

Functional analysis of a cotton glucuronosyltransferase promoter in transgenic tobaccos

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The 5' fragment (1 647 bp) of the cotton glucuronosyltransferase gene (*GhGlcAT1*) was transcriptionally fused to the β -glucuronidase (*GUS*) gene, and functionally analyzed for important regulatory regions controlling gene expression in transgenic tobacco plants. GUS activity analysis revealed that the full-length promoter drives efficient expression of the *GUS* gene in the root cap, seed coat, pollen grains and trichomes. Exposure of the transgenic tobacco to various abiotic stresses showed that the promoter was mainly responsive to the sugars (glucose and sucrose) as well as gibberellic acid. Progressive upstream deletion analyses of the promoter showed that the region from -281 to +30 bp is sufficient to drive strong GUS expression in the trichomes of shoot, suggesting that the 311 bp region contains all *cis*-elements needed for trichome-specific expression. Furthermore, deletion analysis also revealed that the essential *cis*-element(s) for sucrose induction might be located between -635 and -281 bp. In addition, sequence analysis of the regulatory region indicated several conserved motifs among which some were shared with previously reported seed-specific elements and sugar-responsive elements, while others were related with trichome expression. These findings indicate that a 1 647-bp fragment of the cotton *GhGlcAT1* promoter contains specific transcription regulatory elements, and provide clues about the roles of GhGlcAT1 in cotton fiber development. Further analyses of these elements will help to elucidate the molecular mechanisms regulating the expression of the *GhGlcAT1* gene during fiber elongation.

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Introduction

The UDP-glucuronosyltransferases (UGTs) are members of a superfamily of glycosyltransferases, and catalyze the transfer of a glucuronosyl group (GlcA) from uridine 5'-diphosphoglucuronic acid to a range of acceptor substrates, which could be aglycones, such as xylan, anthocyanin and

pectin. In higher plants, glucuronic acid is an important constituent of hemicellulose in cell walls. Cell walls can be divided into two types: primary walls (I) and secondary walls (II). Primary walls are formed during cell growth, and are both mechanically stable and completely extensible for cell expansion to avoid the cracking of cells under their turgor pressure. Secondary cell walls, following the cessation of expansion and division, are synthesized within the bounds of the primary walls, and confer higher mechanical stability on specialized cell types such as xylem cells. There is evidence that the glucuronic acid units of glucuronoxylans in type I walls are added to every sixth xylosyl unit of the backbone [1]. Furthermore, the α -D-glucuronic acid units can be attached to the O-2 position of the xylan backbone as side groups [2]. Additionally, Sawada *et al.* [3] demonstrated that BpUGAT (*Bellis perennis* glucuro-

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Abbreviations: GlcAT, glucuronosyltransferase; DAF, days after flowering; 4-MU, 4-methylumbelliferone; Suc, sucrose; Glu, glucose; Fru, fructose; Man, mannitol; Sor, sorbitol; Eth, ethylene

nosyltransferase) catalyzes the regiospecific transfer of GlcA to the 2-hydroxyl group of the 3-glucosyl moiety of the substrate anthocyanidin. These results suggest that the UGTs may be important in regulation of differentiation and secondary metabolism [4-6].

Plant UGTs belong to a multigene family [7] and have complicated expression patterns. Waldron [8] reported that a UGT activity was mainly present in the secondary wall and highest expression was detected during the elongation of the pea epicotyls. Further analysis showed that the glucuronic acid was added by the UGT during the elongation of xylan [9]. Zhong *et al.* [10] also found that a UGT (FRA8) was specifically expressed during secondary wall thickening in fibers and vessels. Woo *et al.* [11] reported that a *Pisum sativum* UGT catalyzes conjugation of UDP-glucuronic acid to an unknown compound, and its expression is correlated with mitosis and is strongly induced in dividing cells. Inhibition of the UGT expression by constitutive expression of its antisense mRNA markedly retarded the growth and development of transgenic alfalfa. Iwai *et al.* [12] studied a UGT mutant called *nolac-H18* in tobacco (*Nicotiana plumbaginifolia*) generated by T-DNA insertion. The mutation was generated in the *NpGUT1* gene, which functions during pectin biosynthesis. The *nolac-H18* mutant had reduced intercellular attachment. In addition, our recent work showed that a cotton UGT gene, named *GhGlcAT1*, was expressed in fiber and stem; and its transcript was most abundant at 15 days post-anthesis in the fiber, but was not detected in ovule, flower, seed, root and leaf, indicating that the expression of *GhGlcAT1* is regulated developmentally [13]. These findings have provided important insights into functions of the UGTs in plant metabolism, growth and development. However, very little work has been carried out on the structural organization and regulatory elements of their promoters that are important in controlling the expression of these genes.

