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Cloning and characterization of rice *RH3* gene induced by brown planthopper

WANG Xiaolan, WENG Qingmei, YOU Aiqing, ZHU Lili & HE Guangcun

Key Laboratory of the Ministry of Education for Plant Developmental Biology College of Life Sciences, Wuhan University, Wuhan 430072, China

Correspondence should be addressed to He Guangcun (e-mail: gche@whu.edu.cn)

Abstract **Experiments have showed that the histone *H3* gene is correlated with development, cell speciality and stress response. The *RH3* full-length cDNA was isolated from the cDNA library of rice infested by brown planthopper (BPH) with EST (Accession no. BU572343) screened from rice SSH library as probe. This gene encodes histone H3 protein including 136 amino acids, with one amino acid different from a kind of disease resistance-related protein in rice (AF467728). At the position 126, the aspartic acid is replaced by lysine. The time course results showed that the expression of the *RH3* began to increase at 8 h after BPH-feeding, and got to its peak at 96 h. Regulations of the gene expression in treatments with stress/defense signal molecules were analyzed by Northern blot. Water deficit and *Pyricularia grisea* increased the expression of *RH3* while ABA down-regulated the gene. The enhanced accumulation of *RH3* transcripts in the vascular bundle and short cell of stem after BPH feeding was revealed by RNA *in situ* hybridization. It is the first time to report that *RH3* is correlated with the response of rice to BPH.**

Keywords: brown planthopper, histone H3, RNA *in situ* hybridization, defense response.

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Histones are basic low molecular weight proteins found in all eukaryotic genomes. The histones include five classes of basic proteins (H1, H2A, H2B, H3 and H4) that interact with each other and nuclear DNA to form the nucleosome. The H3 and H4 histone proteins are highly conserved and form the central tetrameric block of the core-nucleosome. Histone H3 has several post-transcriptional modifications such as methylation, acetylation, phosphonation, and ADP-ribosylation and it plays important roles in the course of gene transcriptional regulation[1]. For instance, the methylation of Lysine 9 in histone H3 made it as the specific binding site for heterochromatin protein 1 (HP1)[2].

The histone *H3* genes have been isolated from a number of higher animals and plants. And it has been verified that the expression of histone *H3* gene is correlated with development[3,4], cell and tissue speciality[5—7] and stress responses[8]. Plant histone genes have been studied in depth on the structure[9] and the functional studies are mainly focused on the acetylation and deacetylation of histones recently[10].

Wu et al.[11] cloned two histone *H3* genes from a rice genomic library. The two genes exhibit very little homology in the flanking sequences except for several conserved sequence motifs in the 5' flanking regions. Despite the high conservation in the coding region (94%) and the similar amino acid, these clones differ from previously published rice H3, *pRH3-2* by three amino acids[12]. Raghavan[5] studied by *in situ* hybridization the cell- and tissue-specific expression of a rice histone *H3* gene during anther and pollen development in henbane (*Hyoscyamus niger*). They found that the histone mRNA differentially accumulated during anther and pollen development. The hybridization signal was present more or less in the same density in all the cells of the anther primordium. During later development of the anther, there was a sequential accumulation of histone mRNA beginning in the epidermis on the outside and ending in the tapetum inside. It is necessary to isolate these important genes from plants to study the structure, function and regulatory mechanisms.

Brown planthopper (BPH), *Nilaparvata lugens* Stål., is a typical specialist herbivore of rice that sucks assimilates from the phloem of plants and causes severe lost of rice grain yield. In genomic study, rice is a model organism, because of its small genome size, availability of high-density genetic and physical map, and high degrees of synteny with other cereal plants. Rice and brown planthopper have become an ideal model system for studying the interaction between plants and sucking insects. Understanding the mechanism of rice-BPH interaction will help to develop resistant rice variety and environment friendly insecticide.

