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In situ localization of proteinase inhibitor mRNA in rice plant challenged by brown planthopper

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Abstract **Proteinase inhibitor (PI) mRNA was localized by *in situ* hybridization in tissue sections of root, stem and leaf of the resistant rice (B5) plant fed by brown planthopper nymphs. In the rice material without BPH feeding, PI gene was expressed in the root, stem and leaf, while the abundance of PI mRNA was low. In the rice material fed by BPH, PI gene was expressed substantially in the parenchyma of rice stem and leaf, but weakly in the root. The results indicated that the PI gene was up-regulated in the rice plant challenged by brown planthopper. For the first time, we reported the expression changes of proteinase inhibitor gene in plant which was infested by a piercing/sucking insect.**

Keywords: Bowman-Birk trypsin inhibitor, *in situ* RNA hybridization, brown planthopper, plant defense response.

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Brown planthopper (*Nilaparvata lugens* Stål., BPH) is the most serious pest of rice that severely decreases the yield and quality of rice grain. In rice production, the effectiveness of insecticide is far under the farmers' expectation for BPH control. Furthermore, the application of insecticide results in many by-effects such as environmental pollution, increase of production expenditure and the resurgence of brown planthopper[1]. Understanding the mechanism of rice-BPH interaction will help us to develop the resistant rice variety and environment-friendly insecticides.

Proteinase inhibitor (PI) is one type of low-molecular-weight protein (8—20 kD), which can specially combine with proteinase to inhibit its activity[2]. PI is one kind of pathogen-related proteins, and is part of plant defense systems against herbivore, microbe and nematode[3]. PI proteins were found commonly in mocotyledon and dicotyledon, and the serine proteinase inhibitor has been studied in detail. Bowman-Birk proteinase inhibitor belongs to serine PI family. It was reported that soybean Bowman-Birk PI, with functions in cancer-resistance and inflammation-diminishment, played a key role in plant defense responses[4]. In rice, two Bowman-Birk PI genes, belonging to the same gene family, were cloned from variety Teqing and Nipponbare and sequenced by Chen et al.[5] and Sasaki et al.[6], respectively. By Northern hybridization, Rakwal et al.[7] proved that the expression of Bowman-Birk PI gene (*OsBBPI*) in the leaf of Nipponbare could be up-regulated by Catharidin (CN) and Endothall (EN), two potential inhibitors of jasmonate acid (JA), ethylene and protein phosphatase pp2A. Meanwhile, the induced expression would be inhibited by cycloheximide (CHX). When the plant was fed by chewing insect, the expression of PI gene was enhanced[8]. Zhang et al. studied the expression of Bowman-Birk PI gene E61932 in the BPH-resistant rice B5. They found that the gene expression was up-regulated in rice plant infested with BPH nymphs. 72 h after being fed by BPH nymphs, the gene expression came up to the maximum (data not shown).

RNA *in situ* hybridization is a direct and effective technique to study gene expression temporally and spatially on the tissue or cell level[9]. Compared with Northern hybridization, the technique can show the expression pattern of the target-gene more directly. In this research, RNA *in situ* hybridization was employed to study the expression pattern of the PI gene E61932 in rice root, stem and leaf before or after being fed by BPH nymphs. It was found that the transcript specially accumulated in the stem and leaf of the BPH-fed rice plants.

1 Materials and methods

(i) Materials. B5 is a highly resistant rice variety to BPH[10], which derived its resistance genes from wild rice *Oryza officinalis* Wall ex. Watt. All the materials used in tissue slicing, including roots, stems and leaves, were selected from B5. BPH insects used in the experiments were the second-third instar nymphs reared on the susceptible rice variety Taichung Native 1 in the Genetics Institute of Wuhan University.

PI gene (E61932) was kindly provided by Dr. Sasaki in Japanese Rice Genome Research Program (RGP). The cDNA clone E61932 with a total length of 710 bp, was isolated from rice leaves of cultivar Nipponbare, and was inserted into vector pBluescript SK^+. The gene was predicted to encode a protein containing 188 amino acids. By BLAST analysis in NCBI (http://www.ncbi.nlm.nih.gov)[6], it was revealed that the cDNA sequence was highly identical to a gene named rbbi2-3 (97% in ratio) isolated from rice variety Teqing by Peiking University. Both of them belong to Bowman-Birk trypsin inhibitor.