The economic importance of cotton (*Gossypium hirsutum*) is due to extraordinary elongation and thickening of the secondary wall of ovular epithelial cells; and the fiber provides an excellent system to study cell wall morphogenesis and its polysaccharide biosynthesis [14]. Our previous report suggested that the *GhGlcAT1* gene is expressed in a developmentally regulated pattern and may play an important role in the synthesis of cross-linkage polysaccharides [13]. In order to uncover potentially important regulatory elements in the 5' upstream region of *GhGlcAT1*, we isolated a 1 647 bp fragment upstream of the *GhGlcAT1* coding sequence using an improved PCR-based genomic walking method and characterized its ability to direct temporal, spatial and inducible expression of a reporter gene in transgenic tobaccos. Our results demonstrate that the 1 647 bp fragment of the cotton *GhGlcAT1*

promoter directs specific transcriptional regulation patterns in a model plant, and provide an important insight into the regulatory mechanisms underlying the temporal- and spatial-specific expression and stress responses of the cotton *GhGlcAT1* gene.

Materials and Methods

Plant material and bacterial strains

Cotton (*G. hirsutum* cv. CRI 12) plants were grown in a field. Tobacco (*N. abacum* cv. shanxi) plants were grown in a greenhouse under a 16 h light and 8 h dark, 25 °C. *Escherichia coli* strain DH5a was cultivated in LB medium for vector constructs and DNA manipulation. *Agrobacterium tumefaciens* strain LBA4404 was cultivated in YEB medium for tobacco transformation.

PCR cloning of the *GhGlcAT1* promoter region

Total genomic DNA was extracted from leaf tissue as described by Paterson *et al.* [15], and was digested with suitable restriction enzymes. The promoter region was cloned with an improved PCR-based genomic walking method described previously [16]. Eight adaptor-ligated genomic restriction fragment libraries were generated after digestion with eight restriction enzymes. The corresponding library was subjected to a first round of PCR amplification with the outer adaptor primer (AP1) (5'-GTAATACGACTCTACTATA GGG C-3') and an outer gene-specific primer (GSP1) (5'-ATT GTG TGT AGCAACAGCAGG GC-3'), while the inner adaptor primer (AP2) (5'-ACT ATA GGG CAC GCG TGG TC-3') and inner gene-specific primer (GSP2) (5'-TTC CTT CTT TGT TCT CTC AGC AGA CC-3') were used for the second round of PCR. Major bands were isolated from using the Qiagen Gel extraction III kit (Qiagen, Germany), and the isolated fragments were cloned into a pGEM-T Easy vector (Promega, USA). Recombinant plasmid DNA used for sequencing was prepared using the QIAprep Spin Mini Prop Kit (Qiagen, Germany), and the inserts were sequenced using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, USA) on an ABI PRISM™ 377 DNA Sequencer.

Construction of vectors

With the cloned genomic region as a template, a 1 647 bp fragment (pGhGlcAT1) upstream of the translational start codon of *GhGlcAT1* gene and its 5' deletion derivatives were generated by PCR with seven forward primers (pF1: 5'-CAAGCTTCA GAC CTG AG-T CAT TC-3'; pF2: 5'-CAAGCTTCA GTC TAA CGG GTT AGT TG-3'; pF3: 5'-CAAGC-TTAA AAC TTA AAC CCC AAT TC-3'; pF4: 5'-CAAGCTTTC TCT ATA TGA TAG TTG-GC-3'; pF5: 5'-CAAGCTTTA AAT GAC AGC ATA GAC TCC-3'; pF6: 5'-CAAGCTTA-C AAA TTA ATA GTT ACC AT-3'; pF7: 5'-CAAGCTTCC AAG CCA ACT CCT GTT AT-3'; the introduced *Hind*III sites are underlined) and one reverse primer (pR1: 5'-CT-GGATC CC TTA TGA GTA AAA TGG AAT T-3', the introduced *Bam*HI site is underlined). The amplified fragments were respectively inserted into the plasmid pBI121 (Clontech) as a *Hind*III-*Bam*HI fragment at the corresponding restriction sites in place of the cauliflower mosaic virus (CaMV) 35S promoter region, resulting in a series of pBI-pGhGlcAT1::GUS vectors. The seven expression vectors were named P1617 (-1617/+30, as the full-length promoter construct in this study), P1355 (-1355/+30), P1049 (-1049/+30),

tobacco samples were incubated in 5-bromo-4-chloro-3-indolyl-D-glucuronic acid (X-gluc) solution at 37 °C from 3 h to overnight until the blue staining had reached sufficient intensity [20]. Photosynthetic tissues were cleared of chlorophyll by passing through a 70-100% ethanol series. Photography was performed with an anatomy microscope (Leica, Germany) or a camera (Nikon 8700, Japan).

