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Molecular cloning and analysis of a receptor-like promoter of *Gbvdr3* gene in sea island cotton

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ABSTRACT. *Verticillium* wilt caused by soil borne fungus *Verticillium dahliae* could significantly reduce cotton yield. The *Ve1* homologous gene *Gbvdr3* is resistant to *Verticillium* wilt. In order to understand of the function of the promoter *Gbvdr3* in *Gossypium barbadense*, the promoter region of the receptor-like gene *Gbvdr3* was obtained by genome walking, and the *cis*-element in the promoter was identified using the PLACE software in this study. The sequence analysis showed that the promoter contained elements related to stress resistance and light regulation. The cloned promoter was fused to the GUS reporter gene and transformed into *Arabidopsis*. GUS expression was specifically detected in roots, flowers, and seeds, suggesting that the expression of *Gbvdr3* is tissue-specific. Separation and characterization analysis of the promoter of *Gbvdr3* provides a platform for further research and application of this gene. Thorough understanding of the function of the *Gbvdr3* promoter is important for better understanding of *Gbvdr3* function. These results indicated that the promoter of *Gbvdr3* was a tissue-specific promoter.

Key words: Cotton; Receptor-like protein; Promoter; GUS staining

INTRODUCTION

The leucine-rich repeat receptor-like protein kinase (LRR-RLK) belongs to the trans-membrane receptor-like kinases in plants. It is made up of leucine-rich repeats (LRRs) domain, a trans-membrane (TM) domain, and a functional protein kinase domain. As a signal recognition receptor, the LRR-RLK participates in signal transduction process of CLAVATA (*CLV*), Brassinosteroid and Activator of Xa21 in different plants, and plays important roles in plant development and cell growth, hormone signaling, and stress responses (Anne and Steven, 2004). The biological function of these genes includes disease resistance response, stress resistance response, and plant morphogenesis. LRR-RLK such as rice OsSIK1 (Ouyang et al., 2010) and *Arabidopsis ARCK1* (Tanaka et al., 2012) and *GHR1* (Hua et al., 2012) play an important role in optimizing plant tolerance to abiotic stress.

The LRR-RLK participates in a diverse range of processes in response to abiotic stress (Huang et al., 2012) and disease resistance (Zhang et al., 2012). Many studies reported LRR-RLK genes that are involved in plant disease resistance response such as tomato *Verticillium* wilt resistance genes *Ve1* and *Ve2* (Kawchuk et al., 2001; Fradin et al., 2011), tomato leaf mold resistance gene *Cf-9* (Jones et al., 1994), tobacco and tomato *Trichoderma viride* resistance gene *LeEIX2* (Ron and Avni, 2004), apple scab resistance genes *HcrVfa1* and *HcrVfa2* (Belfanti et al., 2004; Malnoy et al., 2008), and *Arabidopsis* genes *AtRLP52* and *AtRLP30* (Ramonell et al., 2005). In addition to the resistance in plants, these genes are also involved in organ development, morphogenesis, and so on. Thus, *Arabidopsis CLV2* participates in the formation of shoot tips and root meristem, corn *FEA2* maintains the apical meristem (Taguchi-Shiobara et al., 2001), and *Arabidopsis TMM* participates in control of stomatal distribution and development of stomata (Nadeau and Sack, 2002). Three *Ve1* homologous genes, *GbVe*, *GbVe1* and *Gbvdr5*, have been isolated from *G. barbadense*, and their resistance to various *V. dahliae* strains has been confirmed (Chen et al., 2016). In this study, a promoter of the receptor-like *Gbvdr3* was cloned from a disease-resistant cultivar of *Gossypium barbadense* L. and transferred into *Arabidopsis*, creating transgenic plants with *Gbvdr3* promoter.

