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Cloning and Characterization of an Organ Specific and Pathogen Responsive Promoter from Cotton (*Gossypium hirsutum* L.)

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Abstract: A promoter region was isolated from the 5′ flanking sequence of *GHNBS* in *Gossypium hirsutum* by Tail PCR. CAAT-box, TATA-box as well as several pathogen, SA, MeJA and ethylene responsive elements, such as W box, GT-1, MYB and MYBST1 motifs were identified by PLACE analysis while some root specific motifs were also identified in this region. Three enhancer elements including two as-1 and one EECCRCAH1 motifs that were responsible for the strong expression of *GUS* gene in transgenic plants were located in the far upstream. Fusions of different 5′ promoter deletion derivatives with the coding region of the *GUS* gene were transformed into *Arabidopsis*. Histochemical localization showed strong staining in roots, phloem of the stem and leaf veins. PGN-1559 and PGN-1117 showed organ specific *GUS* staining patterns while PGN-476 did not. The fact that GHNBS promoter could enhance the expression level of the *GUS* gene in transgenic *Arabidopsis* when treated with SA, ABA, MeJA, ethylene, *Fusarium oxyporum* and DC3000 showed that there were some regulatory elements in the 5′ flanking sequence of *GHNBS* and which was most possible a pathogen responsive and organ specific promoter.

Key words: promoter; cotton(*Gossypium hirsutum* L.); pathogen responsive; organ specific

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Plant diseases caused by fungi, bacteria, viruses or nematodes have catastrophic effects on crops. Plants possess complex resistance mechanisms to defend themselves against pathogen attack. Among these defense related components, proteins encoded by disease resistance genes mediate specific molecular recognition of pathogenic micro-organisms and trigger signaling cascades that activate defense reactions. Members of the largest R protein class are characteristic of nucleotide binding sites and Leu-rich repeats (NBS-LRR). In dicots, NBS-LRR proteins can be subdivided into those expressing either putative coiled-coil (CC) domains or a domain with homology to the cytoplasmic tail of animal signaling proteins called TIR at the N terminus.

Promoters of some pathogen responsive genes such as PR-1, Hs1^{pro-1}, and beta-1, 3-glucanase genes have been isolated[1-3]. Most of these promoters were W box containing, elicitor-responsive. The early and specific response of these promoters to pathogen and stress attack demonstrated the potential of these promoter elements for designing new strategies in disease resistance breeding as well as for further investigation on the regulatory components of defense-related genes and their regulation and/or activation mechanisms.

Gossypium hirsutum was one of the most important economic crops in the world. Cotton diseases caused by *Xanthomonas campestri* pv. *Malvacearum*, *Fusarium oxysporum*, *Verticil-*

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lium dahliae, nematodes are the major challenges to cotton growth and may significantly impact lint yield and fibre quality. Cloning of cotton pathogen responsive gene and promoter was of great importance to make clear the interaction of cotton and pathogens and improve the disease resistance of cotton. Cotton RGAs related to NBS domain have already been isolated and mapped[4]. There are, up to now, no reports on the function of any NBS-LRR gene and promoter in cotton. In this study, the promoter of the CC-NBS-LRR gene *GHNBS* (GenBank accession number DQ785169) that cloned in our lab was isolated, the responses of this promoter to SA (salicylic acid), MJ (jasmonate), Ethylene and pathogens such as *Fusarium oxysporum*, *Pseudomonas syringae* pv. *tomato* DC3000 were also studied.

1 Materials and Methods

1.1 Plant materials

A cotton variety (*Gossypium hirsutum* L.) Chang-kang was used to isolate the promoter of GHNBS (GenBank accession number DQ785169). *Arabidopsis thaliana* ecotype Columbia (Col-0) plants were grown in a controlled-environmental chamber with a photoperiod of 130 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 16 h at 22℃ and 65% relative humidity.