Stress treatments

Three-week-old transgenic tobaccos (T2) were used in stress treatments. For chemical treatments, whole plants were floated on MS medium solution containing 200 mM sucrose, 200 mM glucose, 200 mM fructose, 200 mM mannitol, 200 mM sorbitol, 50 μM naphthylacetic acid, 10 μM gibberellic acid (GA₃), 20% PEG8000, 50 μM abscisic acid (ABA), 250 mM NaCl, 50 μM methyl jasmonate (MeJA) and 1 mM ethylene, respectively, in Petri dishes. MS, without additions, was used as control. The GUS activity was measured after samples had been kept in a growth chamber at 25 °C for 24 h in the dark. All data represent mean value ± SE of at least five repeated experiments from different transgenic lines.

For further analysis of sugar induction, transgenic plants of each construct were treated with 0.2 M sucrose in MS solution. The GUS activity was measured after samples had been kept in a growth chamber at 25 °C in the dark for 24 h.

Results and Discussion

Sequence characterization of the *GhGlcAT1* promoter region

A *GhGlcAT1* promoter region was isolated as described in Materials and Methods, and after the second round PCR,

two major bands were amplified from the *Dra* I- and *Hind* III-restriction fragment libraries, respectively, but no bands could be produced from other restriction fragment libraries (data not shown). Sequencing analysis showed that the larger band from the *Hind* III-restriction fragment library overlapped with the smaller band from the *Dra* I-restriction fragment library. The resulting 1 647 bp fragment, upstream of the *GhGlcAT1* gene, was sequenced and designated as pGhGlcAT1, and the fragment also contains 30 nucleotides of the 5' end of the *GhGlcAT1* cDNA. The first base of the cDNA was designated +1 as the putative transcription start site (Figure 1).

A promoter motif search was carried out to define putative *cis*-elements in the pGhGlcAT1 sequence using the software programs PLACE [22] and PlantCARE [23]. A number of potential regulatory motifs corresponding to known *cis*-elements of eukaryotic genes were found (Figure 1). A TATA box was found at the positions -39 to -34 upstream of the putative transcription start site, whereas a CAAT box was observed at positions -74 to -70 (Figure 1); these boxes function as basal promoter elements for transcription. Furthermore, regulatory elements with homology to those identified in other seed/endosperm-specific genes were found in the pGhGlcAT1 region such as an amylase-box, E-box, CARE, GARE, GCN4-motif, ACGT motif and AACA motif (Table 1). The amylase-box was required for high expression of the gene in the seeds

Table 1 Regulatory motifs found in the *GhGlcAT1* promoter region

| Sequence ^a | Position | Name and function | Reference |
|-----------------------|--|--|-----------|
| TAACAAA | -1420 (+), -767 (-), -527 (+) | amylase box, seed-specific | [24] |
| AACAAAC | -768 (-) | AACA motif, endosperm-specific regulation | [31] |
| ACGT | -1403 (+), -1316 (+), -614 (+), 595 (+) | ACGT motif, endosperm-specific regulation | [39] |
| TGAGTCA | -1605 (+), | GCN4, endosperm-specific regulation | [30] |
| CANNTG | -1190 (+), -759 (+), -354 (+), -94 (+) | E-box, seed-specific | [26] |
| CAACTC | -275 (+) | CARE, germinating seeds | [28] |
| TAACAAA | -561 (+), -767 (-), -527 (-) | GARE, seed germination | [29] |
| TGACT | -1603 (-), -1462 (-), -1442 (-), -1325 (+), -1000 (-), -553 (+), -503 (+), -368 (-) | W-box, sugar-responsive elements | [37] |
| TGTCTC | -102 (-) | AuxRE, auxin response element | [38] |
| CNGTTR | -269 (+), -762 (-), -1361 (+), -1350 (-), -1494 (+), -759(+) | Myb recognition site, regulating many genes expression | [43, 44] |
| AGAAA | -328, -25 | Pollen-responsive, pollen expression | [32] |
| AAATGA | -989 (+), -950 (+), -634 (+), 586 (+) | Pollen expression | [33] |
| GTGA | -1550 (+), -1536 (+), -1390 (+), -504 (+), 91 (+) | GTAG motif, pollen expression | [34] |

^a: N indicates A, C, G or T; R indicates A or G.

+: normal sequence; -: complementary sequence.

