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Analysis of rice genes induced by striped stemborer (*Chilo suppressalis*) attack identified a promoter fragment highly specifically responsive to insect feeding

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Abstract The objective of this study was to identify rice gene promoters that are specifically induced by feeding of the striped stemborer (*Chilo suppressalis*). Two PCR-selected cDNA subtractive libraries were constructed from the rice variety Minghui 63. Up- and down-regulated cDNAs induced by *C. suppressalis* feeding were arrayed on nylon membranes. After array hybridization and Northern blot analysis, a cDNA (B1-A04) encoding a putative subtilisin/chymotrypsin inhibitor was found to be rapidly and highly induced by *C. suppressalis* feeding, compared with mechanical wounding. The putative promoter region, spanning from –1,569 to +446 relative to the transcriptional initiation site was isolated, fused to the *GUS* gene (*β*-glucuronidase reporter gene) and introduced by Agrobacterium-mediated transformation to rice. In non-infested plants, the GUS activity driven by this promoter fragment was detected in culms and panicles, but not in leaves and sheaths. At 6 h after insect feeding, GUS activity was significantly induced in sheaths and culms, but not in leaves. GUS activity and native B1-A04 gene were not induced by JA and ABA treatment. A serial deletion analysis revealed two regions (–1,569 to –1,166 and –1,166 to –582) that negatively regulate the gene expression in sheaths of non-infested plants but not in insect-infested plants. An electrophoretic mobility shift assay (EMSA) identified 7 DNA fragments with various binding activities with nuclear proteins from mechanically wounded, insect-infested and untreated plants, and their possible roles in gene regulation were speculated. This promoter fragment should have utility in development of insect resistant transgenic crops.

Keywords *Chilo suppressalis* · Insect responsive promoter · Protease inhibitor · Rice striped stemborer

Abbreviations

GUS	*β*-glucuronidase reporter gene
EMSA	electrophoretic mobility shift assay
JA	jasmonic acid
ABA	abscisic acid

Introduction

Plants are attacked by a diversity of herbivores under natural conditions. With the constant pressure from herbivores, plants have evolved a large array of counter-responses to herbivores during evolution. Besides chemical and physical barriers as preformed defense mechanisms, plants have developed inducible defense systems that are of central importance in reducing damages by herbivores. Herbivores can trigger direct and indirect inducible defense responses in plants (Kessler and Baldwin 2002). Direct plant defense responses result in inhibition of growth and development of herbivores, while inducible defenses involve a broad range of proteins and other molecules whose synthesis is spatially and temporally regulated, in which the jasmonic acid (JA) pathway is known to play crucial roles (Howe et al. 1996; Liechti and Farmer 2002; Reymond et al. 2004).

Insect feeding inevitably causes wounding of the plant. However, little is known about how plants distinguish and

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respond to different lesions caused by mechanical wounding and herbivores feeding. Previous reports showed that some genes could be activated by both mechanical wounding and insect feeding, while others were induced specifically or activated by damage from insects or by mechanical wounding (Halitschke et al. 2001; Hermsmeier et al. 2001; Reymond et al. 2004). Specific elicitors have been found to induce specific responses of the plants against insects, including fatty acid-amino acids such as volicitin (*N*-(17-Hydroxylinolenoyl)-L-Gln) in regurgitant of *Spodoptera exigua* larvae (Alborn et al. 1997), fatty acid-amino acid conjugates in *Manduca sexta* oral secretions (Halitschke et al. 2001), β-glucosidase in the regurgitant of *Pieris brassica* larvae (Mattiacci et al. 1995), and glucose oxidase in the salivary glands of *Helicoverpa zea* larvae (Musser et al. 2002) that leads to hydrogen peroxide production indirectly eliciting plant responses.

Mithöfer et al. (2005) designed a mechanical caterpillar that very closely resembled the herbivore-caused tissue damage in terms of similar physical appearance and long-lasting wounding period on defined leaf areas. They found that while this mode of treatment was sufficient to induce the emission of a volatile organic compound blend qualitatively similar to that as known from real herbivore feeding, there were significant quantitative differences for a number of compounds. Clearly, although both mechanical wounding and herbivore chewing cause wounds on plants, it is difficult to mimic the herbivore attack with mechanical wounding (Korth and Dixon 1997).

The array technology, enabling changes in transcript abundance of hundreds of genes to be monitored simultaneously, provides a possibility to elucidate the molecular mechanism of the specific response of plants against wounding and herbivore, and offers an effective tool for finding potential new genes for insect resistance. This technique has been used to study expression profile of genes in various plant species for wound- and herbivore-triggered responses (Reymond et al. 2000; Hui et al. 2003; Cho et al. 2004; Voelckel and Baldwin 2004; Cho et al. 2005). For instance, DNA microarray analysis in Arabidopsis has shown that mechanical wounding results in a very different transcript profile from damages by larval feeding of the cabbage butterfly (Reymond et al. 2000). In rice, 27 brown planthopper (*Nilaparvata lugens*)-responsive genes were identified using an array analysis (Yuan et al. 2005).

Chilo suppressalis (Walker), or striped stemborer, is one of the most damaging Lepidoptera pests of rice worldwide. Its injury to the rice plant is caused by larval tunneling in the sheaths and culms. Visible symptoms on affected plants vary with the stage when infestation commences. At the tillering stage, the infestation causes ''dead sheaths'' at first, then ''dead hearts'' (death of the innermost leaf of the tiller). At the booting stage, the infestation results in ''dead panicles''. At the heading stage, the infestation leads to ''white heads.''

In this study, we searched rice genes responding to *C. suppressalis* feeding using a macroarray analysis. A gene corresponding to the sequence B1-A04 encoding a putative subtilisin/chymotrypsin inhibitor was identified as exhibiting rapidly and highly induced expression by insect-feeding but very slightly by mechanical wounding. By examining the expression of *uidA* (β-glucuronidase, or GUS) reporter gene driven by the B1-A04 promoter fragments of various lengths resulting from serial 5′-deletions and by performing DNA-protein binding assays, we identified two novel herbivore-responsive regions that negatively regulate B1-A04 expression.

