

## Analysis of Resistance-related Proteins in Rice Against Brown Planthopper by Two-dimensional Electrophoresis

CHEN Rong-Zhi, WENG Qing-Mei, HUANG Zhen, ZHU Li-Li, HE Guang-Cun\*

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

**Abstract:** A recombinant inbred population (RI) was constructed from a cross between B5, an introgression line from the wild rice *Oryza officinalis* Wall. ex Watt, and susceptible cultivar Minghui 63 (*O. sativa* L.). The brown planthopper (BPH) resistances of RI lines were evaluated. Based on bulked segregant analysis (BSA), two protein bulks were made by extracting proteins from equally mixed seedlings of extremely resistant and susceptible plants selected from the RI population, respectively. Two-dimensional electrophoresis was used to detect the changes of polypeptide pattern. Results showed that a protein P40 (pI 6.3, Mw 40 kD) was significantly reduced or vanished after BPH infestation for 48 h in the susceptible bulk, while it remained uninfluenced in the resistant bulk. In connection with the physiological changes of the resistant and susceptible lines subjected to BPH sucking, we suppose that the protein P40 is related to the interaction responses of rice plants to BPH infestation.

**Key words:** rice; recombinant inbred lines; brown planthopper resistance; two-dimensional electrophoresis

Brown planthopper (BPH), *Nilaparvata lugens* Stål, is one of the most serious pests of rice in Asia. BPH feeds mainly on the stems, and suck assimilates from the phloem of rice plants. Feeding by a large number of BPH may result in decrease in leaf photosynthetic rate, drying of the leaves, wilting of the tillers, even drying of the whole plant called hopperburn. In China, BPH causes the damage of rice on 4 million hm<sup>2</sup> to half of the total rice planting area every year. Exploring and growing the resistant varieties is an effective and economical way to control BPH. To develop resistant cultivars, large efforts have been made to identify BPH resistance genes from various sources. According to their reactions to different BPH biotypes, at least 12 resistant genes have been characterized and reported, and some of them have been mapped with the molecular marker analysis<sup>[1,2]</sup>. These are the foundation for cloning of the BPH resistance genes and understanding of the mechanism of BPH resistance in rice.

Efforts have also been made over years to elucidate the mechanism of BPH-rice interaction<sup>[3]</sup>, such as researches on feeding behavior and physiology of BPH<sup>[4]</sup>, the effects of rice varieties with different levels of resistance on growth and development of BPH<sup>[5-8]</sup> and the damage of BPH infesting on rice<sup>[9-12]</sup>. An understanding of the changes of physiological processes of rice plants caused by BPH could help the use of BPH resistance genes of rice varieties and the control of BPH.

Up to date, little is known about the molecular mechanism of the resistance of rice against BPH. Information on the change of proteins in response to BPH infestation in rice plants is not available. In the present study, the bulked segregant analysis (BSA) strategy, originally

designed to find polymorphic DNA markers<sup>[13]</sup>, was used for protein analysis. Recombinant inbred (RI) lines extremely resistant and susceptible to BPH were selected and pooled into the resistant bulk (R bulk) and susceptible bulk (S bulk), respectively. Two-dimensional electrophoresis was used to analyze the changes of protein pattern of the R bulk and S bulk after BPH infestation. The objective of this work was to search resistance-related proteins in rice against BPH, to provide basis for effective cloning of the BPH resistance genes and give clues to the mechanism of the resistance of rice to BPH.

### 1 Materials and Methods

B5, an introgression line derived from a cross between an accession of *Oryza officinalis* Wall. ex Watt collected in China and cultivar Zhenshan 97B (*O. sativa* L.), shows a high resistance to BPH<sup>[14]</sup>. An F<sub>7</sub> RI population of the cross between B5 and susceptible variety Minghui 63 was developed by continuing self-pollination of F<sub>2</sub> plants. Two hundred of lines randomly selected from the RI population were used in this experiment.

#### 1.1 Evaluation of BPH resistance

In the summer of 2000, two methods were employed to evaluate the BPH resistance of 200 RI lines, and each method was repeated three times.