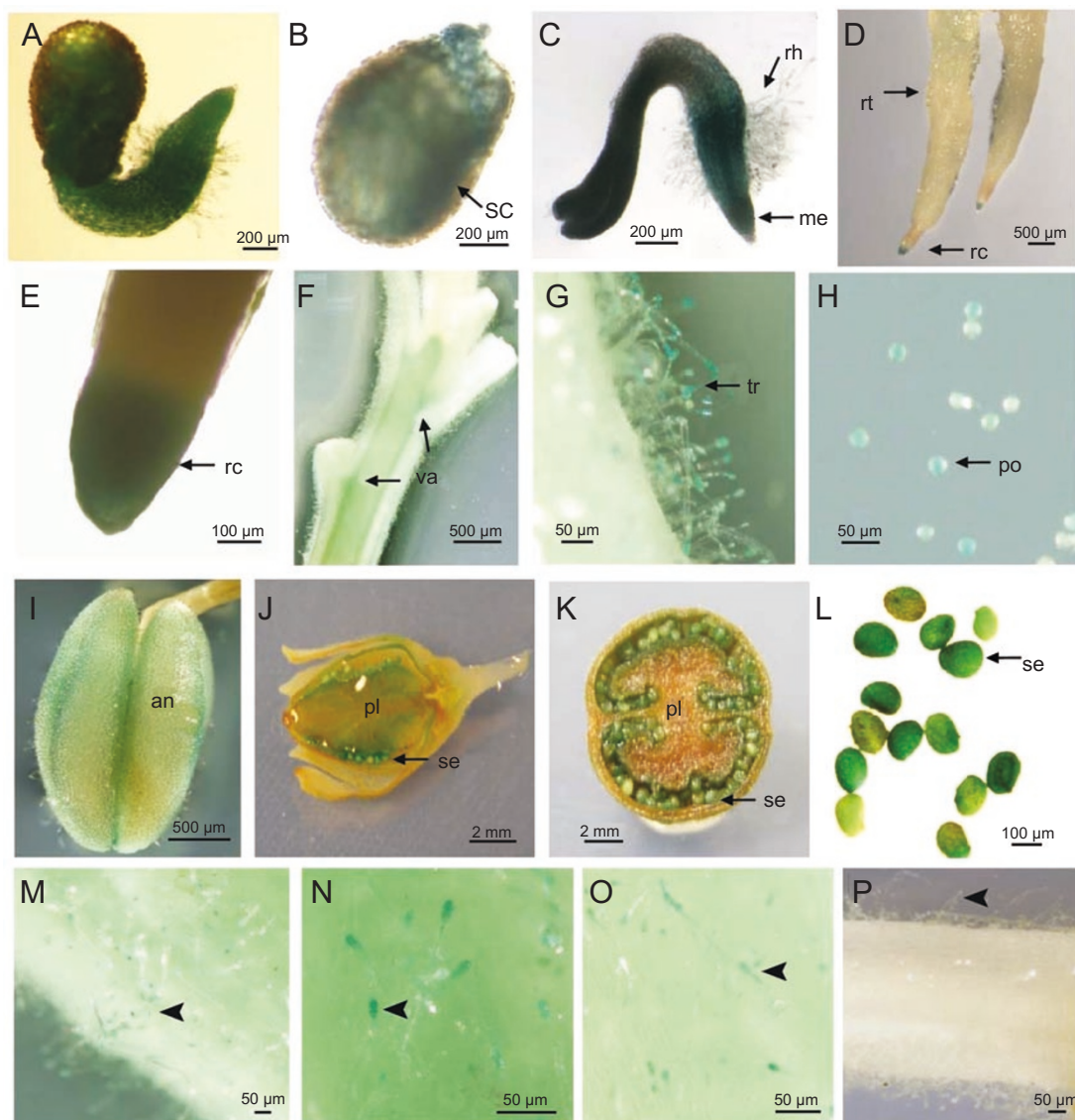


Figure 2 Histochemical analysis of GUS activity in tobacco expressing the *GhGlcAT1* promoter-GUS chimeric gene at different developmental stages. **(A)** GUS is expressed in the 1-day-old germinated seedling. **(B)** Blue staining is present in the seed coat. **(C)** Blue staining is focused on the 2-day-old germinated seedling. **(D)** Blue staining can be observed in the root tip of 3-week-old plant. **(E)** GUS activity is present in the root meristem tissue. **(F)** The longitudinal section of the upper part shoot; GUS expression is in the shoot vascular tissues. **(G)** GUS staining is concentrated in the trichomes. GUS staining is located in pollen grains **(H)** and the anther **(I)**. GUS is expressed in the seeds of longitudinal section of a 10-DAF fruit **(J)**, cross-section of 10 DAF fruit **(K)** and seeds **(L)**. **(M-P)** GUS activity in trichomes derived from a series of pGhGlcAT1 transgenic plants and pBI101 control plants. GUS staining is found in the trichomes of P1617 **(M)**, P457 **(N)** and P281 **(O)** transgenic tobaccos while no staining was found in the trichomes of pBI101 control **(P)**. sc, seed coat; rh, root hair; me, meristem; rt, root; rc, root cap; va, vascular bundle; tr, trichome; po, pollen grain; an, anther; pl, placenta; se, seed.