Materials and methods

Plant materials, treatments and tissue collection

Three seedlings of the cultivar Minghui 63 (*Oryza sativa* L. ssp. *indica*) were grown in pots and protected from herbivore attack with nylon nets. At tillering stage, the first plant was infested with one *C. suppressalis* larva at the 3rd to 4th larval stage, the second plant was wounded by punching with a pair of scissors along the edge of the tiller at 30 min intervals during treatment time, resulting in holes approximately 3 mm wide and 8 mm long, and the third plant was kept as the undamaged control. Rice tissues were harvested at 15 min, 30 min, 1 h, 3 h, 6 h and 12 h after the infestation and wounding treatments, and quickly immersed into liquid nitrogen for RNA extraction.

For fluorometric GUS assay, each transgenic plant was divided into two clones by peeling off and replanting the tillers at tillering stage, one clone for *C. suppressalis* infestation 10 days later and the other as the undamaged control. *C. suppressalis* feeding and material collection were as described above.

For phytohormone treatment, each transgenic plant was divided into three clones by peeling off and replanting the tillers at tillering stage. Ten days later, two of the clones were sprayed with jasmonic acid (JA) or abscisic acid (ABA) at a concentration of 100 μM as described (Kang et al. 2002; Perez-Amador et al. 2002), and the other clone was sprayed with water as the control. Samples were collected at 1, 3, 6 and 12 h after spraying.

Construction of subtractive cDNA libraries

Total RNAs isolated using Trizol reagent (Invitrogen) from sheath tissues of Minghui 63, were used for cDNA library construction. Equal amounts of total RNAs isolated from sheaths at 3 h and 6 h after feeding were mixed, from

which mRNAs were isolated using Dynabeads oligo $dT_{(25)}$ (Dynal A.S.). Two subtractive cDNA libraries were constructed using the PCR-Select cDNA Subtraction Kit according to the manufacturer's instruction (Clontech, USA) with pGEM-T as the vector. Library I was constructed using mRNA isolated from the feeding treatment as the tester with the undamaged control as the driver, and the reverse was done for library II. Thus, library I and II were expected to yield up- and down-regulated genes, respectively. cDNA clones from the subtractive libraries were sequenced with the T7 primer using the BigDye Terminator Cycle Sequencing V2.0 (Applied Biosystems).

cDNA macroarray analysis

The plasmid of each clone was isolated and arrayed in duplicate on a Hybond N+ membrane (Amersham Pharmacia) using a Biomek 2000 robot (Beckman Coulter, USA). A clone of the rice actin gene (GenBank Acc. No. EI077C02) was also arrayed in duplicate as the positive control, and a clone of a porcine glyceraldehydes 3-phosphate dehydrogenase gene (GenBank Acc. No. AF017079) was included also in duplicate as the negative control. Membranes were processed as described by Zhou et al. (2002).

Total RNA (100 μg) from sheaths of the plants at 6 h after feeding and the undamaged control was reverse-transcribed, labeled with ^{32}P-dCTP, and used as the probe to hybridize with the macroarrays. The hybridization was repeated twice independently. After stringent washing as described by Zhou et al. (2002) membranes were exposed to the Storage PhosphorImage Screen for 8 h and scanned with a PhosphorImager SI (Molecular Dynamics). Intensity of the hybridization signal was measured using the ArrayGauge Version 1.0 software (Fuji).

Southern and Northern blot analyses

DNA extraction followed essentially the CTAB method (Murray and Thomson 1980). DNA digestion, electrophoresis and Southern blot hybridization were performed as described by Liu et al. (1997). Northern blot analysis was conducted following the procedures described by Chu et al. (2004). All the hybridization probes were prepared from PCR products. The sequences of PCR primers of all the probes used are listed in Table 1.

Sequence analysis and construction of transgenic vectors for promoter analysis

The full-length cDNA of B1-A04 (see Results) was found in the rice full-length cDNA database (http://cdna01.dna.affrc.go.jp). The putative transcription initiation site in the DNA sequence was predicted using a promoter prediction program (http://www.fruitfly.org/seq_tools/promoter.html) (Reese 2001). The analysis of potential *cis*-regulatory elements in the promoter region was performed with the computer programs PLACE (A Database of Plant Cis-acting Regulatory DNA Elements) Signal Scan (http://www.dna.affrc.go.jp/PLACE/signalscan.html) (Prestridge 1991; Higo et al. 1999).

PCR-amplified fragments of putative promoter regions were cloned into pCAMBIA1381 a, b, c (provided by the Center for the Application of Molecular Biology to International Agriculture, Australia). The promoter region and junction with the *uidA* gene sequences in each construct were verified by DNA sequencing.

Table 1 PCR primer sequence of hybridization probe

Probe	Primer sequence
B1-A04	F: 5′-TTGTAAAACGACGGCCAGTG-3′
	R: 5′-GGAAACAGCTATGACCATGA-3′
A2-C03	F: 5′-TTGTAA AACGACGGCCAGTG-3′
	R: 5′-GGAAACAGCTATGACCATGA-3′
A1-G12	F: 5′-TTGTAAAACGACGGCCAGTG-3′
	R: 5′-GGAAACAGCTATGACCATGA-3′
A1-B01	F: 5′-TTGTAAAACGACGGCCAGTG-3′
	R: 5′-GGAAACAGCTATGACCATGA-3′
GUS	F: 5′-CCAGGCAGTTTTAACGATCAGTTCGC-3′
	R: 5′-GAGTGAAGATCCCTTTCTTGTTACCG-3′
OsATX	F: 5′-ACCTGTGAGAGTCAATCTCT-3′
	R: 5′-ACTGAGGGAGAGTAAGCAAT-3′

Rice transformation

Mature seeds of Zhonghua 11 (*O. sativa* L. ssp. *japonica*) were used for transformation. The transformation procedures followed essentially as described in Hiei et al. (1994) and Wu et al. (2003).

