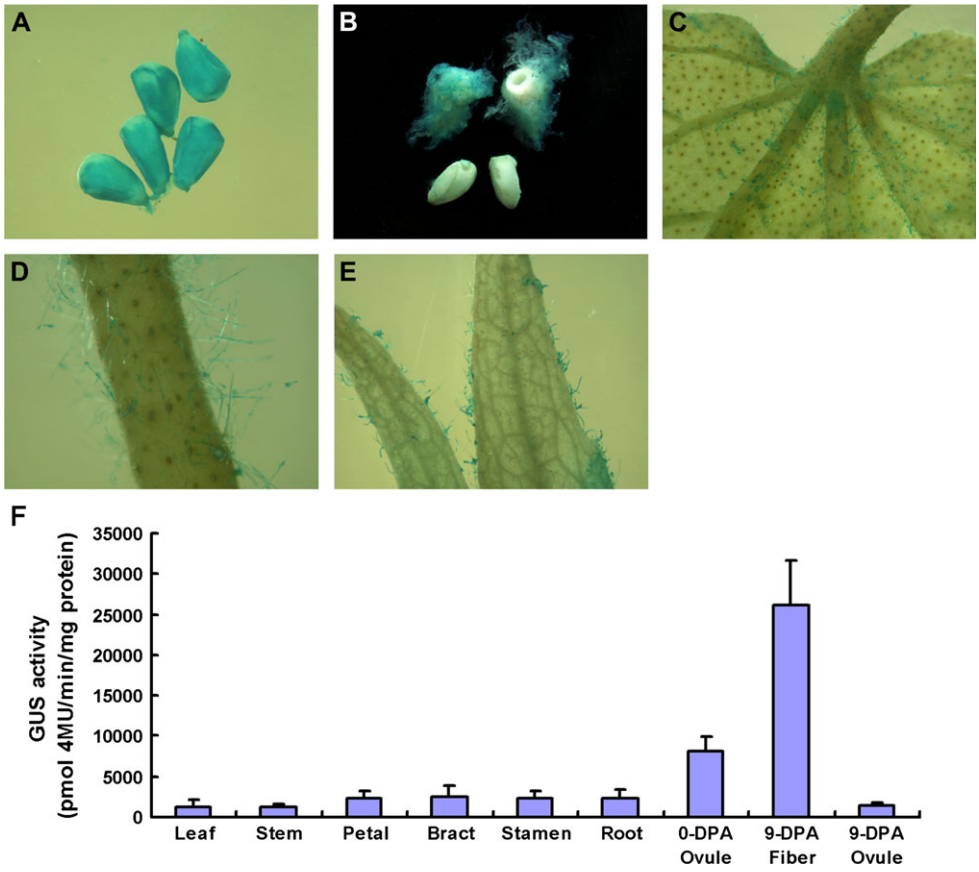

Fig. 1. GUS expression pattern and activities in transgenic cotton (*G. hirsutum*) plants expressing *P-2000::GUS*. (A) 0-DPA ovule; (B) 9-DPA fibre (top) and ovule (bottom); (C) leaf; (D) stem; (E) bract; (F) quantitative analysis of GUS specific activities in different organs.

Histochemical assay of T_1 *P-2000::GUS* plants showed that, in rosette leaves, GUS staining was located exclusively in trichomes (Fig. 2A, B). In stems, the GUS activity was also restricted to trichomes (Fig. 2C). At the flowering stage, GUS activity was again present in the unbranched trichomes of the sepals (Fig. 2D). In 1-week-old seedlings, GUS staining was observable in shoot apical meristems (SAM) and at the margins of the cotyledons, but not in roots (data not shown). Clearly, in *Arabidopsis*, the promoter of this cotton MYB gene drives *GUS* gene expression specifically in trichomes, regardless of their branching status.