MATERIAL AND METHODS

Cloning the *Gbvdr3* promoter

Gbvdr3 promoter was isolated from the *G. barbadense* 'Hai7124' genomic DNA by PCR using primers P1 5'-CAGCTTATCAAACGCAGAAGGAA-3' and P2 5'-CACTTTGACATTGAGCCGAAACC-3'. Taq, buffer, and DNTPs were purchased from SenBeiJia Biological Technology Co., Ltd. (Nanjing, China). The PCR mixture included 15 μL 2X GC buffer I, 5 μL 2.5 mM DNTPs, 1.5 U Taq enzyme, and approximately 1 mg H7124 template genomic DNA, and deionized water was added to make a final volume of 30 μL. The upstream sequence of *Gbvdr3* gene was obtained by genome walking according to the Genome Walker™ (Clontech) instruction. The PCR products were purified from 1.0% agarose gels and then ligated into pGEMT vector (Promega, Madison, WI, USA) and transformed into DH5α bacterial cells. The clones were confirmed by restriction enzyme digestion and sequencing. The *Gbvdr3* promoter sequences were analyzed by the PLACE software (http://www.dna.affrc.go.jp/htdocs/PLACE/).

Plasmid construction and *Arabidopsis* transformation

To construct the vector, the entire coding region of *Gbvdr3* was amplified by PCR using *Sam*I and *Bam*HI linker primers (*Gbvdr3*: 5'-TGAGTCGACCACAGAAGTTGAACAAA CTAGGCAT-3' and 5'-TGAGGATCCAGATAACAATGGCACTAGGTTGA-3') and ligated into the vector pBI121. A 1200-bp fragment of the *Gbvdr3* promoter was used to construct the pBI-*Gbvdr3*-GUS vector. This fragment was amplified by PCR and ligated into the vector. The transformation of female gametes of *Arabidopsis* is accomplished by simply dipping developing inflorescences for a few seconds into a 5% sucrose solution containing 0.01-0.05% Silwet L-77 (v/v) and resuspended agrobacterium cells carrying the genes to be transferred. Treated plants were allowed to set seeds, which were then plated on a selective medium to screen for transformants.

Growth conditions and plant transformation

Cotton seeds of Hai7124, a *Verticillium* wilt-resistant cultivar widely used in China for genetic and breeding studies, were delinted with concentrated sulfuric acid. Wild type *Col*-0 and transgenic plants were grown in potting soil or on half-strength Murashige-Skoog (1/2 MS) medium under 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light in a growth room at a temperature between 22° and 24°C, under a 16 h light/8 h dark photoperiod, and 65% relative humidity. The identified transgenic plants and control plants were allowed to self-fertilize, and the resulting progenies were planted for further use in subsequent experiments.

Histochemical and fluorometric GUS assays

For histochemical staining of GUS, fresh tissue samples were obtained from *Arabidopsis* plants and immediately exposed to X-Gluc solution. A fluorometric GUS assay was performed as described by Jefferson et al. (1987). The fluorescence of 4-methylumbelliferone (4-MU), the product of GUS-catalyzed hydrolysis, was measured using the TECAN GENios system (Tecan Group Ltd., Mannedorf, Switzerland). Protein concentration in the supernatant was assessed by the method described by Bradford (1976), using bovine serum albumin as a standard. GUS activity was normalized to the protein concentration of each supernatant extract and calculated as molar unit (M) of soluble protein per minute.

Expressions of *Gbvdr3* gene in different organs and stages of *G. barbadense* development

Quantitative reverse transcription-PCR (qRT-PCR) for *Gbvdr3* was conducted using a SYBR Premix ExTaqTM II Kit (TaKaRa, Shiga, Japan), the primer pair 5'-TCAGGA TTAAGTAGGGAAGGAGTT-3' and 5'-GTAATACAAGGTGGAAACAGAAGC-3', and cotton polyubiquitin gene *UBQ14* (Artico et al., 2010) as the internal standard. The PCR program consisted of an initial denaturation step of 1 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C, annealing for 20 s at 60°C, and extension for 20 s at 72°C. The real-time PCR thermal cycler qTOWER 2.0/2.2 (Analytik Jena, Jena, Germany) was used to obtain relative expression levels of each sample. All qRT-PCR expression assays were performed independently and analyzed three times under identical conditions.

RESULTS

Construction of the *Gbvdr3* gene promoter and plant expression vector

The 1.2-kb DNA fragment was obtained by two amplifications of genome walking PCR (Figure 1). Compared with the cDNA sequence of *Gbvdr3*, the 1.2-kb DNA fragment has 150 nucleotides in common with the target gene and partially overlaps with the same, suggesting that the fragment was the targeted gene promoter sequence (*Gbvdr3*) (Figure 1). The primers were designed according to the *Gbvdr3* sequences and the recombinant vector of pBI-*Gbvdr3*-GUS (Figure 2) was constructed.