1.2 Isolation of GHNBS promoter

Three nested primers designed according to the genomic sequence of 5′ *GHNBS* (GenBank accession number DQ785169) were 5′- AATTGCAGCTCATCCTGAAGTGATTG-3′, 5′-CCACGAATCAACTGAACTTGTTTTGC-3′, 5′-CGGTTATTATAGATACAATGGCTTCC-3′. Tail PCR was used to isolate the 5′ flanking genomic sequence[5]. Unique amplified products were cloned into the pGEMT-easy vector. The DNA was sequenced and analyzed with the PLACE (http://www.dna.affrc.go.jp) database.

1.3 Construction of GHNBS promoter - GUS fusion vectors and transformation to *Arabidopsis*

To assemble the GHNBS promoter - GUS fusion constructs, the GHNBS promoter fragments covering different regions of the genomic sequence were amplified by PCR. The forward primers designed corresponding to the -1559, -1117, and -476 sequences of the GHNBS promoter were 5′-CGAAGCTTATACGACACACTTAAAAGGAATG-3′, 5′-GCAAGCTTCAACCAAGATTAAGATTCTGTATG-3′, 5′-GCAAGCTTAGAAAAGGGTGTATTGGGTA-3′, respectively. And the reverse primer was 5′-CCGGATCCACTAATTTGCCTTGTTATTTC-AGA-3′. *Hind*III and *Bam*HI sites were introduced into the primers. The three PCR fragments were purified and digested by *Hind*III and *Bam*HI and introduced into the corresponding cloning sites of the pBI101. 1 binary vector[6]. The identities of all the constructs were confirmed by sequence analysis. The resulting constructs were designated as PGN-1559, PGN-1117, PGN-476. All constructs were transferred to the *Agrobacterium tumefaciens* strain LBA4404 by freeze-thaw method. Transgenic *Arabidopsis* plants were performed according to the floral dip transformation procedure. The putative transgenic *Arabidopsis* plants (T_1 plants) were selected by sowing the seeds on an MS plate containing 40 $mg \cdot L^{-1}$ kanamycin. The kanamycin-resistant seedlings were verified by PCR using GHNBS promoter specific primers. The T_2 progenies of the transgenic plants were used for further experiments.

1.4 Analysis of GUS enzyme activity

The GUS activity was estimated using a fluorescence method with 4-methylumbelliferyl glucuronide as the substrate. 4-Methylumbelliferone (4-MU) was quantified using a fluorometer. Protein concentration of extracts was determined by the Bradford methods[6-7]. Histochemical localization of GUS activity was performed with 5-bromo-4-chloro-3-indolyl β-Dglucuronic acid (X-Gluc) as a chromogenic substrate[6]. Different tissues from control and transgenic plants were stained at 37°C in 2 mmol · L^{-1} X-Gluc in 0.2 mol · L^{-1} phosphate buffer.

1.5 Pathogen infection of *Arabidopsis*

Pseudomonas syringae pv. *tomato* DC3000 was incubated overnight in King's B medium plate containing 50 mg · L^{-1} rifampicin. The bacterial cells were harvested, washed twice with 10 mmol · L^{-1} $MgCl_2$, and resuspended. Four week-old *Arabidopsis* plants were inoculated by infiltration with the virulent strain of DC3000 ($10^3 mL^{-1}$), using a needleless syringe[8]. *Fusarium oxyporum* isolate was grown in 300 mL of potato dextrose broth (Difco) in 500 mL Erlenmeyer flasks by inoculating each flask with 1 mL of recovered culture and incubating at 25°C on an orbital shaker at 175 r · min^{-1} for 1 week[8].

1.6 Chemical treatment

SA treatment was carried out by spraying with a 200 μmol · L^{-1} solution containing 0.1% (*W*/*V*) ethanol. For treatment with MJ, a cotton ball containing 200 μL of a 0.5% (*W*/*V*) solution in ethanol was tapped onto the wall of a 20-L airtight container. Ethylene was applied at a concentration of 200 μL · L^{-1} by injection of gaseous ethylene through a rubber septum.