GaMYB2 promoter confers specificity to glandular trichomes in tobacco

Distinguished from cotton fibres and *Arabidopsis* trichomes that are unicellular, tobacco plants have multicellular trichomes, including the non-glandular simple trichomes and the GSTs (Wagner *et al.*, 2004; Shepherd *et al.*, 2005). Most of the tobacco organs are covered with GSTs that have a head of glandular secreting cells and a long or short stalk, but on the base of the anther filaments, trichomes are mainly non-glandular. In order

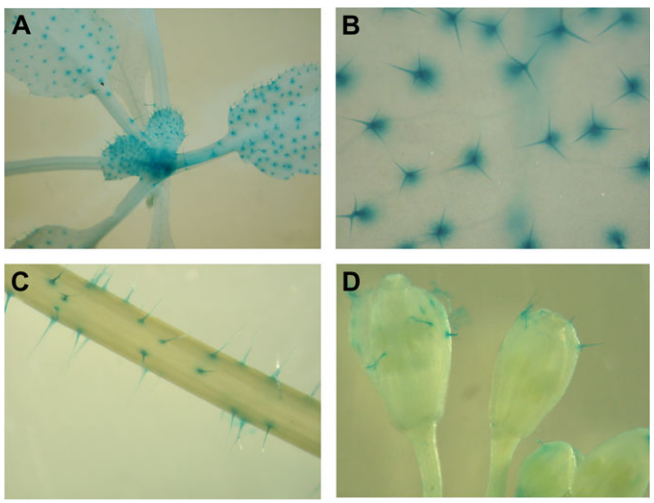

Fig. 2. Histochemical staining of GUS expression pattern in transgenic *Arabidopsis* plants expressing *P-2000::GUS*. (A) 3-week-old seedling; (B) rosette leaf; (C) stem; (D) flowers showing trichomes on sepal.

to test *GaMYB2* promoter activities in the different types of trichomes, transgenic tobacco plants carrying *P-2000::GUS* were generated. GUS staining showed that in

leaves, stems, and bracts, the fusion gene was expressed specifically in the GSTs. Notably, while strong GUS staining was visualized in the glandular head of GSTs, the GUS activity was undetectable in stalk cells (Fig. 3A–C, E). Occasionally faint staining appeared in the stalk cells adjacent to the glandular head, which might be a result of diffusion. No GUS staining was observed in multicellular simple trichomes of the anther filament (Fig. 3D, F). In 1-week-old seedlings which were trichomeless, no GUS staining was observed in the hypocotyl, cotyledon, and root (data not shown). These data demonstrate that, in tobacco plants, the *GaMYB2* promoter drives gene expression only in glandular secreting cells, and it has no activity in other types of trichome cells.

Promoter deletion analysis

To find regulatory regions important for trichome-specific activity of the *GaMYB2* promoter, several DNA fragments of different 5′-deletions were generated by PCR and fused to the *GUS* gene (see Supplementary Table S4 at *JXB* online). These expression cassettes were then introduced into *Arabidopsis* and tobacco plants, respectively. Assay of transgenic *Arabidopsis* plants revealed that a 360 bp fragment proximal to the coding region was sufficient to drive GUS expression in *Arabidopsis* trichomes, with a similar expression pattern and intensity to that of P-2000 plants (Fig. 4A, B). Further deletion of the promoter decreased the gene expression level, as only about 1/3 of the P-220 plants showed weaker GUS staining under the same staining conditions used for P-360 plants (Fig. 4C), and the specific activity of GUS was decreased to about 30% of that of P-360 plants (Fig. 4E). The expression pattern and the intensity of GUS staining were similar among the five promoters ranging from P-2000 to P-360, implicating that the 360 bp fragment of *GaMYB2* contained all the key *cis*-elements conferring trichome-specific activity.

Similar results were obtained with transgenic tobacco plants. The 360 bp fragment directed *GUS* expression in secreting head cells of GSTs (Fig. 5A, B), with a similar level of specific activity in the leaf as that of P-2000 (data not shown). In the transgenic plants of P-220, the GUS signal became very weak (Fig. 5C).