[24], while the E-box was shown to be bound by bHLH (basic helix-loop-helix) transcription factors and to regulate the seed-specific expression of seed storage protein genes and the expression of genes involved in anthocyanin biosynthesis [25-27]. The CARE and GARE boxes were

required during seed germination in *Arabidopsis* and rice [28, 29]. In addition, a combination of GCN4, ACGT and AACA motifs was sufficient to confer a detectable level of endosperm expression in rice [30, 31]. Several motifs including AGAAA, AAATGA and GTGA, and six MYB

response elements are also present in the promoter region (Table 1). The AGAAA, AAATGA and GTGA motifs were reported to relate to pollen grain development [32-34], while MYB response elements were related to trichome development [35, 36]. Additionally, the promoter region also contains several potential motifs with homology to inducible regulatory elements such as the W-box and AuxRE (Table 1). The W-box was reported to be involved in sugar stress response in barley [37], while a reverse AuxRE (GAGACA) was found to bind to auxin response factors [38]. Taken together, sequence analysis of pGhGlcAT1 promoter region suggests that it contains a number of elements that are potentially important in controlling gene expression and that the *GhGlcAT1* gene may be subjected to complex regulations including tissue-specific expression and stress induction.

Histochemical and quantitative analysis of GUS activity

To investigate the temporal and spatial regulation directed by the pGhGlcAT1 promoter region, transgenic tobaccos carrying the *GUS* reporter gene fused to the 1647 bp fragment were grown in the greenhouse, and T1 plants were histochemically assayed for GUS staining. GUS staining was observed deeply in the whole 1-day-old germinated seeds (Figure 2A), and was also strong in the coats of the 2-day-old germinated seeds (Figure 2B). In 2-day-old seedlings, GUS staining was observed in the root cap meristem zones and cotyledons, but less GUS activity was detected in the root hairs (Figure 2C). In the 3-week-old seedlings, GUS activity appeared in the root tip (Figure 2D and 2E) and the shoots (Figure 2F). GUS staining in aerial tissues was found mainly in the vascular tissues and

trichomes of the upper part of the shoots (Figure 2F-2G), but not in the leaves, mature vascular bundles and trichomes of basal shoots (data not shown). Strong GUS staining in the vascular tissues of stems was also consistent with our previous report of *GhGlcAT1* expression by RNA blotting [13]. To monitor low levels of tissue-specific GUS activity, quantitative GUS assays were performed with 4-week-old transgenic T1 plants. As shown in Figure 3, GUS activity was mainly detected in the basal roots and the upper regions of stems, but very low activity was detected in the fourth leaves. The GUS activity in roots (111.94 pmol/mg protein/min) was 5.19-fold higher than that in the leaves. These results were in agreement with analysis of the GUS staining, indicating that the *GhGlcAT1* promoter directed reporter expression in the meristematic and vascular tissues.

In the reproductive growth stage, strong GUS activity was observed in anthers and pollen grains of flower organs (Figure 2H-2I). As they are T1 transgenic plants, only about half of pollen grains (1:1) showed strong GUS staining (Figure 2H), indicating that only one copy of extrinsic DNA was inserted into the tobacco genome. In 10-day-old fruits, GUS staining could be seen in the seeds, but with less activity in the placenta (Figure 2J-2K). Approximately 3/4 of the seed showed GUS staining (Figure 2L). GUS activity was hardly detected in the corresponding tissues of the negative control plants (data not shown). Consistent with the GUS activity levels, the promoter region contains several important elements, such as an amylase-box, CARE, GARE, GCN4-motif, ACGT motif and AACA motif, which are involved in seed-specific and normal embryo-specific expression [24, 28-31, 39]. Quantitative GUS assays showed that the highest expression was in