2 Results

2.1 GHNBS promoter isolation and analysis

The cloned *GHNBS* gene promoter region was 1600 bp in length and no identity was found by Gene Bank blast (Fig. 1). This indicated that it is a new promoter. The promoter region of the *GHNBS* gene has been analyzed to search for the putative cis-acting elements using the PLACE database. A TATA box was located at upstream of the putative transcription initiation site. Several putative regulatory motifs, which are homologous to the cis-acting elements involved in activating the defense genes in plants, were identified in the promoter region of the *GHNBS* gene. Eight copies of GT1 CONSENSUS motifs for GT-1 like protein binding were found in GHNBS promoter, which were reported playing an important role in regulating PR-1 expression (Fig. 1)[9]. The GHNBS promoter was also found containing WBOXATNPR1 and WBOXNTERF box, which are important pathogen-responsive cis-acting elements binding to the WRKY transcription factor and the SA responsive factor[10]. W boxes are present in the promoter of PR1, the hallmark gene associated with the induction of systemic acquired resistance in *Arabidopsis*[11]. Nine copies of root motifs were also existed in GHNBS promoter, which could control and enhance root specific expression. Several dehydration-response elements (MYCONSENSUSAT) and water stress-responsive MYBCORE elements were found in the promoter. Additional regulatory sequence motifs identified in the promoter region were the MYBST1 core, the MYB1LEPR, ASF-1 motif, and the EECCRCAH1 motif (Fig. 1). The ASF-1 element has been shown to be responsive to the defense signaling molecules SA and MJ and has been identified in the promoters of glutathione S-transferase genes and on the 35S promoter of *Cauliflower mosaic virus*[12].

2.2 Analysis of tissue specific expression of the PGNBS promoter -β-glucuronidase gene

To determine which regions of the GHNBS promoter contribute to the regulation of gene expression, the translational fusions of the promoter deletion derivatives were performed with the coding region of the *GUS* gene. The resulting constructs were designated as PGN-1559, PGN-1117, PGN-476. The whole plant, and the root, leaf and stem cross section of the T_2 progenies were used for Histochemical localization. PGN-1559 showed strong staining signal in roots and leaf veins (Fig. 2 A, B and C) and the staining signals in the stem section were limited to phloem (Fig. 2D). Compared with PGN-1559, PGN-1117 presented similar patterns but very weak staining signal. And GUS staining signals in root and stem were hardly detected (Fig. 2 A and B). Interestingly, the staining patterns of PGN-476 were completely different and no organ specific GUS expression was observed. Strong GUS staining signal was not detected in leaf vein but spotted in leaves (Fig. 2 A, B and C). The root was not been stained while GUS staining patterns in a stem cross section was dispersed instead of limited to phloem (Fig. 2D).