Activation of transcription by bHLH protein through binding to T/G-box

PLACE analysis revealed a T/G-box element (AACGTG) present at -226 to -221, which attracted attention. The T/G-motif has been shown to play an important regulatory role in tomato defence genes of proteinase inhibitor II and leucine aminopeptidase (*LAP*). *JAMYC2* and *JAMYC10*, both encoding the basic helix-loop-helix (bHLH) domain-containing transcription factor, specifically recognize the T/G-box motif in the promoter of these two genes and

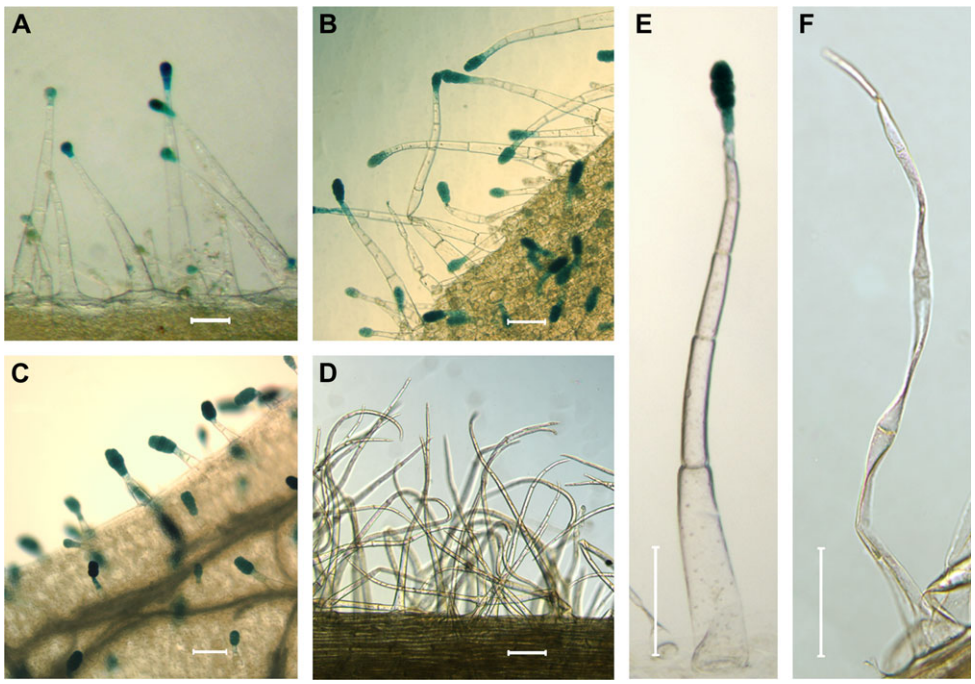

Fig. 3. Histochemical staining of GUS expression pattern in transgenic tobacco plants expressing *P-2000::GUS*, GUS activity was detected in glandular head cells of the multicellular glandular secreting trichome (GST). (A) GSTs on leaf; (B) GSTs on stem; (C) GSTs on flower bract; (D) multicellular simple trichomes on the base of anther filament; (E) magnified view of a leaf GST; (F) magnified view of an anther filament simple trichome. Bar = 100 μm.