Fluorometric GUS assay and histochemical GUS staining

Fluorometric GUS assays of rice sheaths and leaves were performed as described by Jefferson et al. (1987). GUS activity was determined with a DyNAQuant 200 fluorometer (Hoefer). Protein concentrations of plant extracts were determined by the dye-binding method of Bradford (1976).

For histochemical GUS staining, the sheaths, leaves, culms and panicles of at least 10 transgenic lines were cut off and submerged in GUS staining buffer (Jefferson et al. 1987) at 37°C overnight. Stained samples were cleared of chlorophylls in 75% ethanol, observed under a Leica MZFLIII dissection microscope (Leica Microsystems), and photographed with a Nikon E5400 digital camera.

Electrophoretic mobility shift assay (EMSA)

Approximately 100 g sheath of Minghui 63 at tillering stage was harvested 6 h after *C. suppressalis* infestation and wounding, with untreated plants as the control. The nuclear protein isolation and EMSA were conducted as described previously (Qiu et al. 2007).

Results

Macroarray analysis revealed both up- and down-regulated transcripts by *C. suppressalis* feeding

A total of 864 clones were arbitrarily selected from the two subtractive libraries with 528 clones from the subtractive library I and 336 from library II. Sequencing of these clones identified 271 unique sequences with the length ranging from 101 bp to 866 bp. These 271 clones and the positive and negative controls, together with 109 additional EST clones from a normalized cDNA library of the same variety Minghui 63 (Chu et al. 2003), were arrayed on nylon membranes. The transcript levels of 17 clones were altered by at least 1.5-fold in both hybridizations in sheaths after the feeding challenge. The predicted functions of these differentially expressed genes are shown in Table 2.

Among the 17 putative differentially expressed sequences, 12 were induced and 5 were repressed by *C. suppressalis* feeding. About 10 of the 12 up-regulated genes were from the subtractive library I (Table 2), while 4 of the 5 down-regulated genes were from library II. Such results suggested that the subtractive libraries enriched cDNA clones of genes responsive to *C. suppressalis* feeding.

A sequence specifically induced by *C. suppressalis* feeding encoded a putative subtilisin/chymotrypsin inhibitor

To search for candidates of *C. suppressalis*-inducible genes, 4 of the up-regulated genes were randomly selected for further analysis. The expression level of these four genes at different time points after *C. suppressalis* challenge was examined using Northern blot analysis (Fig. 1). Three of the sequences (A1-G12, A1-B01 and A2-C03) appeared to be highly induced by both wounding and feeding, although the induction levels and timing varied between the treatments. The wound-induced expression of A1-B01, encoding an oryzacystatin, followed the same

Table 2 Functional categories of the ESTs that showed differences of 1.5-fold or more in signal intensity in the treatment of 6 h after insect-feeding compared with the undamaged control in both hybridizations

Clone ID	GenBank Acc. No.	Library[a]	Ratio[b]	*E*-value[c]	Functional category
B1-A04	DT806917	I	5.04	1e-21	Subtilisin/chymotrypsin inhibitor—*Zea mays*
A2-B07	DT806831	I	2.63	2e-78	SalT protein precursor—*Oryza sativa*
B2-B06	DT806984	I	2.48	1e-122	Probable serine/threonine-specific protein kinase—*Arabidopsis thaliana*
B2-A08	DT806937	I	2.36	0.0	Cullin-like protein—*Arabidopsis thaliana*
A1-C06	DT806831	I	2.29	4e-78	Salt-stress induced protein—*Oryza sativa*
A1-G12	AU164638	N	2.18		No hits found
A1-B01	DT806878	I	1.88	6e-42	Oryzacystatin—*Oryza sativa*
A2-C03	DT806849	I	1.82	6e-25	Guanine deaminase-like—*Bacillus subtilis*
A1-C07	DT806859	I	1.75	0.0	Mitochondrial ADP, ATP carrier protein—*Oryza sativa*
A1-C08	DT806881	I	1.62	0.0	Polyubiquitin—*Zea mays*
A2-E10	DT806861	II	1.56	2e-59	H+-transporting two-sector ATPase chloroplast chain I—*Triticum aestivum*
A1-A05	DT806960	I	1.51	1e-169	phosphate transport protein, mitochondrial—*Zea mays*
B1-C05	DT807032	II	0.64	1e-79	Ribosomal protein L17—*Zea mays*
A1-A10	DT806913	I	0.59	2e-28	Small nuclear riboprotein Sm-D1-like protein—*Arabidopsis thaliana*
B1-D11	DT806916	II	0.51		No hits found
B1-D10	DT807058	II	0.43	5e-29	DNA-binding protein MNB1B (HMG1-like protein)—*Zea mays*
B1-D09	DT806930	II	0.42	2e-62	Hypothetical protein p85RF—*Prunus armeniaca*

[a] I, the cDNA library constructed using mRNA isolated from the feeding treatment as the tester with the undamaged control as the driver; II, the cDNA library constructed in the reverse way as for library I; N, a normalized cDNA library of Minghui 63 (Chu et al. 2003)

[b] Average ratio of treatment against control in two hybridizations

[c] *E*-value resulted from BLASTx search

time course as that by insect feeding, but the induction intensity of wound is higher than that of insect feeding. The wound and insect-feeding induced expression of A1-G12, a sequence with unknown function, also seemed to follow a similar time course, whereas the induction intensity by wound is lower than that by insect feeding (Fig. 1). A2-C03, encoding a guanine deaminase-like protein, was highly activated 1 h after wounding and then dropped to the background level. But the induction of insect feeding sustained from 1 h to 12 h after feeding.

There was very low level of induction of the fourth mRNA (B1-A04) after wounding. However, it was highly (more than 5-fold) induced at 3 h after feeding, and the induction was further increased at 6 h and 12 h after feeding (Fig. 1), indicating that the gene corresponding to clone B1-A04 more specifically responded to *C. suppressalis* feeding than to mechanical wounding.