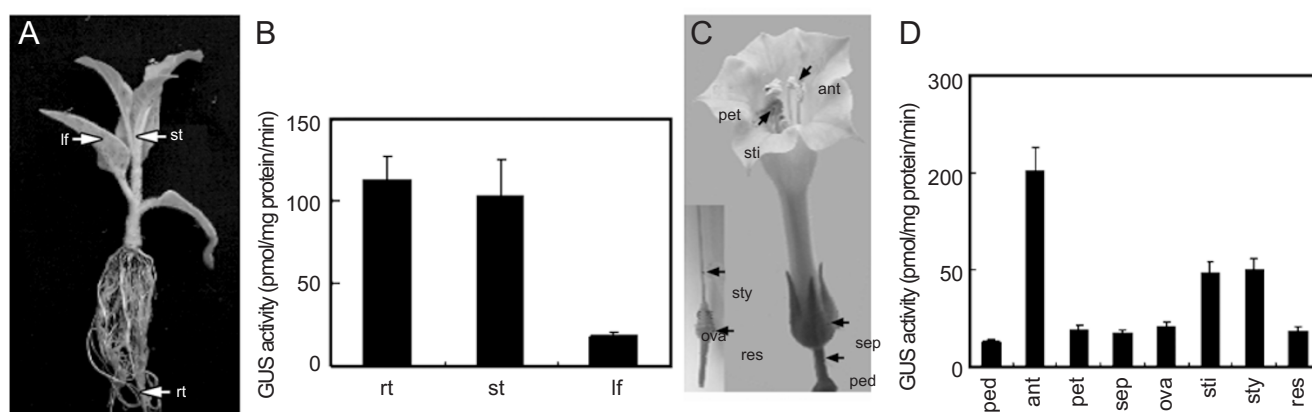


Figure 3 GUS activity assay in transgenic tobaccos. Roots (rt), stems (st) and leaves (lf) are indicated in the transgenic tobacco plants (A) and were assayed for GUS activity (B). Anthers (ant), stigmas (sti), styles (sty), sepals (sep), petals (pet), ovaries (ova), receptacles (rec) and pedicels (ped) from mature plants (C) were sampled and assayed for GUS activity (D). Mean values are expressed in pmol MU per mg protein per min with data from five independent lines. Error bars indicate SE values.

the anthers, with relatively high expression in the stigmas and styles. However, GUS activity was detected at lower levels in the sepals, petals, pedicels, receptacles and ovaries (Figure 3). The average GUS activity in the anther was 203.12 ± 5.42 pmol/mg protein/min, at least 6.1-fold higher than that in pedicels, and two-fold of that in styles (Figure 3). High GUS activity in anther was also consistent with GUS staining in anther and pollen grains. Several kinds of pollen development-related motifs (AGAAA, AAATGA and GTGA) present in the pGhGlcAT1 promoter region are likely related to strong GUS expression in anther and pollen grains.

A *PsUGT1* gene encoding a *Pisum sativum* UGT was reported to be expressed in root cap meristem [11]. Inhibiting *PsUGT1* expression by the constitutive expression of its antisense mRNA (under control of the 35S promoter) markedly retarded the growth and development of transgenic alfalfa, especially the floral development. The floral buds from antisense transgenic plants were short and thick, with stamens producing less pollens. These results indicate that *PsUGT1* plays a role during the development of pollen or other flower organs. Furthermore, the *PsUGT1* promoter::GUS fusion gene was found to have a high expression in active cell division regions [40]. In addition, an *NpGUT1* (tobacco glucuronyltransferase 1) was also expressed predominantly in shoot and root apical meristems [12], while the expression of antisense *NpGUT1* RNA produced shoots with reduced cell adhesion. Analysis of the *nolac-H18* mutant tissues showed that *NpGUT1* is involved in pectin biosynthesis.

In the present work, the *GUS* gene controlled by the *GhGlcAT1* promoter region was also highly expressed in root and shoot apex meristem tissues, pollen grains as well as pistils. These patterns were similar with *PsUGT1* and *NpGUT1* expressions in other plant species, suggesting that *GhGlcAT1* might also function during differentiation of meristem tissues, development of pollens and cell division biosynthesis.

Effects of abiotic stress on *GhGlcAT1* promoter in transgenic tobacco

The 3-week-old transgenic tobaccos from the T2 homozygous seed were used for various abiotic treatments, and then their GUS activity was examined. As shown in Figure 4, glucose and sucrose treatment could obviously raise the GUS activity, which was about 88.1% and 79.5% higher than that of non-treated control plants, respectively. However, GUS activity was only slightly raised (by about 13.8%) in fructose-treated plants, and no increase in GUS activity was detected in mannitol- and Sorbitol-treated plants (Figure 4). Thus the treatments of glucose and sucrose had a greater effect on *GUS* gene expression than