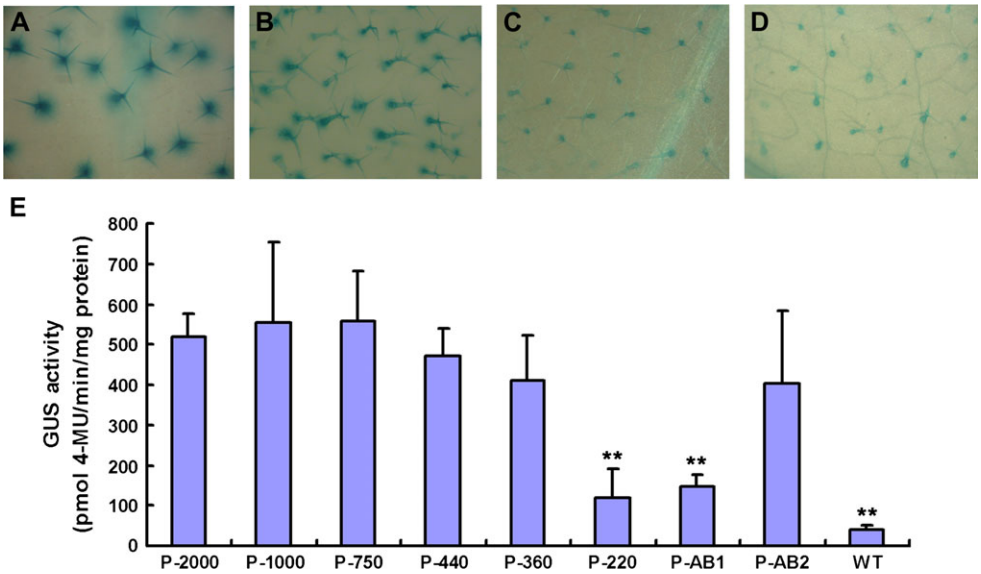

Fig. 4. Analysis of *GaMYB2* promoter activities with different deletions, GUS activities in transgenic *Arabidopsis* plants were assayed. (A–D) GUS staining of rosette leaves of P-2000 (A), P-360 (B), P-220 (C), and P-AB1 (D) plants; (E) quantitative analysis of GUS specific activities in rosette leaves of *Arabidopsis* transformed with *GUS* gene driven by different versions of *GaMYB2* promoters as indicated (see also Supplementary Table S4 at *JXB* online). Leaves of 3–4-week-old plants of ten different transgenic lines were used for GUS assay. $^{**}P \leqslant 0.01$ versus P-2000 plants.

transactivate their expression (Boter *et al*., 2004). Similar to these MYC proteins, GL3, a key regulator of *Arabidopsis* trichome development, also contains a conserved bHLH domain (Payne *et al*., 2000). A recent report showed that GL3 is able to bind to the promoter sequence and activate transcription of MYB transcription factor genes, such as *CPC* and *ETC1*, which are negative regulators of trichome development (Morohashi *et al*., 2007). It was then asked if GL3 could activate the *GaMYB2* promoter.

First, a modified promoter, P-AB1, that lacked the T/G-box was generated by removing a 20 bp fragment (-233 to -214) from P-440. *Arabidopsis* plants harbouring *P-AB1::GUS* exhibited a strong reduction of the GUS signal in the trichome (Fig. 4D) and the specific activity of GUS in leaf proteins was dramatically decreased (Fig. 4E). A similar reduction of promoter activity was detected in P-AB1 tobacco plants, in which the GUS staining in GSTs was very faint (Fig. 5D). Another promoter, P-AB2, was then made in which a 87 bp fragment (-317 to -231) was removed from P-440, while the T/G box was intact. Analysis of *Arabidopsis* plants revealed that deletion of this 87 bp sequence resulted in only a marginal loss of activity (Fig. 4E). These deletion results suggest that the 17 bp region between -230 and -214 plays an important role in activating the *GaMYB2* promoter, further supporting the assumption that the T/G-box present in this region may serve as a *cis*-acting element conferring promoter activity in trichomes.