The B1-A04 sequence was localized on chromosome 12 by BLAST search of the Nipponbare (*O. sativa* L. ssp. *japonica*) sequence (International Rice Genome Sequencing Project 2005). It corresponded to a single copy gene in the rice genome as determined by both Southern blot (not shown) and genomic sequence analysis. A sequence of full-length rice cDNA from etiolated shoots corresponding to this sequence was found in the GenBank database (Acc. No. AK063833). It encodes a putative subtilisin/chymotrypsin inhibitor, belonging to the protease type I of the serine protease inhibitor family (Ryan 1990).

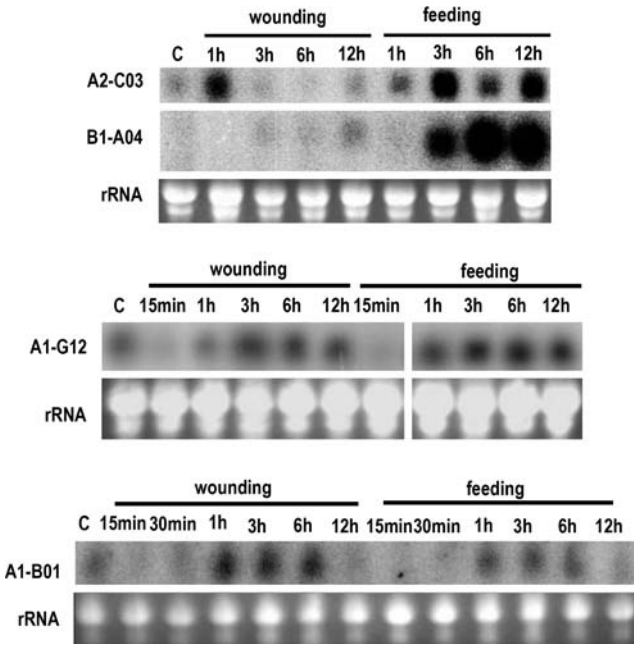

Fig. 1 RNA gel blot analysis of four up-regulated ESTs in the sheaths of Minghui 63 at tillering stage. The samples were isolated at different time points after mechanical wounding and *C. suppressalis* feeding. Lane C was loaded with RNA from undamaged controls

Expression profile of the B1-A04 sequence at rice tillering and booting stages

In order to characterize the expression profile of the sequence B1-A04, the putative promoter region, spanning from –1,569 to +446 relative to the transcriptional initiation site thus containing part of the 5′-end coding sequence of the gene, was isolated by PCR amplification using a clone (18M07) from a BAC (bacterial artificial chromosome) library of Minghui 63 (Peng et al. 1998) as the template. This fragment, referred to as –1,569 for ease of description, was fused in frame with the GUS gene and transformed to rice cultivar Zhonghua 11 (Fig. 2). Since tillering and booting are the main stages for *C. suppressalis* infestation, *GUS* expression in transgenic rice plants was examined mostly in tissues harvested at these two stages. Without infestation, GUS activity was not detectable by histochemical staining in leaves and sheaths at either stage, but it could be detected in culms and panicles at booting stage (Fig. 3A–F). At 6 h after *C. suppressalis* feeding, *GUS* expression was detected in sheaths, panicles and culms, but still not in leaves (Fig. 3G–L). Northern blot results showed that the *GUS* mRNA levels increased significantly in sheaths and culms after *C. suppressalis* feeding (Fig. 4), in accord to the histochemical staining. The expression pattern of endogenous gene corresponding to the sequence B1-A04 is essentially the same as the *GUS* expression driven by the cloned promoter fragment (Fig. 4). Thus, this gene had an organ-specific (i.e., culm and panicle) as well as developmental stage-specific (booting stage) expression pattern. These results also indicated that the cloned promoter fragment was sufficient to respond to the challenge by *C. suppressalis* feeding.

JA and ABA were not involved in the insect feeding response of this promoter fragment

It was known that JA and ABA are involved in the plant defensive pathway activated by herbivores (Peña-Cortés et al. 1989; Herde et al. 1999; Casaretto et al. 2004). To investigate whether these two phytohormones are involved in the induction of the B1-A04 promoter fragment by *C. suppressalis* feeding, GUS activity and *GUS* gene expression were assayed in leaf and sheath tissues sampled at 1, 3, 6 and 12 h after the phytohormone treatments of the transgenic plants harboring the *GUS* gene driven by the –1,569 promoter fragment. The water treatment was used as the negative control, and *OsATX*, a single copy rice gene that can be effectively up-regulated by either JA or ABA treatment (Agrawal et al. 2002), as the positive control. No induction of the GUS activity or *GUS* expression was detected with either of the treatments in any of the time points, neither was the expression of the native B1-A04