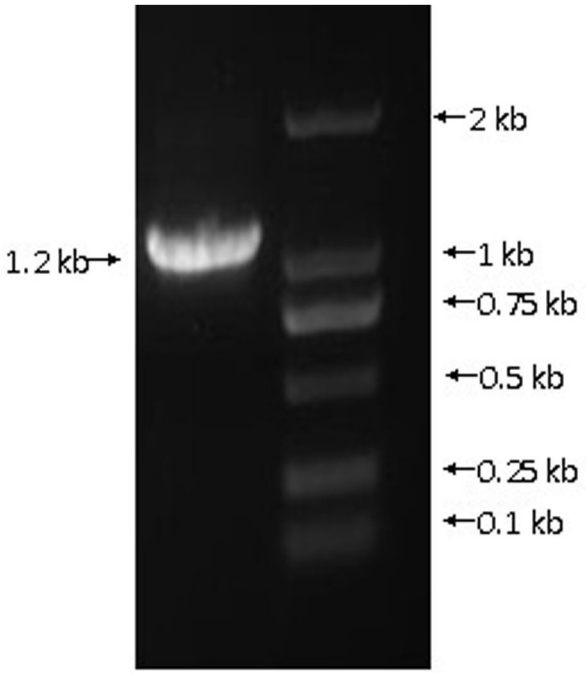

Figure 1. Promoter fragment of *Gbvdr3* isolated by genome walking.

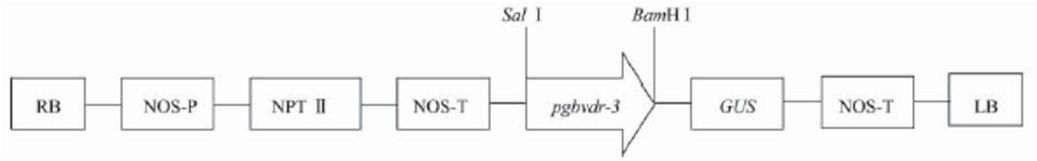

Figure 2. Diagram of pBI- *Gbvdr3*-GUS.

Gbvdr3 gene promoter sequence

The *Gbvdr3* promoter sequences were analyzed by the PLACE software (Figure 3). The promoter contained multiple light regulatory elements such as GATABOX (GATA) EBOXBNNAPA (CANNTG), GT1CONSENSUS (GRWAAW), IBOXCORE (GATAA), INRNTPSADB (YTCANTYY), and so on. Many researchers suggested that the disease resistance of plants is bound with light (Asai et al., 2000; Brodersen et al., 2002; Fryer et al., 2003). The *Gbvdr3* promoter also contained a number of defense related components such as BIHD1OS (TGTCA), ELRECOREPCRP1 (TTGACC), GCCCORE (GCCGCC), HSELIKENTACIDICPR1 (CNNGAANNNTTCNNG), SEBFCONSSTPR10A (YTGTCWC), WBOXATNPR1 (TTGAC) and others, indicating that the promoter may be induced by pathogens. Furthermore, the promoter also contained multiple hormone response elements such as auxin and salicylic acid induction component ASF1MOTIFCAMV (TGACG), gibberellin induction component CAREOSREP1 (CAACTC), and ABA induction component DPBFCOREDCDC3 (ACACNNG). In addition, we also found many organ-specific components such as pollen-specific expression components POLLEN1LELAT52 (AGAAA), root hair-specific components RHERPATEXPA7 (KCACGW), and root expression element ROOTMOTIFTAPOX1 (ATATT).

Figure 3. Sequence of the *Gbvdr3* promoter.

Transgenic *Arabidopsis* with *Gbvdr3* promoter

The 35S:*Gbvdr3* construct was introduced into *Arabidopsis* ecotype *Col*-0 and eight independent, fertile primary *Arabidopsis* transformants were regenerated. The eight positive transgenic lines obtained by *Agrobacterium*-mediated transformation were confirmed by PCR (Figure 4).