(ii) Methods

(1) Rice materials and preparation of tissue sections. The seeds of rice B5 were sown in the plastic pots in 40 cm diameters. At three-leaf stage, the seedlings were infested with BPH at 5 insects per seedling, then the pots were caged with screen to prevent the BPH escape. In the other pots, the same treatment was adopted but without

BPH infestation as the control. 72 h later, the roots, stems and leaves of both the treatment plants and control plants were collected and fixed in FAA (10% formaldehyde, 50% ethanol, 3% acetic acid), dehydrated in tertiary butyl alcohol (TBA) and embedded in paraffin (Paraplast Plus, Sigma, USA). The embedded tissues were sliced into serial 8 μm (roots) or 13 μm (stems and leaves) sections.

(2) mRNA *in situ* hybridization. RNA *in situ* hybridization essentially followed the method by Chen et al.[11].

E61932 plasmid was linearized with *Sca* I (MBI) and transcripted *in vitro* into mRNA using T3 and T7 polymerases (Promega, USA) with DIG labeling (recorded as E61932 T3 and E61932 T7) respectively. E61932 T3 and E61932 T7 were used as probes in RNA *in situ* hybridization of the tissue sections. Immunological reaction was conducted with anti-DIG-antibody (Roche, Switzerland) conjugate followed with several rinses. Color reaction was carried out with nitro-blue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Sections were sealed by Neutral Balsam and examined under a microscope with blue-purple color as the positive reaction.

2 Results

(ⅰ) Determination of antisence RNA probe. Vertical sections of stem, from rice B5 plants infested by BPH, were *in situ* hybridized with the probes of E61932 T3 and E61932 T7, respectively. Sections hybridized with E61932 T3 showed no color reaction except a faint background (Fig. 1(a)), but those hybridized with E61932 T7 were stained blue-purple in color (Fig. 1(g)). So the antisense RNA E61932 T7 was used as the probe to carry out *in situ* hybridization of the tissue sections in the following experiments.

(ⅱ) Expression of PI gene in B5 fed by BPH. The tissue sections of root, stem and leaf, collected from B5 materials 72 h after BPH infestation or without infestation, were *in situ* hybridized with probe E61932 T7. By color reaction, expression manners of E61932 homologous gene in B5 were exhibited in different tissues under conditions of BPH infestation or not (Fig. 1(b)—(i)).

In situ hybridization results of horizontal sections of leaf indicated that, whether conducting BPH infestation or not, E61932 mRNA was expressed strongly in the leaves, of which the expression abundance of the mRNA in mesophyll (me) was particularly significant (Fig. 1(h)). In mesophyll of the materials with BPH infestation, the accumulation level of E61932 mRNA was higher than those without BPH infestation (Fig. 1(e)), showing that the gene was up-regulated. In addition, some of the bulliform cell (bu) showed strong positive reaction in the leaf without BPH infestation (Fig. 1(e)), but lower expression was observed in those with BPH infestation. The gene expression was not detected in both epidermis (ep) and bundle sheath (bs) of leaf.

Lowlevel expression of PI gene was exhibited in root (Fig. 1(b) and (c)), which was mainly distributed in the dermis parenchyma (de) and bundle parenchyma (bp). There was no difference between the materials with BPH infestation and those without. Results showed that infestation with BPH did not influence the expression of PI gene in the rice root.

In rice stem, substantial difference of expression level of PI gene was caused by BPH feeding. Hybridization results of vertical sections of stem with BPH infestation indicated that the gene was expressed strongly in some tissues, including blue cells (bc), parenchyma (pa), etc., and was expressed weakly in epidermis. But in bundle sheath and vessel element (ve), the mRNA was not expressed. Compared to the hybridization results of the stem sections without BPH infestation, parenchyma of stem with BPH infestation showed more positive reaction pots, and these pots were dispersedly distributed in the cells (Fig. 1(d) and (g)). Hybridization results of horizontal sections of stem (Fig. 1(f) and (i)) were similar to those of vertical ones.