The binding activity of GL3 to the T/G-box motif of *GaMYB2* promoter was then tested by the yeast one-hybrid assay, using a 60 bp DNA fragment containing 4× T/G-box (four tandem repeats of the T/G-box element and its flanking sequence). It was found that only the yeast clones harbouring the pGAD-GL3 plasmid grew on the medium used for the assay (Fig. 6A), indicating that *Arabidopsis* bHLH protein GL3 is indeed

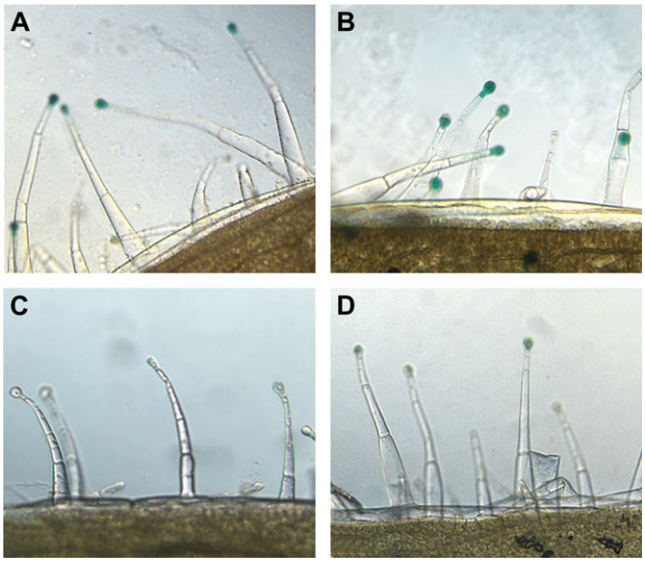

Fig. 5. GUS staining of tobacco plants transformed with the *GUS* gene driven by the *GaMYB2* promoter with different deletions. (A–D) GSTs on leaf of the P-2000 (A), P-360 (B), P-220 (C), and P-AB1 (D) plants. All the plants assayed were at rooting stage.