In order to study the defense mechanism of rice to brown planthopper, we have constructed an SSH library of rice challenged by brown planthopper insects, and a specific expressed rice histone *H3* EST (GenBank Accession No. BU572343) was found to be up-regulated. In this study, the isolation and characterization of *RH3* gene in rice were reported; RNA gel blotting and RNA *in situ* hybridization were employed to tell when and where the gene was induced. Expression regulatuons of this gene in response to hormones and abiotic stress treatments were also reported.

1 Materials and methods

(ⅰ) Materials. Rice B5 (*Oryza sativa* L.) is highly resistant to BPH, which derived its resistance genes from wild rice *Oryza officinalis* Wall ex. Watt. All the materials used in tissue slicing, including stems and leaves, were selected from B5. BPH insects used in the experiments were the second-third instar nymphs reared on the suscep-

tible rice variety Taichung Native 1 in the Genetics Institute of Wuhan University.

(ii) Probe preparation. The *RH3* EST from SSH library was signed as P1 and used to screen cDNA library. P2, the PCR production of *RH3* cDNA, was used as probe in Southern and Northern blot analysis. *RH3* plasmid was linearized with *Sca* I (MBI) and transcripted *in vitro* into mRNA using T3 and T7 polymerase (Promega, USA) with DIG labeling (recorded as RH3 T3 and RH3 T7) respectively. *RH3 T3* and *RH3 T7* were used as probes in RNA *in situ* hybridization of the rice tissue sections.

(iii) Methods

(1) Plant growth condition and treatments. Twenty seeds of B5 were sown in plastic pots 10-cm in diameter and grew under normal management. Three-leaf seedlings were subjected to various treatments. For BPH feeding experiments, 2nd and 3rd instar nymphs of BPH were collected on susceptible rice variety Taichung Native 1 and put on rice seedlings at a density of 10 insects per plant. The pots were placed inside clear nylon net to prevent insect from escaping. Mechanical damages were caused by puncturing the stem three times with a needle. For salt treatment, rice seedlings were grown hydroponically at 26℃ under a photoperiod of 12 h (light): 12 h (dark), and the seedlings were watered with 300 and 400 mmol/L NaCl for 24 h. Rice seedlings were sprayed with solutions that contained MeJA (150 μmol/L), ethephon (50 μmol/L, ETH), SA (100 μmol/L), ABA (100 μmol/L), or GA3 (10 μmol/L), respectively, and grown for 24 h. The seedlings were infiltrated with 10^6 colony-forming units/mL *Pyricularia grisea*, kept in dark for 36 h and harvested at 48 h, uninfected shoots were harvested simultaneously. B5 rice plants were subjected to water-deficit by not watering for 5 days, while the control was watered everyday. For all the treatments and corresponding controls, the plant parts above ground were harvested and frozen immediately in liquid nitrogen for RNA extraction.

(2) Construction and screening of cDNA library. Poly $(A)^+$ RNA was isolated from rice plants that had been exposed to BPH nymphs for 48 h using TRIZOL and MESSAGE-MARKER (Gibco BRL, USA). The cDNA library was prepared using Uni-ZAP XR library kit (Stratagene, USA). Library construction, screening, and *in vivo* excision of pBluescript SK followed the instruction manually supplied with the kit. The *RH3* EST was used to screen for corresponding full-length cDNAs. The probes were labelled with [α-^{32}P] dCTP using the Prime-a-Gene labeling system (Promega, USA). About 50000 plaques of the cDNA library were transferred onto duplicate Hybond N^+ membrane, (Amersham-Pharmacia, USA). The blots were hybridized with probe having followed the method by Chen et al.[13].

(3) Northern blot analysis. RNAs were isolated from samples of each treatment and control with a TRIZOL reagent (Gibco BRL, USA), and dissolved in DEPC-treated water. RNA was quantified with PERKIN-ELMER LAMBDA BIO 20/1.0 nm UV/VIS spectrophotometer (Perkin-Elmer, USA). For Northern blot analysis, total RNAs (20 μg) were separated on a denaturing 1.5% formaldehyde agarose gel then transferred to Hybond N^+ membrane (Amersham-Pharmacia, USA). The probe was *RH3* cDNA isolated from the cDNA library. Hybridization was performed for 10 h at 65℃. Membranes were washed with 1×SSC and 0.2% SDS at 65℃ for 15 min, and then with 0.5×SSC and 0.1% SDS for 15 min.