3 Discussion

Through the RNA *in situ* hybridization, we concluded that PI gene could be expressed in the tissues of root, stem and leaf of rice, infestation with BPH substantially affected the gene expression in the stem. In detail, E61932 mRNA accumulation was enhanced significantly in the B5 stem 72 h after being fed by BPH. In the process of experiment, we ensured that the conditions for plant growth, seedling age, times to treat, etc., were identical for the treatment and the control. The enhanced expression of PI gene in the treated materials, therefore, was related to the infestation of BPH nymph, in other words, it was the result of responses of B5 plant against BPH feeding.

Coincidently, the enhanced expression of PI gene induced by BPH feeding mainly occurred in the stem of rice, which is the region for piercing and sucking of the insects. Research on the habit of BPH feeding showed that BPH sucked juice from the bundle sheath by piercing its stylet into phloem of rice stem to form saliva sheath[12]. The spatial accumulation of E61932 mRNA in stem, where BPH fed on, further confirmed that the higher expression of PI gene was induced by BPH feeding.

Trypsin is the main digestive enzyme in the gut of chewing insect. PI protein acts as deterrent and protects plant from damaging by insect. After feeding tissues containing PI, the insect cannot take up enough nutrients for maintaining its growth and development, because the digestive enzyme in insect gut combines with PI and the digest function is reduced. In the other aspect, after prote-