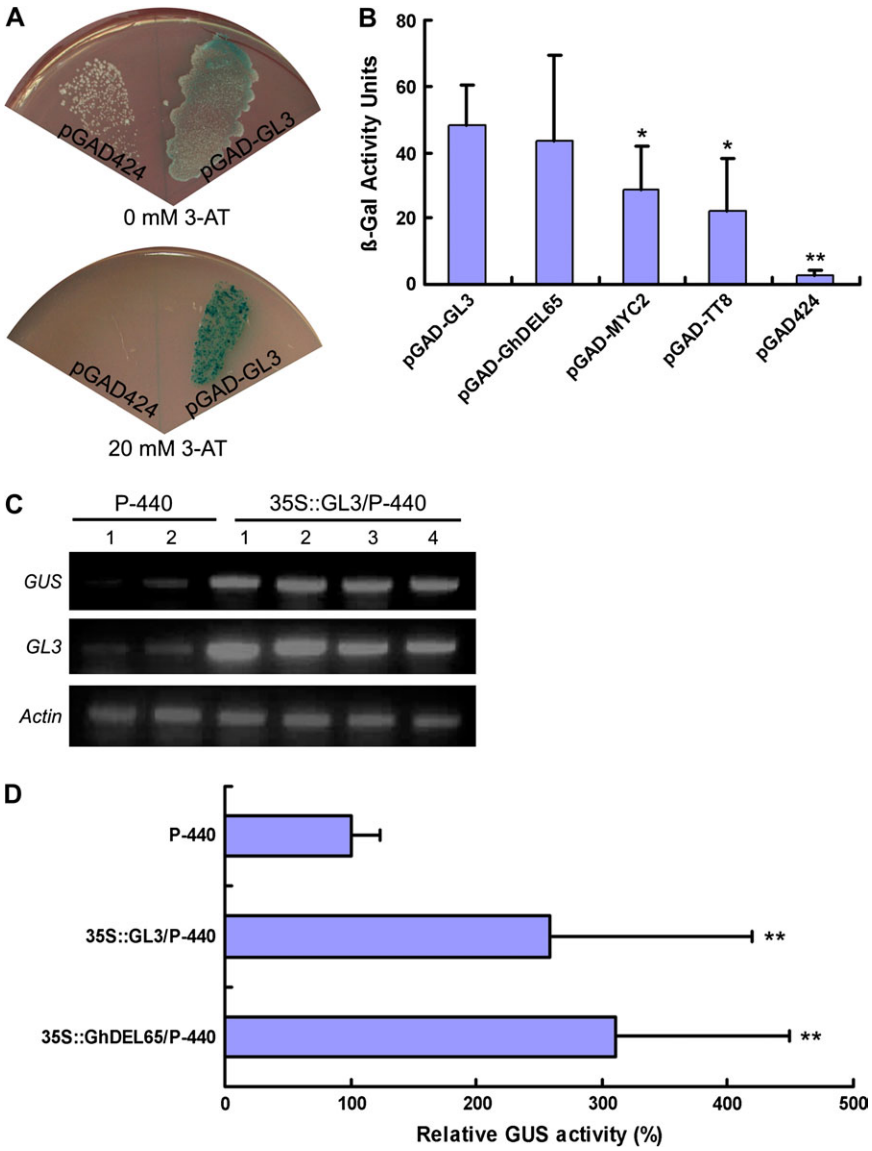

Fig. 6. Transcriptional activation of *GaMYB2* promoter by bHLH protein. (A) DNA–protein interaction in a yeast one-hybrid system. pGAD-GL3 and pGAD424 plasmids were transformed into a yeast strain carrying dual report genes under the control of four-time tandem repeats of the T/G-box element. The transformants were examined for growth in the presence of 3-AT and β-galactosidase (β-Gal) activity using X-Gal as substrate. Only the yeast clones harbouring pGAD-GL3 grew on the -His-Ura-Leu synthetic dextrose (SD) base containing 20 mM 3-AT and also showed a high β-Gal activity (blue staining), demonstrating binding activity of GL3 to the T/G-box. (B) Comparison of T/G-box binding activity of four bHLH proteins. The DNA–protein interaction was determined by β-Gal activity measured through an ONPG assay. $^{**}P \leqslant 0.01$ and $^{*}P \leqslant 0.05$ versus pGAD-GL3 binding activity. (C) RT-PCR analysis of expression of *GL3* and *GUS* genes in *35S::GL3/ P-440::GUS Arabidopsis* plants. Total RNA was isolated from leaves of 4-week-old plants, *Actin2* (At3g18780) was amplified as an internal control. (D) Enhanced activation of the *GaMYB2* promoter by constitutive expression of *GL3* and *GhDEL65* in P-440 plants. Leaves of 4-week-old plants (eight transgenic lines) were used for the GUS activity assay, the mean value of GUS specific activities of P-440 plants was set as 100% and used to define the relative GUS activity of *35S::GL3/ P-440::GUS* and *35S::GhDEL65/P-440::GUS* plants, respectively. $^{**}P \leqslant 0.01$ versus P-440 plants.

able to recognize and interact with the T/G-box motif, and function as a transcriptional activator, at least in yeast.

The *Arabidopsis* genome encodes more than 160 bHLH transription factors, which act as important regulatory components in diverse biological processes (Bailey *et al*., 2003; Toledo-Ortiz *et al*., 2003). Among them TT8 shares 30% amino acid sequence identity with GL3, and it plays a role in regulating the flavonoid pathway by forming a ternary complex with TT2 (a MYB) and TTG1 (a WD-repeat protein) (Baudry *et al*., 2004). Another bHLH transcription factor of *A. thaliana*, AtMYC2, is 26% identical to GL3 based on amino acid sequences. AtMYC2 is an important regulator in the jasmonic acid