Expression pattern of the *Gbvdr3* promoter in *Arabidopsis*

After the GUS staining, the roots were dark in color, indicating that *Gbvdr3* promoter was highly expressed in the roots (Figure 5A). Additionally, in other plant parts, *Gbvdr3* promoter was significantly expressed in floral organs (style and stamens), there was

no expression in the pod, and some level of expression was detected in seeds (Figure 5). Therefore, the *Gbvdr3* promoter displayed a tissue-specific expression pattern. These plants exhibited constitutive GUS expression.

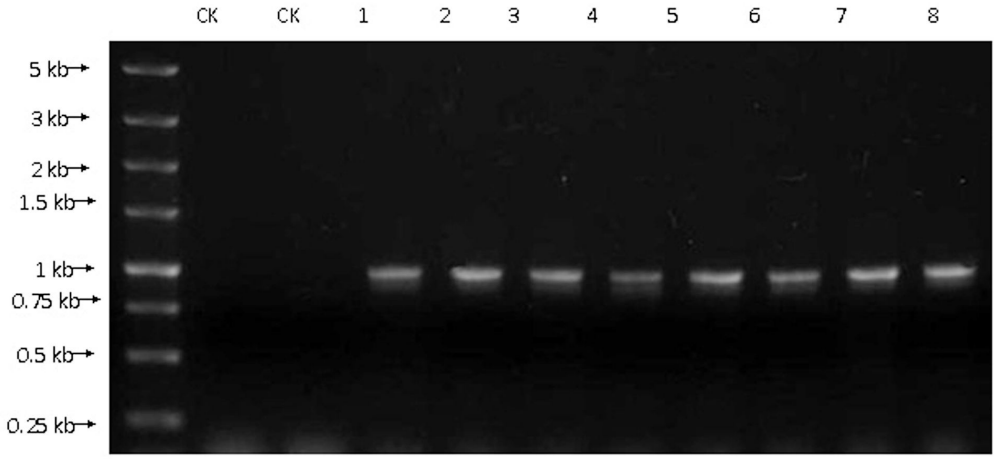

Figure 4. PCR identification of *Gbvdr3* transgenic lines. CK, untransformed plants; 1-8, transformed plants.

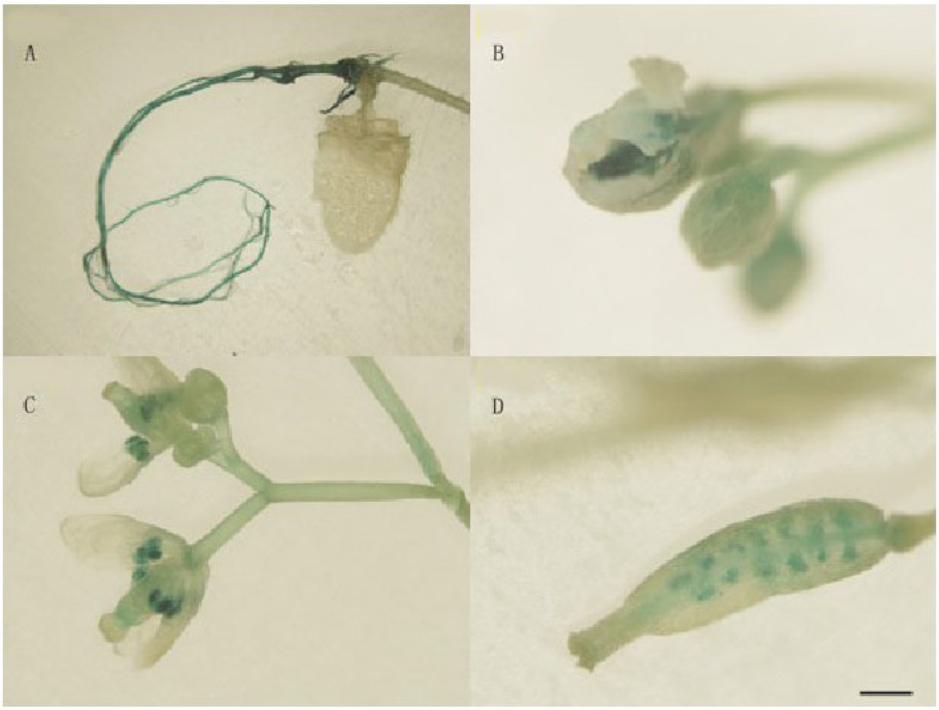

Figure 5. GUS expression in transgenic *Arabidopsis*. Bar = 100 mm. **A.** root; **B. C.** flower; **D.** pod with seeds.