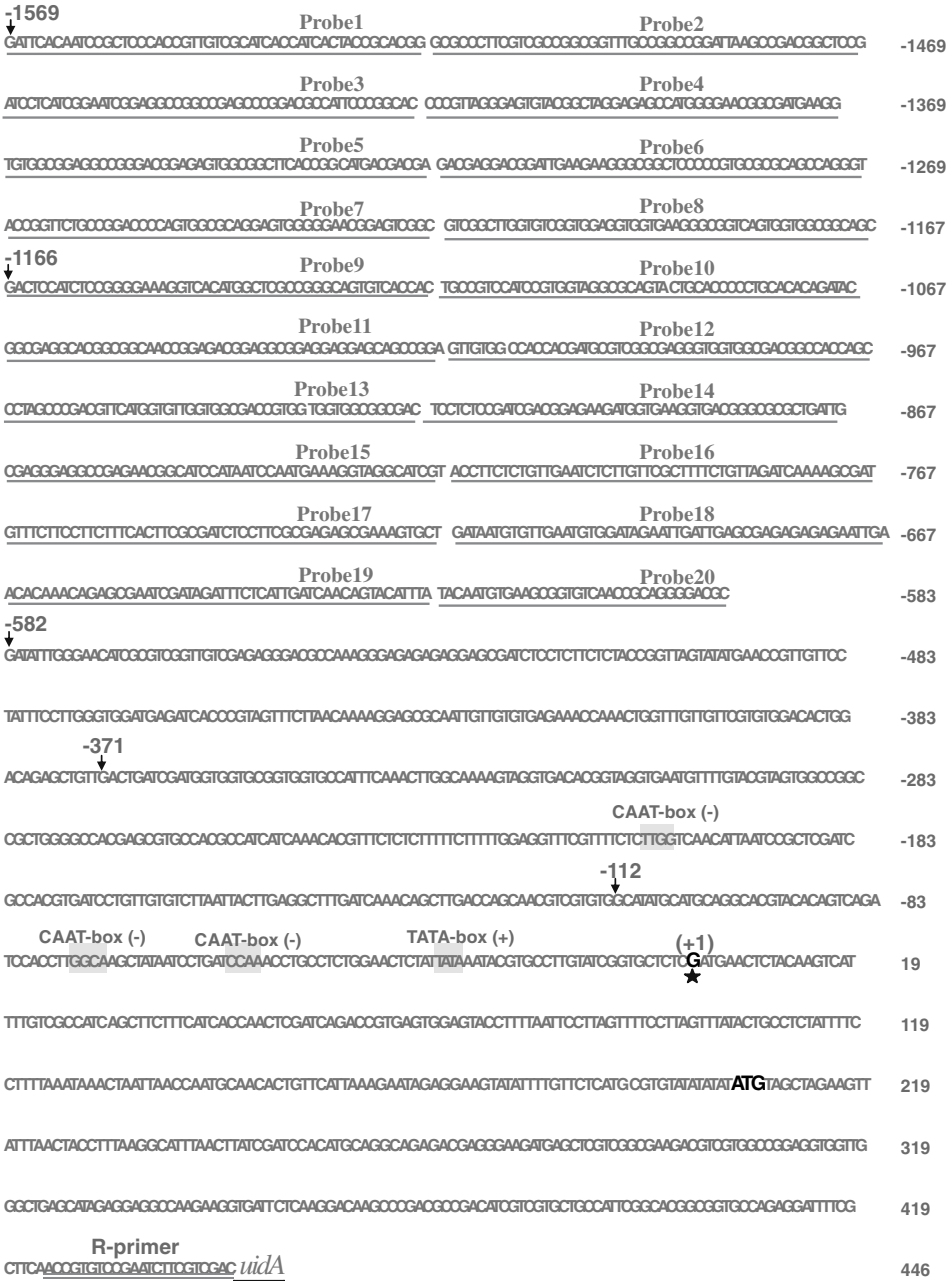

Fig. 2 Nucleotide sequence of B1-A04 promoter fragment. Nucleotides are numbered relative to the putative transcription start site (+1) labeled with an *asterisk*. The predicted motifs are shaded in *grey*, and their orientations are shown in parenthesis. The positions of five 5′-deletions for the translational fusion with *GUS* reporter gene are arrowed. The start codon ATG is in *bold*. The 20 underlined DNA regions indicate the probes used for EMSA. The R primer is the primer anchored at the 3′-end to amplify the different 5′-deletion fragments

gene detected. In contrast, the *OsATX* was highly induced by both JA and ABA treatments in both leaf and sheath tissues from 3 h to 12 h after treatments (Fig. 5). Thus, the induction of this promoter fragment is unlikely regulated by JA or ABA.

The B1-A04 promoter fragment contains two regions that negatively regulate the gene expression in non-infested plants but not in insect-infested plants

In order to define promoter regions responsible for the expression pattern observed above, fragments with serial deletions were isolated by PCR amplifications of the genomic DNA with BAC clone 18M07 as the template, using primers amplifying regions from –1,166, –582, –371 and –112 to +446 (referred to as fragments –1,166, –582, –371 and –112, respectively). These fragments were fused with the *GUS* gene and introduced into Zhonghua 11. The expression patterns in transgenic plants driven by these truncated fragments were compared with that by the –1,569 fragment. GUS activity in leaves and sheaths of at least 15 independent transgenic lines per construct at tillering stage was assayed (Fig. 6A). Without insect feeding, the GUS activity in all the plants transformed with the truncated fragments was higher than transformants of the –1,569 fragment. The deletion to –1,166 increased the promoter