(JA) and abscisic acid (ABA) signalling pathways (Abe *et al*., 2003; Boter *et al*., 2004), and has been reported to bind to the MYC-site (CACATG) in the *Arabidopsis RD22* gene promoter (Abe *et al*., 1997) and T/G-box (AACGTG) motif in the tomato *LAP* promoter (Boter *et al*., 2004). A search of the NCBI database for cotton homologues of *Arabidopsis* GL3 retrieved a putative bHLH protein, GhDEL65, which shares ~50% sequence identity with GL3, and is more distantly related to TT8 and AtMYC2 with sequence identities of 35% and 20%, respectively. To see if the *GaMYB2* T/G-box motif was specifically recognized by a certain type of bHLH protein, the β-galactosidase activities of yeast cells expressing each bHLH proteins was compared, respectively, in a yeast one-hybrid system. It was found that while GL3 and GhDEL65 were equally active in interacting with the *cis*-elements, TT8 and AtMYC2 had significantly lower activities (Fig. 6B).

Recognition of the T/G-box motif by GL3 and GhDEL65 prompted the question whether both transcription factors would activate the *GaMYB2* promoter *in planta*. The coding region of the two genes under the control of the 35*S* promoter was introduced into T_2 plants of P-440, respectively. RT-PCR analysis of individual *35S::GL3* transformants showed that the *GUS* transcript level was markedly increased in plants overexpressing *GL3* (Fig. 6C). In comparison with P-440 plants, GUS activities were elevated by about 2.6-fold due to *35S::GL3* expression and about 3.1-fold due to *35S::GhDEL65* expression (Fig. 6D). The trichome specificity, however, was not changed. Therefore, in transgenic *Arabidopsis* plants, constitutive overexpression of *GL3* or *GhDEL65* strongly enhanced the *GaMYB2* promoter. These results suggest that cotton bHLH proteins homologous to GL3 may be involved in regulating *GaMYB2* gene expression during cotton fibre development.

Discussion

It has been shown that a promoter of a cotton fibre MYB gene, *GaMYB2*, directs reporter gene expression specifically in trichomes of *Arabidopsis* and GST head cells of tobacco. Plant GSTs produce and accumulate a rich plethora of specific metabolites, particularly secondary metabolites, and are considered ideal plant cell factories for metabolic engineering (Verpoorte *et al*., 2000; Wagner *et al*., 2004; J Liu *et al*., 2006). Promoters of several cotton genes highly expressed in fibre cells have been reported, and those of *LTP3*, *LTP6*, *FSltp4*, *GhGlcAT1*, and *GhRGP1* genes were examined using transgenic tobacco plants. Although active in GSTs as well, they are less tissue-specific (Hsu *et al*., 1999; Liu *et al*., 2000; Wu *et al*., 2006, 2007; Delaney *et al*., 2007). The high specificity of the *GaMYB2* promoter makes it a valuable tool not only for engineering cotton fibre traits but also for modification of GST metabolism.

In tobacco, which has both simple and glandular secreting trichomes, activity of the *GaMYB2* promoter is restricted to GST head cells. It is inactive in other types of cells, including GST stalk cells and multicellular simple trichomes. This intriguing pattern seems to suggest that unicellular trichomes of cotton and *Arabidopsis* share with tobacco GST head cells a conserved molecular machinery in regulating the expression of a set of genes, but this machinery is not operating in either GST stalk cells or multicellular simple trichomes of tobacco. In *Arabidopsis*, multimeric complexes of MYB-bHLH-WD40 play a key role in regulating trichome patterning and development (Payne *et al*., 2000; Ramsay and Glover, 2005; Serna and Martin, 2006), as well as anthocyanin and flavonoid biosynthesis (Hartmann *et al*., 2005; Koes *et al*., 2005). Recently, the bHLH transcription factor GL3 was shown to bind the promoter of *GL2*, *ETC1*, and *CPC*, a group of genes involved in the development and patterning of trichomes, and directly activate their expression (Morohashi *et al*., 2007). Our results that overexpression of *GL3* or its cotton homologue *GhDEL65* enhanced the *GaMYB2* promoter activity suggest that, in cotton, bHLH transcription factor(s) are probably involved in up-regulating expression of *GaMYB2* and possibly other functionally related R2R3 MYB genes.