**1.1.1 Seedling bulk test** Seventeen seeds of each RI line were sown in a row of 20 cm in length in a plastic box. The distance between rows was 2.5 cm. Two lines of B5, Minghui 63 and Taichung Native 1 were randomly planted among the RI lines as controls. At the three-leaf stage, the seedlings were infested with the third-instar nymphs of BPH at ten insects per seedling. When all of the seedlings of Taichung Native 1 died, the plants of the

RI lines were examined and the resistance level of each RI line was scored as described previously<sup>[2]</sup>.

**1.1.2 Honeydew excretion measurement<sup>[15]</sup>** Five third-instar nymphs of BPH were starved but water-satiated for 5 h, then were caged on a tiller of rice plants and allowed to feed for 24 h. The honeydew excreted was collected on filter paper at the base of the plant. The filter papers were stained with 0.05% ninhydrin in acetone solution. The area of magenta color developed on the filter paper was read into computer by a scanner. The pixels were taken to represent the stained honeydew area. The resistance level of each RI line was categorized based on the ranges of the stained honeydew area.

### 1.2 Selection of the extreme bulks

According to the results of the 200 RI lines' BPH resistance evaluation 21 RI lines extremely resistant to BPH and 21 RI lines extremely susceptible to BPH were selected to form the R bulk and S bulk. Five seeds per line in each bulk (R bulk and S bulk) were sown in a plastic pod respectively. At the three-leaf stage, the seedlings were infested with BPH. After BPH infestation for 48 h, equal seedlings per line in each bulk were pooled to form the sample of R bulk and S bulk respectively. The corresponding samples without BPH infestation were used as control.

### 1.3 Protein extraction

Proteins were extracted following the procedure described in He *et al.*<sup>[16]</sup> and Damerval *et al.*<sup>[17]</sup>.

### 1.4 Protein determination

Ten microlitres of protein sample were added in a 0.2 mL eppendorf tube. Then 40  $\mu$ L deionized water and 50  $\mu$ L 20% trichloroacetic acid were added in and the tube was allow to incubate on ice for 30 min. After spun in microfuge at 4  $^{\circ}$ C for 15 min, the supernatant was aspirated off. The pellet was washed in cold acetone, air dried and finally resuspended in 100  $\mu$ L PBS buffer. The protein concentration was determined by Bradford<sup>[18]</sup> method using bovine serum albumin as standard.

### 1.5 Two-dimensional electrophoresis and silver staining

Two-dimensional electrophoresis as described previously<sup>[16]</sup> with a protein load of 80  $\mu$ g and silver staining of the gel following Blum *et al.*<sup>[19]</sup> with some modifications were carried out. All steps were done with gentle shaking of the tray. The isoelectric point (pI) was measured according to the Wang and Tong<sup>[20]</sup>. The molecular weight of P40 was determined by comparing with the *Arabidopsis* reference map provided by SWISS-2D PAGE database<sup>[21]</sup>.

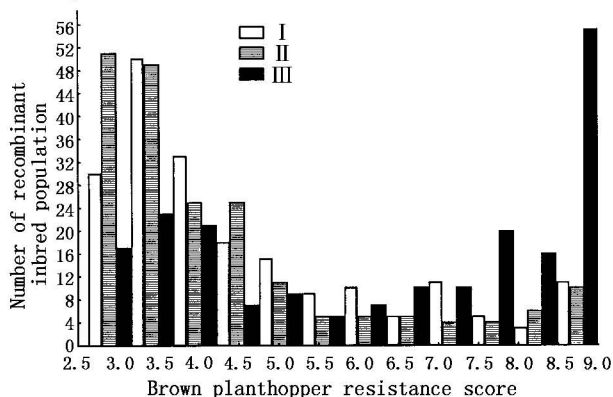
## 2 Results

### 2.1 Evaluation BPH resistance

Two screening methods, the seedling bulk test and honeydew excretion measurement, were used to evaluate the BPH resistance of 200 RI lines.