(4) Southern blot analysis. Genomic DNA was extracted from rice plants of B5 following CTAB (cetyltrimethyl ammonium bromide) procedure[14]. The DNA was digested with restriction endonucleases *Eco*R I, *Eco*R V and *Dra* I, then was electrophoresized on 0.8% agarose gels and transferred onto Hybond N^+ membrane. Probe labeling, hybridization and washing condition were the same as reported[15].

(5) mRNA *in situ* hybridization. Seeds of B5 were sown in the plastic pots in 40 cm diameters. At three-leaf stage, the seedlings were infested with BPH at 10 insects per seedling. In the other pots, the same treatment was adopted but without BPH infestation as the control. Stems and leaves of both the treatment plants and control plants were collected 48 h later and fixed in FAA (10% formaldehyde, 50% ethanol, 3% acetic acid), then dehydrated in tertiary butyl alcohol (TBA) and embedded in paraffin (Paraplast Plus, Sigma). The embedded tissues were sliced into serial 10 μm sections. RNA *in situ* hybridization followed essentially the method described by Chen et al.[16].

2 Results

(i) Construction of cDNA library and isolation of *RH3* gene. A cDNA library including 1.1×10^6 clones was constructed by using B5 exposed to BPH 48 h as materials. A recombinant test was completed by color selection. According to the different response of recombinant and background to Xgal and IPTG, the recombinant ratio of this library was 96% and the titer was 5.0×10^{11} pfu/μL after amplification. By PCR analysis of the inserted fragments of 10 randomly selected white plaques, the results showed that every plaque has inserted cDNA and the sizes ranged from 0.5 to 3.8 kb. Screening the cDNA library using the *RH3* EST as probe, a total of 5 clones containing 0.9 kb insertion were got. The cDNAs were sequenced and the nucleotide and deduced amino acid sequence of *RH3* are showed in Fig. 1. The full length cDNA includes a 5′ non-coding sequence of 57 nucleotides, an open reading frame of 408 nucleotides which encodes a putative protein of 136 amino acid residues, and a 3′ non-

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   2 GGCACGAGGCCCGATTCGAAGCGTGAAGAGAGGAGCGGCGCTTGCGAGAGGAGAGAGATGGCCCGTACCAAGCAGACCGCTCGTAAGTCC  91
   1                                                   M  A  R  T  K  Q  T  A  R  K  S   30

  92 ACAGGAGGAAAGGCTCCCAGGAAGCAGCTTGCAACCAAGGCTGCTCGTAAGTCTGCTCCCACCACTGGAGGAGTTAAGAAGCCCCACCGT 181
  31 T  G  G  K  A  P  R  K  Q  L  A  T  K  A  A  R  K  S  A  P  T  T  G  G  V  K  K  P  H  R   60

 182 TACCGCCCTGGAACTGTTGCCCTCCGTGAGATTCGCAAGTACCAGAAGAGTACTGAGCTTTTGATCAGGAAGCTGCCCTTCCAGAGGCTT 271
  61 Y  R  P  G  T  V  A  L  R  E  I  R  K  Y  Q  K  S  T  E  L  L  I  R  K  L  P  F  Q  R  L   90

 272 GTTAGGGAAATTGCACAGGACTTCAAGACCGATCTGCGTTTCCAGAGCCATGCTGTCCTTGCCCTCCAGGAGGCTGCGGAGGCATACCTT 361
  91 V  R  E  I  A  Q  D  F  K  T  D  L  R  F  Q  S  H  A  V  L  A  L  Q  E  A  A  E  A  Y  L  120

 362 GTTGGTCTCTTCGAGGACACCAACCTGTGCGCTATTCATGCCAAGCGTGTGACCATCATGCCTAAGGACATTCAGCTGGCTAGGAGGATT 451
 121 V  G  L  F  E  D  T  N  L  C  A  I  H  A  K  R  V  T  I  M  P  K  D  I  Q  L  A  R  R  I  150