−1600	AGTGGAGTAGCAAAGGAAAGGAACAGATGGAGGGACCTGTCATACGACACACTTAAAAGG
−1540	AATGCAACGTCAATGGCGATGCCTAATTGTTACTTACAGCAGGTTCCAAAGATTTGTTCC
−1480	ACCACGCGTGAAGGAATCAGATTCGACTGCTCCCAACTTACATGCAACGTCAATATCAAT
−1420	CCTCATATAAAGCTGATTAAAAGTTGATTTTTAATATTAAGAATAGCATCCCATTCACCT
−1360	CCCATAACAGTGATCATTTTGTAACATTTTAAAGTTAAGTGACTAAAACATAAATTTACT
−1300	GTTAATTTAGTGATCTTGGATGTAATTTATCCATATTTATGTTACATGCACGGTGAAGAC
−1240	TTCCTGCATTTGGGAACATTTTCACTTGTCTTTCTTTACTTCATTCTTGTACTTTAATCT
−1180	ATCTCAACCAAGATTAAGATTCTGTATGTTGAGAACTTTGGTTAGTTTATATTTGAAAAA
−1120	AATTATTGATATTATTAAAAAAAATAACTATAAATTTAAAAAATATTAAAAACATGTACA
−1060	ACCAAAACATCTATTTTAAATGAAGCATTTCGAAAGCGAATTTGATTAAAAAAGGATAGC
−1000	CATGTGAAAGTCAACTTTGAGCATGTGAACAACTTATTGAAATCATTTATCATTTTCAAT
−940	TAGTTGAAAGTCCTAAATTTATTTTTTAAAAATCGGTTTGTTTAAAACTAAGGTGTTGAT
−880	AGAATAAGTATTTAATTAAATGCATTATTTTTAGTGTAAGAAAAATAATAATTCAGTATG
−820	CCATGTAATTGATGTGGTACGATTAAGTTCGAGCAACAAATCGAAAGGAGGCGGTTTTTG
−760	GGCATGCTATAGGGCATTTGGTTCCGTACAAATATGCAAATAAGTTTAAATGTAATTTTG
−700	ATGGGGCCCTCTCTGTTGACAGTGGGTATTCGGGGTGAATTTGTGCGGTGCATCTCGAGT
−640	TTTATAAAGATCGCAAAGTCCATCTTTCTGCGGTGGAAGTCATGTCTACTCATCATTGCT
−580	TTATTGAAAGCAAACTACTTTGAATGTATTTCTTATCCTGTTTTATCATTCATACTGTTA
−520	ATGTTGATAAATTGTTACAACAGGATGAAAATAAGATAGGACTTAGAAAAGGGTGTATTG
−460	GGTAAGCCGGTTGATTTGGATTTAATCTGTACCACTTATTTTCATTTTGTTTTCTTTTTA
−400	TCAGTTTTACTGGTTTAATGAATTTGATTTTATATTTAAAAAAAATCAAATATAAATGTT
−340	TAGTTGCCATCAGCATTAGGATATCAACCACATGTCAAGCATGATTGCTTAATTTGATTT
−280	AAATATATATAATAATAAATGAAAATTGACTTTTTGGTTGGATGATAACATCACTTATGT
−220	AAGCTAAAATTGCAATTTAAAATCAGAAAATGAAATCCTCGAGTTCTTTTTCATTCATAC
−160	TTTCATAAATTCAACTATAGTTAATAATAGATCATAAATGCAGTCCAGTTTCTTATAATA
−100	AAAAAACAAGGTAGGTCTTAAATTCTTATAA**ATG**CAAGCAGTCCAGTTTCTGAAATAACA
−40	AGGCAAATTAGTTTTCTGAGTGACTAAAAAGTTAGGGAGGATGGAAGCCATTGTATCTAT