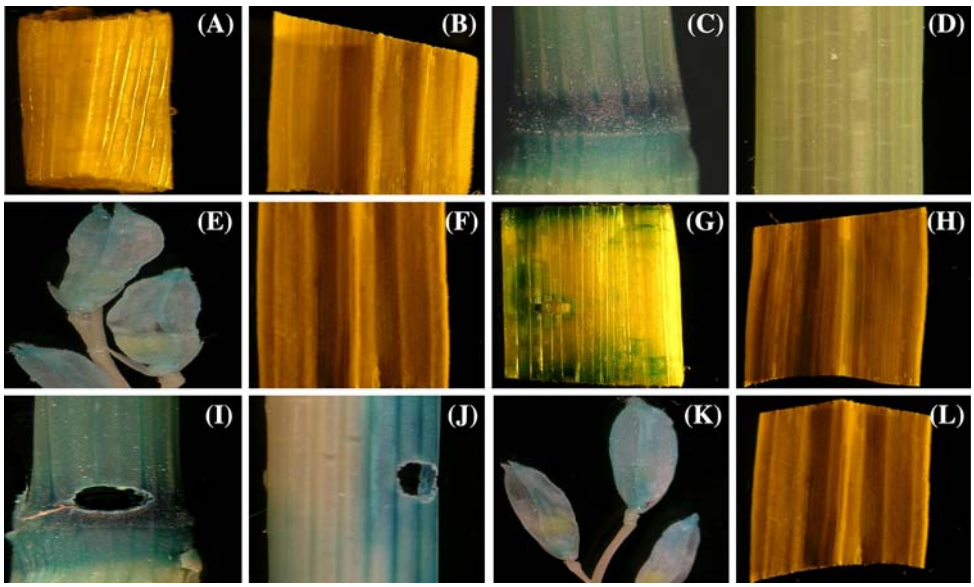

Fig. 3 GUS staining of transgenic rice plants driven by the –1,569 promoter fragment. At least 10 independent transgenic lines were assayed. (**A**–**F**) Non-infested tissues: sheath (**A**) and leaf (**B**) at the tillering stage; culm (**C**), sheath (**D**), panicle (**E**) and leaf (**F**) at booting stage. (**G**–**L**) *C. suppressalis* infested tissues: sheath (**G**) and leaf (**H**) at the tillering stage; culm (**I**), sheath (**J**), panicle (**K**) and leaf (**L**) at booting stage

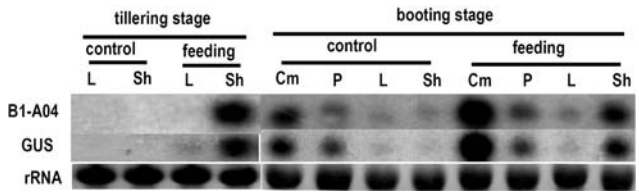

Fig. 4 Expression pattern of the endogenous B1-A04 sequence compared with the *GUS* gene driven by the -1,569 promoter fragment in transgenic plants at the tillering and booting stages, with and without insect feeding. Tissues were sampled from transgenic plants treated with *C. suppressalis* feeding for 6 h. L, leaf; Sh, sheath; Cm, culm; P, panicle.

activity by about 3-fold, statistically significant ($P < 0.01$) as assessed by the *t*-test. Further deletion to –582 resulted in about 10-fold increase of the *GUS* expression compared to that of the –1,569 fragment ($P < 0.01$), and 3-fold increase compared to that of the –1,166 fragment ($P < 0.01$). The GUS activity remained at a similar high level in transformants with the –371 and –112 fragments as in the –582 transformants. These results suggested that two or more negative *cis*-acting element(s) existed between –1,569 and –582 relative to the transcription initiation site in the B1-A04 promoter fragment that repressed the gene expression in non-infested sheaths and leaves.

To identify promoter regions responsible for the induction by *C. suppressalis* feeding, GUS activity of at least 5 independent transgenic plants per construct was examined 6 h after feeding. As shown in Fig. 6B, GUS activity in infested sheaths of plants transformed with the –1,569 fragment increased by 4- to 10-fold, and those transformed with the –1,166 fragment by 2- to 4-fold ($P < 0.01$ in both cases), compared with the non-infested controls. However, there was no significant change of the GUS activity between leaves of infested and non-infested plants (data not shown). A further deletion to –582 abolished the effect of negative regulation of the *GUS* expression by the promoter region resulting in equally high level of GUS activity between infested and non-infested plants (Fig. 6B), as was the case for deletions to –371 and –112. These results indicated that the 403 bp region from –1,569 to –1,166 and the 584 bp region from –1,166 to –582 contained *cis*-elements responsible for the induction of the B1-A04 promoter activity in sheaths by *C. suppressalis* feeding.

EMSA identified regions putatively containing the *cis*-acting elements

In order to validate the existence of *cis*-acting elements between –1,569 and –582, 20 DNA probes of 34–54 bp in length were synthesized (by Shanghai Sangon Biotechnology Co., Ltd., China) covering the entire region from –1,569 to –582 (Fig. 2). These probes were used to interact with nuclear proteins isolated from sheaths of rice plants treated with *C. suppressalis*-infestation and wounding with the untreated as the control, and subjected to EMSA to analyze their protein binding activity. Thirteen of the DNA probes showed no binding activity to nuclear proteins from any of the tissues (data not shown), while the remaining 7 probes displayed various patterns of binding activities to nuclear proteins of these tissues (Fig. 7). Two probes, probe6 and probe7 located in –1,319 to –1,219, showed intensive binding to proteins from the control, mechanically wounded and *C. suppressalis*-infested plants. Three probes, probe11, probe12 and probe13 located in –1,067 to –917, bound intensely to proteins from the control but not

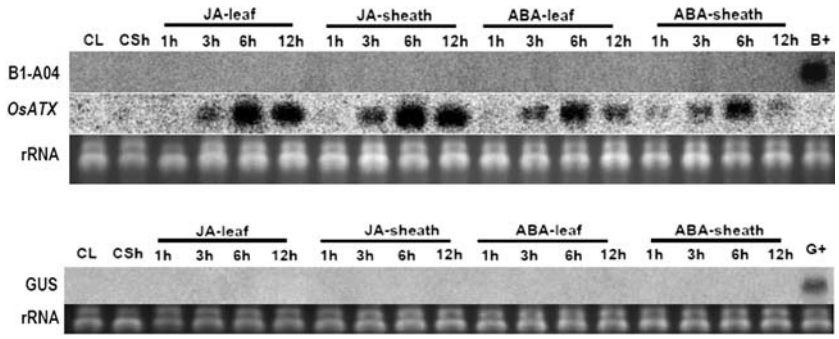

Fig. 5 Northern blot analysis of *GUS* and endogenous B1-A04 gene expression after JA and ABA treatments at the tillering stage in rice plants transformed with the –1,569::*GUS* construct, with water sprayed transgenic plants as the control. RNA samples were isolated from leaves and sheaths of the transgenic plants treated with JA or ABA at 1, 3, 6, 12 h after treatments. CL, leaves of the control plants; CSh, sheaths of the control plants; JA-leaf, leaves of the JA-treated transgenic plants; JA-sheath, sheaths of the JA-treated transgenic plants; ABA-leaf, leaves of the ABA-treated transgenic plants; ABA-sheath, sheaths of ABA-treated transgenic plants; G+, total RNA of sheaths of plants transformed with the –112::*GUS* at tillering stage as positive control; B+, total RNA of sheaths of Minghui 63 treated with *C. suppressalis* feeding for 6 h at tillering stage as positive control