Expression of the *Gbvdr3* gene in different organs and stages of *G. barbadense* development

The results of the qRT-PCR showed that at the seedling stage expression of *Gbvdr3* was higher in the leaves than in the root, and the lowest expression was detected in the stem at the seedling stage. At the flowering stage, expression of *Gbvdr3* in the flower was higher than that in the stem, and it was the lowest in the flower bud (Figure 6).

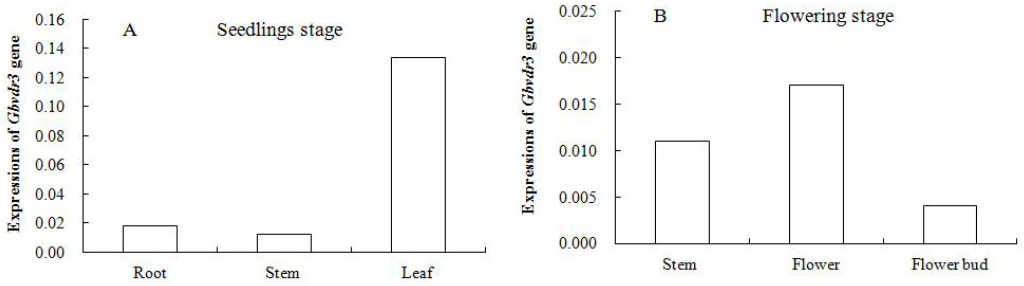

Figure 6. Expression of *Gbvdr3* in different organs at different growth stages of the seedlings.

DISCUSSION

Efficient genetic modification of cotton for agronomic traits requires the use of regulatory sequences known as promoters that drive the expression of downstream transgenes in specific plant tissues. Currently, the widely used promoter for gene expression in dicotyledons including cotton is the cauliflower mosaic virus (CaMV) 35S promoter, which provides strong constitutive expression (Song et al., 2000). However, to develop transgenic cotton with *Verticillium* resistance, a larger arsenal of constitutive and tissue-specific promoters is required. The need for more developmentally or environmentally regulated promoters has become evident and considerable effort has been allocated into the discovery of specific tissue or biotic and hormonal or abiotic stress-responsive genes and promoters (Potenza and Aleman, 2004). Constitutive promoters of actin, ubiquitin, and tubulin gene have been widely used in various plant species for expressing transgenes or selectable markers, such as *RbcS* gene promoter (Tanabe et al., 2015), potato tuber tissue-specific promoter of *GBSS* (Chen et al., 2011), and *Arabidopsis thaliana pyk10* gene promoter (Lǔ et al. 2011). The *Gbvdr3* promoter displayed a tissue-specific expression pattern. The transgenetic *Arabidopsis* exhibited constitutive GUS expression, light regulatory elements, defense related components, multiple hormone response elements, and organ-specific components (Chen et al., 2016).

Gbvdr3 and the reported *Ve1* homologous genes in sea island cotton, including *GbVe* (Zhang et al., 2011), *Gbve1* (Zhang et al., 2012), and *Gbvdr5* (Yang et al., 2014), suggest that the inheritance of cotton resistance to *Verticillium dahliae* may be controlled by multi-dominant resistant genes, which have a unique resistance specificity due to the recognition of different effectors. The promoter region (1.5 kb upstream) of the *Gbvdr3* was cloned. The results of the histochemical staining confirmed that the *Gus* activity was specifically localized in the roots and flowers of the transgenic plants. These results indicate that *Gbvdr3* can be

induced by defense signaling molecules and infection by *V. dahliae* in vascular regions of the roots and stems. *Gbvdr3* is regulated by some complex factors involved in cotton resistance and stress-induced processes (Zhao et al., 2013). Better understanding of the functional activity of the *Gbvdr3* promoter important for further studyies of *Gbvdr3* gene function.

In conclusion, the cloned promoter was fused to GUS reporter gene and transformed into *Arabidopsis*. Histochemical staining revealed that GUS expression was specifically detected in roots, flowers and seeds, indicating that *Gbvdr3* is a tissue-specific promoter.

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