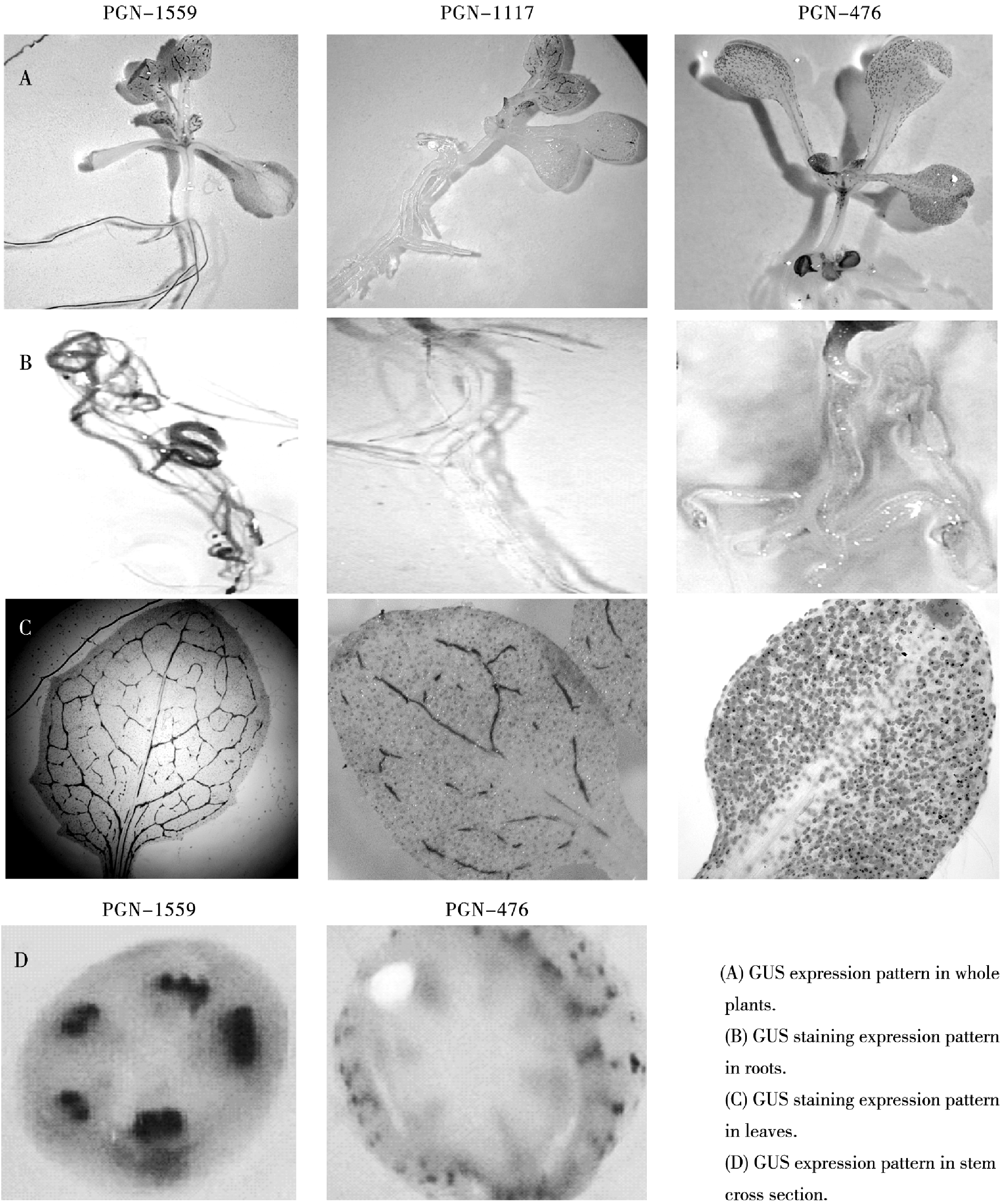
The motifs with a significant similarity to the previously identified cis-acting elements are shown underline. The putative transcription start site is shown in bold type. The box indicates the translational start site. Root specific motif: ATATT; ASF-1: CGTCA; GT1: GRWAAW; WBOXHVISO1: TGACT; WBOXATNPR1: TTGAC; WBOXNTERF3: TGACY; MYBST1: GGATA; MYBPZM: CCWACC; EECCRCAH1: GACTTCC.

Fig. 1 Nucleotide sequences of GHNBS promoter region and putative cis-acting element

2.3 Analysis of GUS activity of the PGNBS promoter -β-glucuronidase gene

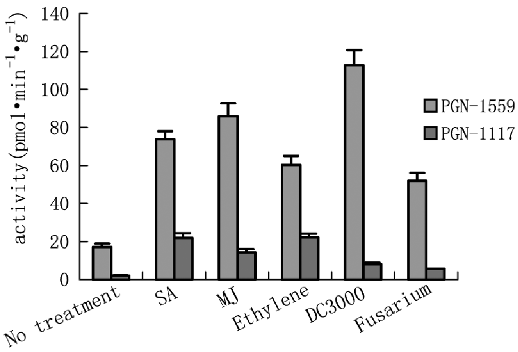
In order to understand the regulated patterns of this promoter, the phloem specific transgenic plants PGN-1559 and PGN-1117 were further studied. The PGN-1559 showed 8-fold GUS activity as that of PGN-1117. This indicated that a reduction in the promoter length resulted in the negative regulation of GUS expression. A highly significant 6.55-fold increase in GUS activity was observed in the PGN-1559 *Arabidopsis* after inoculation with the bacterial pathogen *P. syringae* pv. DC3000, while compared with the untreated seedlings (Fig. 3). Compared with that of PGN-1559, the induction of GUS activity of PGN-1117 construct was quite similar in the bacteria-infiltrated treatment, despite the relatively low level of promoter activity (4.00-fold induction). GUS activity of both PGN-1559 and PGN-1117 showed up to 3.00-fold increase after inoculation with *Fusarium oxyporum* strains. This indicated that the DNA elements of the promoter may contribute to the *GHNBS* gene expression in response to virulent pathogen infection.