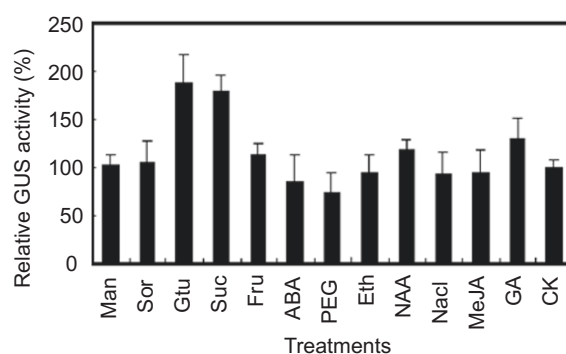


Figure 4 GUS activity in 3-week-old pBI-pGhGlcAT1-1647::GUS transgenic tobaccos under abiotic stress, including sorbitol (Sor), mannitol (Man), glucose (Glu), sucrose (Suc), fructose (Fru), ABA, PEG8000 (PEG), ethylene (Eth), naphthylacetic acid (NAA), NaCl, MeJA, GA, and MS medium (CK), respectively. MS treatment was used as a control and taken as 100%. Each value represents the average of at least five transgenic individuals. Error bars indicate SE values.

those of fructose, mannitol and sorbitol. One possible explanation may be that glucose and sucrose might contribute to the pool of GlcA, one of the substrates for UGTs. It is possible that pGhGlcAT1 might be regulated by different types of sugar. In addition, GA and NAA (which are associated with cell division) treatment also enhanced GUS activity, which was about 31.7% and 18.6% higher than that in the non-treated control plants, respectively. However, treatments with Eth, NaCl and MeJA did not cause any significant changes in the GUS activity compared with the control plants (Figure 4), while treatments with ABA and PEG8000 decreased the GUS activity (by about 14.3% and 25.3%, respectively). The possible reasons might be that ABA and PEG8000 stress could quickly repress cell meristems and growth in these tissues.

On the other hand, the *GUS* gene was expressed in root cap meristematic zones and upper part of the shoot (Figure 2D-2F), and the GUS activity increased in response to GA and NAA treatments (Figure 4). It is well known that GA can promote shoot elongation and cell wall expansion, while the shoot apical meristem is the main site of auxin biosynthesis. Therefore, *GhGlcAT1* expression in those locations might be controlled by the fine regulation of plant hormones in those tissues. Additionally, meristematic zones are also the main sites of primary cell wall biosynthesis, while vascular bundles are mainly associated with the synthesis of the secondary cell wall. The high expression in those tissues indicates that the *GhGlcAT1* gene might participate in primary cell wall and initial secondary cell wall synthesis. Therefore, the results presented in this paper,

together with the data from Northern blot analysis [13], strongly suggest that *GhGlcAT1* might be related to sugar and hormone responses in fiber development.

Deletion analysis of the promoter in transgenic tobacco

In order to localize promoter regions important for the transcriptional control of the *GhGlcAT1* gene, the full-length promoter (P1617) and a series of its 5'-truncated fragments (P1355, P1049, P813, P635, P457 and P281) were fused to the *GUS* reporter gene (Figure 5). The up-ground shoots of five individual 3-week-old transgenic T1 tobacco plants were analyzed. The results showed that *GUS* activities were slightly higher in plants containing P1049 (-11049/+30) compared to P1617 (-11617/+30). This suggests that there might be repressor elements in the region upstream of -11049. *GUS* activities were decreased in the transgenic plants harboring the P813 (-813/+30) and P635 (-635/+30) constructs, while there were less changes comparing the plants harboring P635 and P281 (-281/+30) constructs (Figure 5). It was remarkable that a 311 bp (-281/+30) promoter fragment could still drive the expression of high *GUS* activity. Furthermore, *GUS* staining could still be observed in the trichomes of transgenic tobacco apical shoots containing P457 (-457/+30) and P281 constructs as well as in the full-length construct (Figure 2M-2O), while no *GUS* staining could be observed in the trichomes of pBI101 transgenic tobaccos (Figure 2P). Therefore, the elements needed for trichomes-specific expression might be located in this 311 bp fragment (-281/+30). Further sequencing analysis found that there was an MYB recognition site and an E-box in this region. Fiber-specific promoter should contain critical element(s)