The yeast one-hybrid assay showed that GL3 and GhDEL65 have higher binding activities to the *GaMYB2* T/G-box than AtMYC2 and TT8, suggesting that the *GaMYB2* promoter is prone to the recognition by GL3-type bHLH transcription factors. TT8 is similar to GL3 in working mechanisms, as both are recruited to the MYB-bHLH -WD40 activation complex. While GL3 is a trichome and non-root hair cell regulator, TT8 is involved in regulating anthocyanin and flavonoid biosynthesis (Payne *et al*., 2000; Zhang *et al*., 2003; Ramsay and Glover, 2005). The detectable binding activity of TT8 protein to the T/G motif of the *GaMYB2* promoter, although comparatively low, provides a possibility that TT8 homologues in tobacco glandular trichomes, which function in secondary metabolisms, could participate in the activation of the reporter gene expression specifically in glandular cells.

Our previous analysis of the cotton *GaRDL1* promoter showed that the L1-box and MYBCORE are two *cis*-elements conferring trichome specificity to this promoter. Mutation of either element reduced the *GaRDL1* promoter activity in *Arabidopsis* trichomes. Furthermore, expression of MYB (*GL1* or *GaMYB2*) and HOX (*GL2* or *GaHOX3*) transcription factors, responsible for binding the MYBCORE and L1- box, respectively, induced a strong ectopic expression of the reporter gene in non-trichome cells (S Wang *et al*., 2004). The *CYP71D16* promoter of tobacco was able to direct *GUS* gene

expression in glandular cells of GSTs in transgenic tobacco plants (Wang *et al*., 2002), and this promoter has been used for engineering plant defence against aphid infection (E Wang *et al*., 2004). As the promoter deletions progressed, GUS activity decreased and the expression pattern extended. Although still trichome-specific, GUS staining was concentrated in the lowest gland cell and stalk cells, and the MYB-like sequence (CAACAG) between -56 and -51 was speculated be important for trichome specificity (Wang *et al*., 2002). *SaPIN2b*, a nightshade (*Solanum americanum*) proteinase inhibitor II gene, containing six MYB-binding motifs and an L1 box in its promoter region, was constitutively expressed in GSTs; similar to the *CYP71D* promoter, when *SaPIN2b* promoter deletion proceeded a small portion of the trichomes showed a shift of GUS activity to the stalk cell (J Liu *et al*., 2006). The *Arabidopsis OASA1* promoter was reported to direct gene expression in GSTs and simple trichomes, and the MYB motifs located in the promoter and the first intron region of this gene may act as enhancer elements in trichome cells (Gutierrez-Alcala *et al*., 2005). *AtTSG1* promoter also showed trichome-specific activity in *Arabidopsis*; deletion analysis of this promoter indicated that the MYB-like recognition site (AACCAAAC) was a putative element for trichome specific expression of this gene (Ni *et al*., 2008). In tobacco, the promoter of the *T-phyllopanin* gene directed reporter gene expression specifically in short procumbent trichomes, which could explain the biosynthesis of T-phylloplanin proteins only in this particular type of glandular trichome (Shepherd *et al*., 2005). Despite these interesting findings and speculations, *cis*-elements and the related transcription factors conferring glandular trichome expression await further identification.

Although the fragment of -233 to -214 containing a T/G-box was important for the activity of the *GaMYB2* promoter in trichome, removal of this *cis*-element decreased the promoter activity greatly, but did not change the GUS staining pattern. Furthermore, ectopic expression of *GL3* or *GhDEL65* under the control of the 35*S* promoter resulted in enhanced, but not the ectopic expression of the reporter gene. It is reasonable to assume that this T/G-box serves as an enhancer, and other *cis*-element(s) exist that confer trichome-specificity to the *GaMYB2* promoter. Identification of such *cis*-element(s) should help to dissect the molecular mechanisms regulating cotton fibre and tobacco GST development.

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