 452 CGTGGTGAGAGGGCTTAAATTCCCCTCGGCGACTCCTTTGACAAATGAACATGCGTCGTAGTGTTAGTAGTGGGTTTAATCTTTTGCTTA 541
 151 R  G  E  R  A  *                                                                          180

 542 TAAgaacaatctgagtagggtgtattttgtggaacaatatgtttctctctgtgacatgatggtgctgtattcgtcttattggtggatctg 631

 632 tcaaaaatactcagaatattgtcagtgtgtttggtgactcgtattttgttagtcggattcgtttgcgtcttattctagcggtgctgcctc 721

 722 tttggcactgtgcatggctagatacaattacattgtaattcacctgtctctaatccgtgttaatgtaatgttccgttgctgttcgtgtga 811

 812 AAAAAAAAAAAAAAAACTCGAGGGGGGGC
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Fig. 1. Nucleotide and deduced amino acid sequence of *RH3*. The stop codon is indicated with an asterisk.

coding sequence of 341 nucleotides followed by a 3′ polyA tail. The stop codon TAA is located at position 479—481. The polyadenylation signal AATAAA is not present in the 3′ non-coding region. Whereas the putative AATAAA like near-upstream element (NUE), AATGAA is present in the position from 495 to 501. In pea rbcS mRNA and maize 27 kD zein mRNA this AATGAA motif serves as NUE and is involved in the process of mRNA 3′ end formation[17]. Blast searching in Genbank (http://blast.cbi. ku.edu.cn) found the scaffold 2410 with length 23688 bp homologous to *RH3* gene. The genomic sequence of *RH3* gene is composed of four exons and three introns. In the deduced RH3 amino acid sequences from the BPH response only one amino acid is different from a kind of disease resistant related protein (Accession No. AF467728). At the position 126 of *RH3*, the aspartic acid is replaced by lysine. RH3 protein shows 100%, 99%, 86%, 85%, 84%, and 82% identity with histone H3 proteins of *Lycopersicon esculentum* (Accession No.X83422), *Oryza sativa* (Accession No. AF467728, *Gossypium hirsutum* (Accession No. AF024716), *Nicotiana tabacum* (Accession No. AB015760), *Lolium temulentum* (Accession No. X79714), *Medicago sativa* (Accession No. U09460, U09465), respectively.

(ii) *RH3* mRNA expression after different treatments. Rice shoots were harvested at 0, 2, 6, 8, 16, 24, 36, 48, 72 and 96 h after addition of BPH insects and RNAs were isolated and transferred to membrane. Distinct expression patterns were demonstrated when hybridized with the *P2* probe. The expression of *RH3* changed after the rice plants were fed by BPH. It decreased in 2 h after BPH feeding then elevated after 8 h. The expression of *RH3* was stronger than the control after being exposed to BPH insects for 48 h, and it increased further till 96 h (Fig. 2). To determine whether this BPH-regulated gene responded to known modulators in biotic, abiotic stress and defense signal transduction pathways, transcripts accumulation in plants treated with wounding, *P. grisea* infection, and defense signaling molecules such as MeJA, ethephon, SA and ABA was investigated (Fig. 3). Compared to the corresponding control, RH3 expression level in rice increased obviously after water-deficit treatment. *RH3* expression was down-regulated in ABA treatment, but up-regulated when exposed to SA. After wounding and *P. grisea* infestion, the expression level of *RH3* elevated. The other treatments such as NaCl stress, did not show an obvious effect on the gene expression. These data indicate that RH3 was down-regulated by ABA signal pathway.

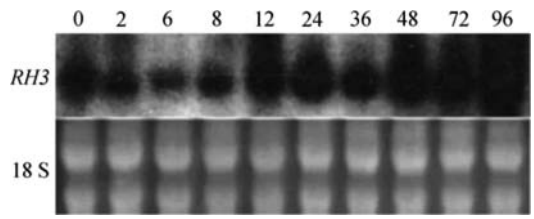

Fig. 2. Transcript accumulation of *RH3* in different time courses. The number from 0 to 96 shows the hours fed by brown planthopper.