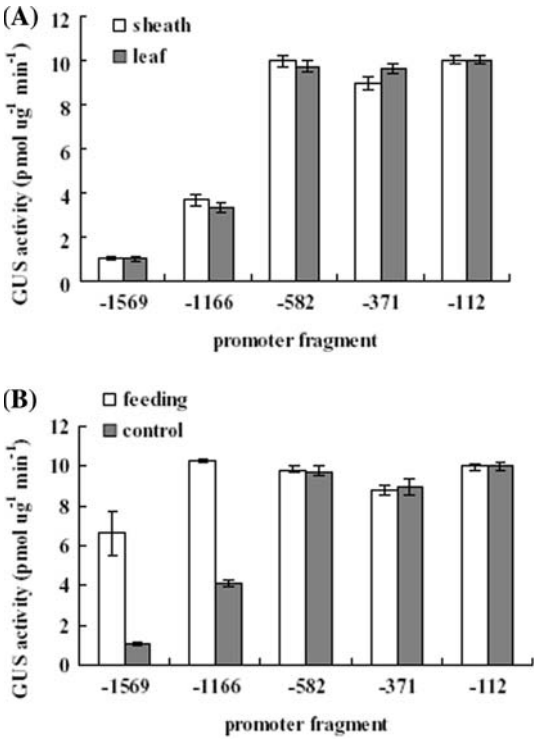

Fig. 6 GUS activity in sheaths and leaves of transgenic plants with promoter fragments of various lengths resulting from serial 5′-deletions. (**A**) Without insect feeding, the measurements for each construct are based on 15 independent transgenic plants. (**B**) After *C. suppressalis* feeding treatment, the measurements for each construct are based on 5 independent transgenic plants. The measurement was from two repeats for each plant. *Error bars* indicate the standard errors.

mechanically wounded or *C. suppressalis*-infested plants. Both probe3 and probe4, located in –1,469 to –1,369, bound intensively to proteins from mechanically wounded plants. However, probe3 also bound to proteins from the control, while, in contrast, probe4 bound highly intensely to proteins from the insect-attacked plants but not the control plants (Fig. 7).

Taken together, all the 7 probes, except probe4, bound to proteins from the control, 4 probes (3, 4, 6 and 7) bound to proteins from the mechanically wounded plants, and 3 probes (4, 6 and 7) had binding activities to proteins from the insect-fed plants.

To assess the specificity of the protein-DNA binding, the above 7 probes were subjected to competitive binding with 50× excess of unlabeled DNA fragments of the respective probes. The protein-binding intensity of all the 7 probes was greatly reduced presumably due to competition by unlabeled probes (Fig. 7), indicating that the binding activity detected for each of the probes was highly specific.

Discussion

Using a combination of subtractive cDNA libraries and DNA macroarray strategy, we identified 17 sequences whose expression was altered by *C. suppressalis* feeding. Northern blot analysis of four randomly selected clones revealed that three of them were induced by both wounding and feeding, although the induction level and time course varied among the clones and between the treatments. One sequence (B1-A04) responded rapidly and intensely to *C. suppressalis* attack, but showed only very low response to mechanical wounding, suggesting that distinct mechanisms exist in responses to challenges posed by mechanical wounding and *C. suppressalis* feeding. Similar findings were also reported in other plants. In Arabidopsis (Reymond et al. 2000), a microarray analysis revealed that 27 of the 150 defensive genes were responsive only to mechanical wounding, 1 gene (Hevein-like protein) was