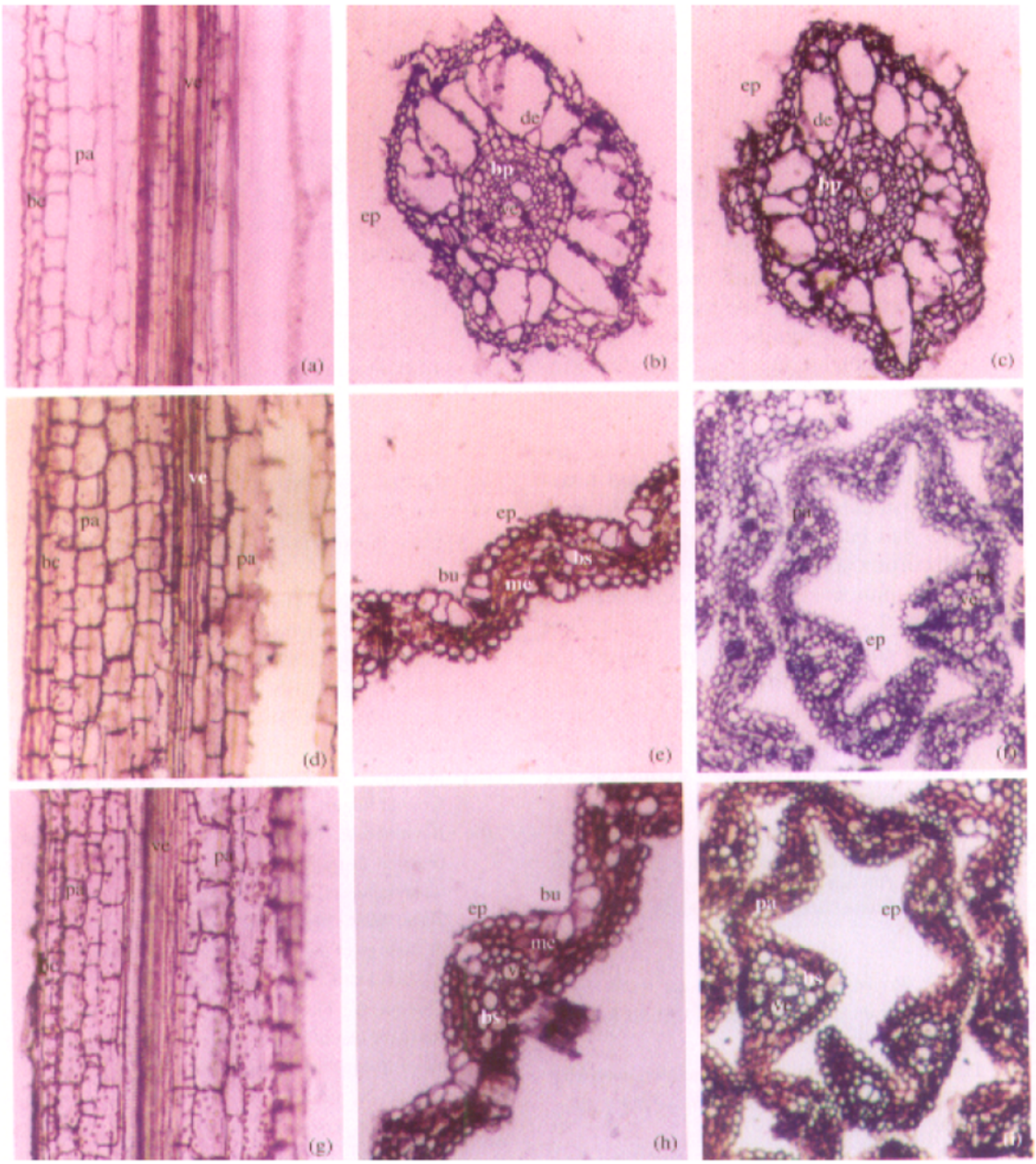

Fig. 1. *In situ* localization of E61932 mRNA in rice tissues. (a) *In situ* hybridization with sense RNA E61932 T3 as probe; (b)—(i) *in situ* hybridization with anti-sense RNA E61932 T7 as probe. (a) Vertical section of stem with BPH infestation (control), × 10; (b) horizontal section of root without BPH infestation, × 10; (c) horizontal section of root with BPH infestation, × 10; (d) vertical section of stem without BPH infestation, × 10; (g) vertical section of stem with BPH infestation, × 10; (e) horizontal section of leaf without BPH infestation, × 10; (h) horizontal section of leaf with BPH infestation, × 10; (f) horizontal section of stem without BPH infestation, × 10; (i) horizontal section of stem with BPH infestation, × 10. bc, blue cell; bp, bundle parenchyma; bs, bundle sheath; bu, bulliform cell; de, dermis; ep, epidermis; me, mesophyll; pa, parenchyma; ve, vessel element.

ase enzyme is inhibited or hydrolyzed, more of them will be produced for compensation in the insect body. As a result, it causes more serious scarcity of amino acids[13—15]. PI genes, such as *CpTI* from cowpea, have been widely used in crop transformation for insect resistance and exhibited satisfactory resistance to elytra, lepidoptera and orthoptera insects[16]. As a defensive protein, PI is induced to be expressed strongly when plant was challenged by pathogen, chewing insect or mechanical wounding. And the expression is controlled by the signal molecules as salicylic acid (SA), auxin, abscisic acid (ABA)[17,18]. In this study, PI expression was enhanced systematically in the rice stem and leaf except the root when the plant was fed by BPH.

Different to the chewing insects, piercing/sucking insects have been thought to lack digestive proteolysis[19,20]. But cDNAs for a cathepsin B-like protease and a trypsin-like protease were isolated and characterized from a cDNA library of brown planthopper gut tissue by Fiossac et al.[21]. And Lee et al.[22] reported that transferring soybean Kunitz trypsin inhibitor (*SKTI*) gene into japonica rice Nagdongbyeo would cause the BPH death rate raising and the BPH ovipositor rate and hatching rate reducing. These latest research results suggested that digestive proteolysis would make a significant contribution to nitrogen source for brown planthopper, and the direct inhibition of digestive proteolysis by the PI may play a role in rice BPH resistance response.

B5 is a stably resistant rice line to BPH and no obvious damage appearance occurred in the repeated resistance evaluation experiments over years[10]. Two main effect resistant genes had been identified and located on chromosome 3 and chromosome 4[23,24], respectively. In addition, another two minor effect loci were recently found on chromosome 2 and chromosome 9 (data not shown). The results in this experiment have led to the connection of PI gene with BPH-resistance in rice. Whether the enhanced expression of PI gene has direct resistance to BPH, or it is just one part of the plant defense responses formed through long-term evolution, remains as an important subject.

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