(iii) Southern blot analysis. To investigate the copy number of *RH3* gene in rice, genomic DNA was

digested with *Eco*R Ⅰ, *Eco*R Ⅴ or *Dra* Ⅰ respectively, and hybridized with P2 probe. As showed in Fig. 4, a single band was detected with all three restriction enzymes. The results suggest that *RH3* exists in a single copy in the rice genome. The Southern blot analysis is consistent with the research result in rice genome database, in which only one scaffold showed homologue to *RH3* gene.

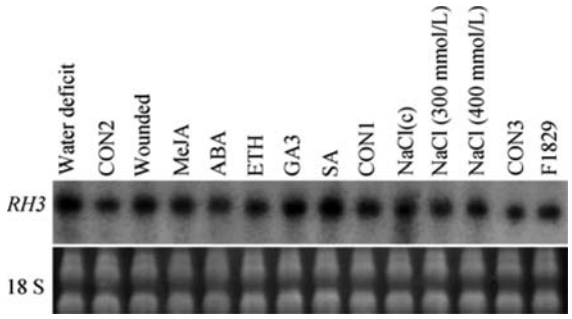

Fig. 3. Expression of *RH3* in response to wound, defense and stress signals. Total RNA was isolated from three-leaf stage B5 plants after different treatments MeJA (150 μmol • L^{-1}) to leaves (MeJA), exposure to ethephon (50 μmol • L^{-1}, ETH), SA (100 μmol • L^{-1}), ABA (100 μmol • L^{-1}), GA3 (10 μmol • L^{-1}), their control lanes correspond to untreated plants (CON1). Three leaf stage B5 plants were subjected to five days of water-deficit stress, three-leaf stage B5 plants were wounded after 24 h, the control of water-deficit and wounded treatments correspond to untreated plants (CON2). Three-leaf stage B5 plants were infiltrated with 10^6 colony-forming *Pyricularia grisea* (units/mL), darkened for 36 h and harvested at 48 h, uninfected shoots were harvested simultaneously (CON3). Three-leaf stage B5 plants were watered with 300 and 400 mmol • L^{-1} NaCl, harvested at 24 h, untreated shoots were harvested simultaneously. The rRNA was used as control. The "BPHi" from the gene indicated was omitted.

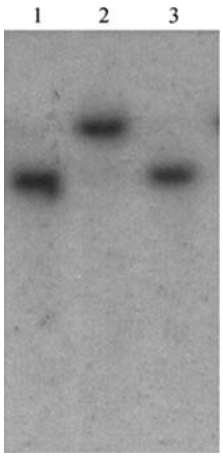

Fig. 4. The Southern blot result in rice genome. Lanes 1, 2, 3 were digested by *Eco*R Ⅰ, *Eco*R Ⅴ, *Dra* Ⅰ respectively.

(ⅳ) *RH3* mRNA *in situ* hybridization. Cross sections of B5 stems of rice plants infested by BPH were *in situ* hybridized with the probes of *RH3 T3* and *RH3 T7*, respectively. Sections hybridized with *RH3 T3* showed no color reaction except for a faint background (Fig. 5(a)), but those hybridized with *RH3 T7* were stained blue-purple in color (Fig. 5(b)—(f)). So the antisense RNA *RH3 T7* was determined as the probe to carry out *in situ* hybridization of the tissue sections. *In situ* hybridization results indicated that, whether subjected to BPH infestation or not, *RH3* mRNA expressed in the rice stems (Fig. 5(b) and (c)). In the vascular bundle of the BPH-infested stem (Fig. 5(c)), the accumulation level of *RH3* mRNA was higher than that without BPH infestation (Fig. 5(b)), showing that the gene was up-regulated in vascular bundle system. Compared to the hybridization results of stem sections without BPH infestation, the level expression of *RH3* gene in parenchyma (pa) exhibited down-regulation upon BPH infestation (Fig. 5(b) and (c)). BPH feeding leads to enhancement of RH3 expression in the short cells in rice stem. Signals appearing as "X" were observed in the vertical sections of the stem fed by BPH for 48 h, but not in the control (Fig. 5(e)). The results indicated that the accumulation level of *RH3* mRNA was higher in the short cells after being challenged by BPH (Fig. 5(d), sc). *In situ* hybridization results for leaf cross sections indicated that, whether subjected to BPH infestation or not, *RH3* mRNA expressed strongly in mesophyll (me) (Fig. 5(f)). The results indicated that the *RH3* mRNA expressed in vascular bundle system and short cells suggested that *RH3* might play roles in the defense response in rice to BPH feeding.