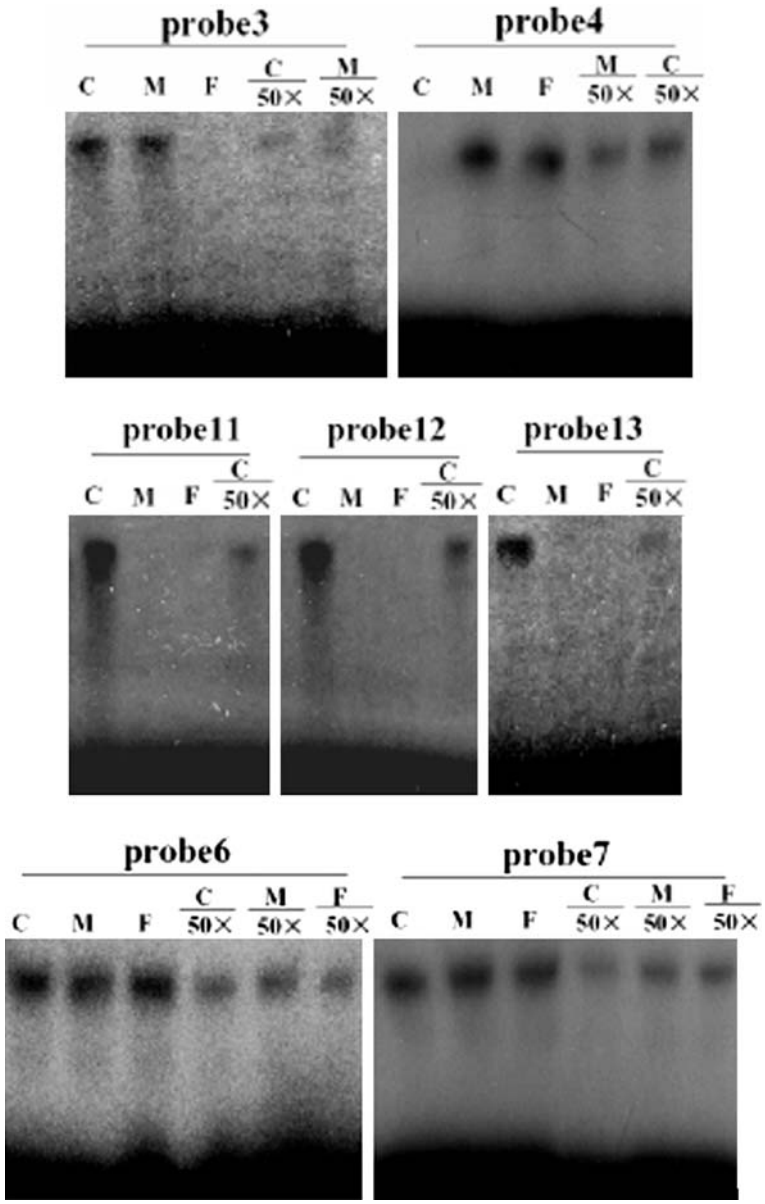

Fig. 7 EMSA of the **7** DNA fragments showing various degrees of binding activity with nuclear proteins. Nuclear proteins were isolated from rice sheath of Minghui 63 at 6 h after *C. suppressalis* feeding (F), mechanic wounding (M) and untreated control (C). 50×, binding assay using the labeled probe mixed with 50-fold unlabeled probe

only induced by *Pieris rapae*, and 16 genes by both wounding and *P. rapae* attacking. But most of the 16 genes were induced to a higher level by wounding than *P. rapae* attacking. The results of Hermsmeier et al. (2001) and Schittko et al. (2001) indicated two types of wound-responsive genes in *Nicotiana attenuate*. One type of the genes was induced by continuous mechanic wounding while repressed by mechanic wounding supplied with regurgitant of *Manduca sexta*, and the other type acted in a reversed fashion. All these results indicate that plants can distinguish between mechanic wounding and insect attacking and adjust their defensive system in a very delicate way.

Protease inhibitors are important defensive proteins or polypeptides that occur naturally in a wide range of plants and are considered to be an essential part in the plant direct defense against herbivores. They are often induced by both herbivory and wounding and can cause inefficient protein digestion in insects, leading to reduced growth, delayed development and lower fecundity (Koiwa et al. 1997; Tamayo et al. 2000; Zavala et al. 2004). The present study identified a gene encoding a putative subtilisin/chymotrypsin inhibitor, belonging to protease type I of the serine protease inhibitor family (Ryan 1990), as responsive to insect feeding. Serial deletion analyses indicated two regions containing *cis*-regulatory elements, which repress the gene transcription in non-infested sheaths and leaves, but not in insect-infested sheaths.

Based on the results of EMSA in conjunction with the promoter activity data from serial deletion (Fig. 6), the

following conjectures may be made. First, the sharp difference of the GUS activity between the insect feeding-treated and control plants harboring the –1,166 fragment suggests that the region marked by probes 11, 12 and 13 that bind to proteins from the control but not insect-fed plants contains one or more negative regulatory element(s) reducing the expression of the gene in the control plants. Second, the lower GUS activity of the –1,569 promoter fragment than that of the –1,166 fragment indicates that the region covered by probes 6 and 7 that bind to proteins from both the control and inset-fed plants plays a general role of negative regulation. Third, the bindings of probe3 to proteins from the control and probe4 to proteins from insect-fed plants suggest the possibility of opposite roles of the two regions, such that probe3 negatively regulates the expression in the control plants while probe4 positively regulates the gene expression in the insect-fed plants. Finally, the binding of probe3 to proteins from mechanically wounded plants but not from insect-fed plants may account for the differential induction of the gene expression in the insect-feeding treatment. However, the details of the elements and modes of actions involved in the regulation remain to be characterized in future studies.

There have been many reports that JA and ABA can activate the expression of plant protease inhibitors. It is known that expression of many protease inhibitor genes induced by wounding and insect attacks is controlled by the JA pathway (Farmer et al. 1992; Farmer and Ryan 1992; Casaretto et al. 2004). ABA is another signal known to be involved in the inductive expression of protease inhibitors. ABA-deficient mutants of potato and tomato show a drastically reduced induction of protease inhibitor II genes in response to wounding, and high levels of protease inhibitor II gene expression are observed in mutants and wild-type plants upon exogenous application of ABA (Peña Cortés et al. 1989; Herde et al. 1999). However, unlike other protease inhibitor genes, the rice subtilisin/chymotrypsin inhibitor gene as identified in this study was not responsive to the two phytohormones, although highly responsive to *C. suppressalis* feeding.

Another feature of the B1-A04 sequence is that it did not seem to be expressed in a detectable level in non-infested sheaths and leaves at the tillering stage, but was expressed in young panicles and culms at the booting stage. When challenged by *C. suppressalis* feeding, the expression levels drastically increased in sheaths and culms but not in leaves, suggesting that the response was not systemic. These phenomena are consistent with the feeding habit of *C. suppressalis*. In nature, *C. suppressalis* only infests and damages sheaths at the tillering stage, but never the leaf tissues. Sheath is a vegetative organ, of which the host can tolerate the damage by compensatory growth including increasing photosynthetic capacities, enhancing nutrient uptake and promoting tillering (Stowe et al. 2000). Thus it is not critical for the protease inhibitor to be constitutively produced in sheaths, provided that it can be rapidly induced upon infestation by the insect. This seems to be an economic way for the plant to produce the protease inhibitor only in infested sheaths. At the booting stage, *C. suppressalis* can damage young culms and panicles. As the damage is irremediable, the plant needs to produce the protease inhibitor even before infestation and drastically increase its production in culms upon infestation. Such inducible expression of a protease inhibitor gene may lessen the ability of *C. suppressalis* to adapt to host defenses. Thus, the expression profile of this gene probably reflects the outcome of evolution during long-term interactions between the insect and rice under natural conditions.

Presently, there has been very little work on the characterization of herbivore-responsive promoters in plants. The identification of the promoter fragment and the regulatory regions as accomplished in this work may have a great potential to be applied to insect-resistance breeding in rice as well as other crops.

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