conferring specific expression in trichomes of model plants, as cotton fibers are unicellular trichomes that are derived from the extraordinary elongation of epidermal cells of cotton ovules [41], while the regulation of trichome development in plant is dependent on a number of MYB transcription factors [42-44]. It is believed that these two motifs might be related to trichome-specific expression. In addition, trichome development was also regulated by GL3, which is related to the MYC transcription factor. GL3 contains a conserved bHLH domain in its C terminus, which could bind to the E-box [45]. GL3 regulates trichome development in *Arabidopsis* via interaction with GL1 and TTG1. The *GhGlcAT1* gene is highly expressed in fiber cells at the elongation stage [13], and its promoter sequence contains six MYB recognition sites and four E-boxes (Table 1), which might be responsible for directing *GUS* reporter expression in tobacco trichomes. To avoid the difficulties in the regeneration of cotton plants, several fiber-specific promoters have been studied in model plants such as tobacco [46-48] and *Arabidopsis* [36, 41]. The results showed that these promoters contain critical element(s) conferring preferential expression in trichomes of model plants, as cotton fibers are unicellular trichomes that are derived from the extraordinary elongation of epidermal cells of cotton ovules [41]. Based on our results, the promoter of *GhGlcAT1* may also function in a similar way as the *GhGlcAT1* gene is preferentially expressed in cotton fiber [13].

As pGhGlcAT1 was induced by sucrose and glucose stresses, an attempt was also made to uncover the *cis*-regulatory elements in response to sucrose induction. *GUS* activities could be strongly induced in transgenic plants

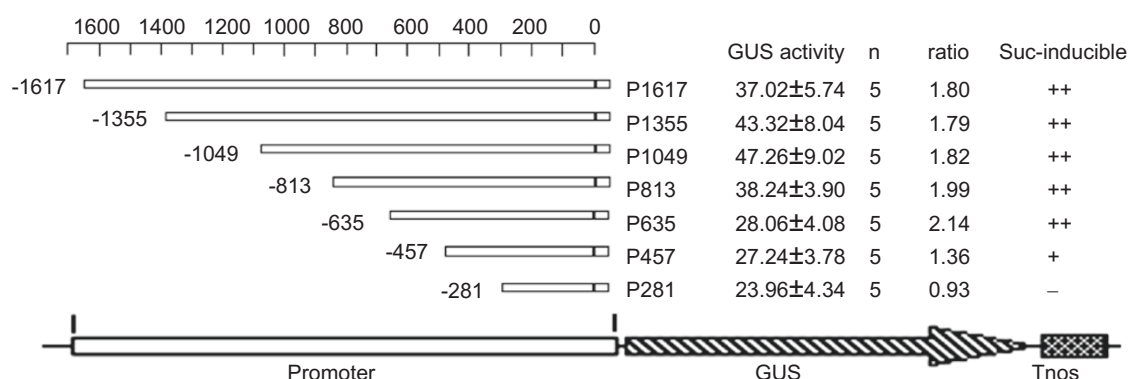


Figure 5 Structure of *GUS* fusion constructs and determination of *GUS* activity in transgenic tobaccos. The bars represent various deletion fragments of the *GhGlcAT1* promoter, numbering started with the transcription start site (+1), and is indicated by a line. *GUS* activities presented above were the mean values (pmol MU per mg protein per minute) of (n) individual plants including SD (\pm), and the ratio of *GUS* activities between sugar-induced and uninduced plants were calculated. “+” indicates inducible and “-” uninducible.

harboring five of the promoter constructs when plants were placed on MS medium supplemented with sucrose (Figure 5). The highest GUS induction was found in transgenic plants containing P635 (2.14-fold). However, the GUS activity was only slightly increased (1.36-fold) in transgenic plants containing P457, while the induction was completely lost in the plants harboring P281 (Figure 5). These results indicated that the *cis*-element(s) required for sucrose induction might be mainly located between -635 bp and -281 bp. Sequencing analysis revealed that there are eight W-boxes in the *GhGlcAT1* promoter region, and W-boxes were reported to be associated with sugar responses [37]. Two W-boxes and one reverse W-box are located in the region between -635 and -457 bp, and -457 and -281 bp, respectively; and no W-boxes are present in the region between -281 bp and the translation start site (Figure 1). Our results suggested that W-boxes in the *GhGlcAT1* promoter might play an important role in sugar responses.

In conclusion, the present work using *GUS* expression assay indicated that a 1 647 bp fragment upstream of *GhGlcAT1* coding sequence confers a high level of *GUS* expression in trichomes, vascular tissues, pollen grains, seeds and meristematic zones of roots, and in response to sugar (glucose and sucrose) stresses in transgenic tobaccos. The results demonstrated the spatial and temporal regulation of a cotton promoter in a model plant, and provided a useful foundation to understand the biological roles of this glucuronosyltransferase in cotton fiber development. To demonstrate the contribution of the various identified elements in the promoter to tissue-specific and inducible gene expression, further study by loss of function analysis is needed including deletions analysis, linker scanning and/or point mutations.

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