3 Discussion

Four types of histone proteins are known (H2A, H2B, H3, and H4) to assemble into an octamer core of the nucleosome. Every core histone has two domains: histone-fold domain, which has been known to associate with interaction between histones and DNA coiling; the other is lysine enriched N-terminal domains, like a tail, which is the site of acetylation. Acetylation and deacetylation of histone tails are critical steps for transcriptional activity of many genes. Lam et al.[11] verified that tetramer of a 21-base pair synthetic element conferred transcriptional enhancement in seeds in response to water stress. In this RH3 anticipated BPH response, it is unknown whether the BPH resistance in B5 was enhanced by the accumulation of RH3 or it used the similar mechanism as that in water stress. However, from the fact that *RH3* expression level increased obviously we can deduce that the RH3 was correlated with the defense response in rice against BPH.

The *RH3* expression elevated between 4—6 h after submergence, and then decreased[18], while the *RH3* expression to BPH response reduced between 2 and 6 h then increased, and got to its maximum at 96 h. This result showed that *RH3* played different roles in different stresses. Additionally, the *RH3* isolated from the BPH-induced cDNA library share 99% homology with gene for a disease related protein in rice. The expression level of *RH3* was up-regulated in water deficit and pathogen

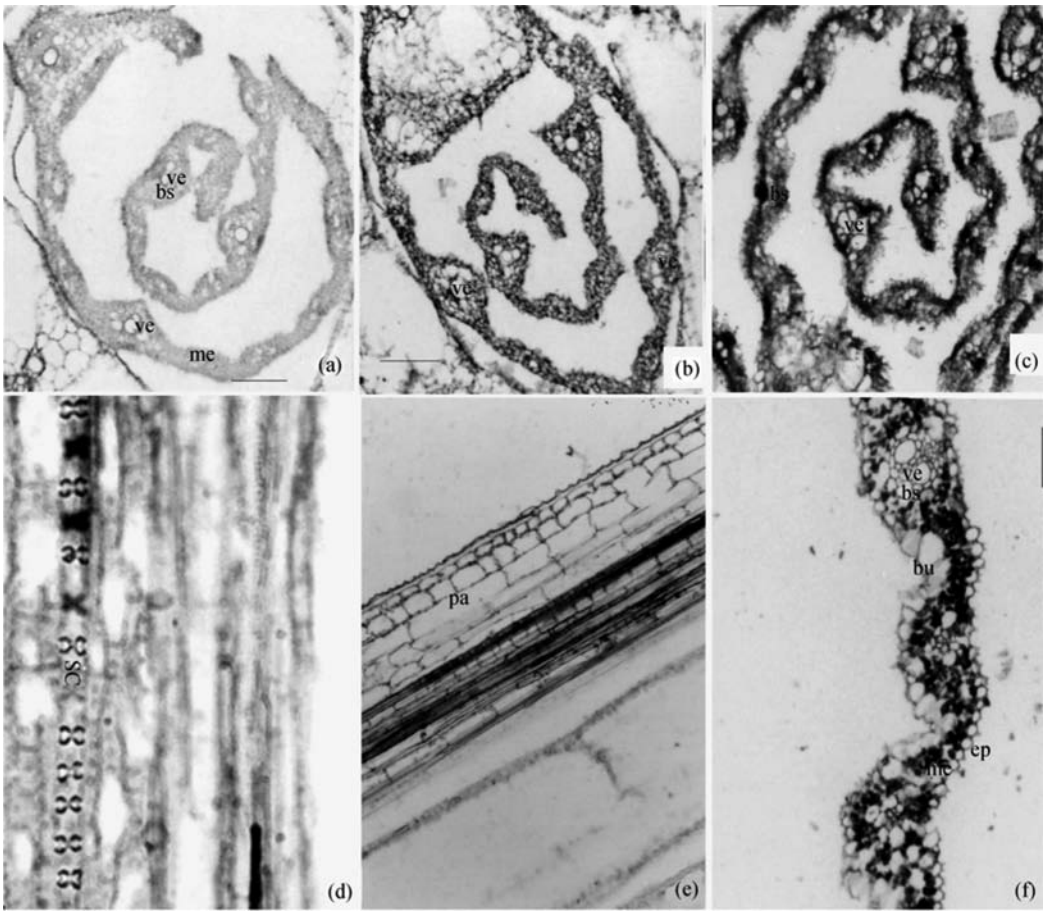

Fig. 5. *In situ* localization of *RH3* mRNA in rice stems. (a) *In situ* hybridization with sense RNA *RH3 T3* as probe; (b)—(f) *In situ* hybridization with anti-sense RNA *RH3 T7* as probe. (a) Cross section of leaf without BPH infestation (control), ×10; (b) cross section of stem with BPH infestion, ×10; (c) cross section of stem without BPH infestation, ×10; (d) vertical section of stem with BPH infestation, ×10; (e) vertical section of stem without BPH infestation, (f) cross section of leaf with BPH infestation, ×10. bs, bundle sheath; bu, bulliform cell; ep, epidermis; me, mesophyll; pa, parenchyma; sc, short cell; ve, vessel element.

treatments, which indicated that the signal pathways among water deficit, pathogen and BPH crosstalked. Through the RNA *in situ* hybridization, we concluded that *RH3* gene could express in rice stem. The enhanced expression of *RH3* gene mainly occurred in the vascular-bundle of the stem. Experiments on the feeding habit of BPH showed that BPH sucks juice from the boundle sheath by piercing its stylet mouthparts into phloem of rice stem. The fact indicated that the high expression of *RH3* mRNA vascular bundle was induced by BPH feeding.