Transgenic plants from the two chimeric constructs were also tested for the inducibility of GUS expression by applying ethylene, SA and MJ to the leaves. GUS activity of PGN-1559 increased 4.25 fold and 5.02 fold respectively after SA and MJ treatment. While PGN-1117 showed 11.00-fold and 7.15-fold increase after SA and



(A) GUS expression pattern in whole plants.
(B) GUS staining expression pattern in roots.
(C) GUS staining expression pattern in leaves.
(D) GUS expression pattern in stem cross section.

Fig. 2 Gus expression patterns of PGNBS promoter in transgenic *Arabidopsis thaliana* plants



No treatment: GUS expression without treatment; DC3000: Pseudomonas syringae pv.DC3000 treatment; ethylene : treated with ethylene; SA: salicylic acid treatment.

Fig.3 GUS activity of the PGN 1559 and PGN 1117 transgenic *Arabidopsis* after pathogen infection or salicylic acid, ethylene and MJ treatments

MJ treatment. The exogenous application of ethylene sharply activated both the PGN-1559 and PGN-1117 genes expression up to 3.51-fold and 11.08-fold respectively compared with the basal level (Fig. 3).

3 Discussion

Organ specific promoter plays an important role in plant genetic engineering such as oil quality improvement[13]. GHNBS promoter proved to be expressed in root, phloem of stem, vein of leave. Some organ-specific expression pattern has been reported in other pathogen related genes. In *N. tabacum*, for example, the promoter of a basic PR1 gene drove the expression of a β-glucuronidase (GUS) reporter gene in floral organs, roots and stems[14]. Histochemical studies of these transgenic plants revealed that GUS expression in stems was restricted to the vascular tissue[14]. The Hs1^{pro1} promoter is functional and drives a nematode responsive and feeding site-specific GUS expression[2]. Nine copies of root motif in GHNBS promoter may contribute to the strong GUS expression in root. It is still unclear that which elements regulate the phloem specific and vein specific expression in stem and leaves, respectively.

Sequence analysis found two ASF-1 enhancer elements in the full length GHNBS promoter PGN-1559. The ASF-1 element has been shown to be responsive to the defense signaling molecules SA and MJ[12]. The ASF-1 may also play some role on increasing basal Gus activity in PGN-1559 transgenic *Arabidopsis*.

A SA-dependent network is thought to mediate resistance against biotrophic pathogens such as *Peronospora parasitica*, whilst a MeJA-dependent network is thought to engage resistance against necrotrophic pathogens such as *Alternaria brassicicola*[14]. The tobacco pathogenesis related PR-2d gene encodes an acidic beta-1, 3-glucanase. Expression of the PR-2d: uidA (GUS) chimeric gene is induced in leaves undergoing the hypersensitive resistance response to tobacco mosaic virus and after treatment with salicylic acid (SA) results show up to 20 fold increase of GUS activity by application of SA[15]. In our experiment, GUS activity of PGN-1559 increased 4.25 fold, 5.02 fold and 3.51 fold respectively after SA, MJ and ethylene.

In summary, *GHNBS* gene promoter was organ specific expression and positive regulation upon pathogen inoculation, SA, MeJA and ethylene application, suggesting that *GHNBS* gene and its promoter may play an important role in cotton disease resistance and signal transduction.

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