Functional classification showed that the fold domain at histone N-terminal is necessary for the suppressor binding to DNA and protein-protein interaction. The rich region of essential amino acid, and arginine and glycine rich regions increase the activity of suppression response[19]. Methylation of lysine residues at the specific sites in the nucleosome core histone influences the function of chromatin[20]. In the deduced RH3 amino acid sequence, the lysine at the position of 126 amino acid was replaced by aspartic acid. Whether this kind of replacement would produce novel regulation in response of rice to brown planthopper deserves further study. Southern blot analysis and BLAST research both confirmed that only one copy of *RH3* exists in rice genome, but possible being various types. Wu et al.[11] and Peng et al.[6] cloned two histone *H3* genes from a rice genomic library respectively. The sequences of the genes are different from the BPH-responsing *RH3* gene. It is interesting to know if there is any evolutional or functional significance of the polymorphism of plant histone *H3* genes. Isolation of more histone genes from plants will help to elucidate these problems.

The brown planthopper is one of the most serious sucking insects of rice; it causes direct tissue damage to rice crops but also is the vector of rice viral diseases [21]. Knowledge of the mechanisms of plant-insect interactions and the molecular and physiological changes in plant after

sucking insect infestation is limited. Characterization of BPH resistance-related gene from rice provides new cue for further research on the phloem-sucking insects. The gene isolated from B5 cDNA library verified that histone H3 participated in rice response to BPH. Furthermore, this *RH3* is homologous to a disease resistant related protein in rice, indicating that the signal pathways between pathogen and insect overlapped. Now, the studies to histone are mainly focused on post-transnational modification such as acetylation of the protein. In our experiments, the *RH3* transcripts accumulated the vascular bundle system of rice stem after being infested by BPH, which demonstrated that histone *H3* gene possibly participated in defense response by adjusting the level of expression.

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