The BPH resistance of each RI line could be evaluated by the degree of injury of the seedling by BPH

infestation in the seedling bulk test. Similar results were obtained for three seedling bulk tests. The correlation coefficient was 0.70 between T1 (result of the first test, and T2, T3 represent results of the second and the third tests) and T2, 0.62 between T1 and T3, and 0.59 between T2 and T3. These represented a significant positive correlation. The average severity score of B5 was 3.69 and that of Minghui 63 was 7.53. The severity scores of the 200 RI lines infested with BPH showed a continuous distribution, ranging from 2.20 to 9.00, with a valley around 7.0 in the distribution curve (Fig.1). A line was classified as resistant if the average severity score was less than 7.0, and as susceptible if the average severity score was between 7.0 and 9.0. Among the 200 RI lines, there were 151 resistant plants and 49 susceptible plants. Segregation of resistant and susceptible plants fitted a 3:1 ratio in the RI population ( $\chi^2 = 0.027$ ,  $P > 0.75$ ). This confirmed the results that there were two BPH resistance genes in B5<sup>[1,2]</sup>.



**Fig.1.** Distribution of brown planthopper resistance scores in 200 recombinant inbred lines from a cross between Minghui 63 and B5. I, II, III represent the results of three seedling bulk tests respectively.

Honeydew excreted by BPH has been used as a criterion for determining the amount of sap ingested by the insect on rice plants. On the susceptible cultivars, BPH feeds more sap and excretes more honeydew. While on the resistant cultivars, BPH feeds and excretes less. After the honeydew was collected on filter paper and stained with ninhydrin acetone solution, the color area represented the amount of excretion. In our experiment, the average stained honeydew area of the three tests was taken to represent the resistance levels. The average stained honeydew areas of B5 and Minghui 63 were 9 520 (the pixels of the stained honeydew area in each stained filter paper) and 29 145, respectively. The average stained honeydew area of the RI lines was 20 696. The correlation coefficient between the results of the seedling bulk test and the honeydew excretion measurement was 0.61, indicating that the results of these two screening methods were comparable and reliable.

According to the results of BPH resistance evaluation in RI population, we selected 21 extremely resistant RI lines to form the R bulk and 21 RI lines extremely susceptible to BPH to form the S bulk. In the R bulk, all RI

lines were below 3.82 in severity score, and the stained honeydew area was less than 12 328. The average severity score was higher than 7.86, and the stained honeydew area was more than 30 000 in the S bulk. The RI lines selected as the extremely resistant bulk or susceptible bulk in the seedling bulk test also exhibited extreme resistance or susceptibility in honeydew excretion measurement, indicating that the bulks we had chosen were reliable.

## 2.2 Two-dimensional electrophoresis

In our experiment, we made some improvements in protein determination, which eliminated the interference by the combined effect of urea, detergents, carrier ampholytes, and thiol compounds. The assay allows accurate determination of the solubilized protein in sample prior to electrophoresis, thereby equivalent amounts of protein from biologically differing sources can be compared. The improved silver staining method showed increased sensitivity, higher resolution and reproducibility on the 2D gels.

Results from two-dimensional electrophoresis showed that, without BPH infestation, two-dimensional electrophoresis map of the resistant bulk (Fig. 2a) was similar to that of the susceptible bulk (Fig. 2c). No difference in protein patterns had been detected. After BPH infestation for 48 h, a protein of the susceptible bulk was significantly reduced or vanished (Fig. 2d), while this protein remained uninfluenced in the R bulk (Fig. 2b). The molecular weight and the isoelectric point (pI) of the protein was 40 kD and 6.3, respectively, thus the protein was named as P40.

## 3 Discussion

In this research, we used the seedling bulk test and honeydew excretion measurement to evaluate the BPH resistance of RI lines. The results of the two methods showed a high correlation coefficient. The repeated screenings guaranteed the accuracy of the BPH resistance evaluation, and thus assured the reliability of the results. Based on these data, BSA<sup>[13]</sup> was used in the subsequent study. According to the phenotypes of RI lines, 21 RI lines that were inferred to be extremely resistant to BPH were selected, and equal amounts of seedlings from each of these lines were mixed to form a protein sample of resistant bulk. Similarly, equal amounts of seedlings from each of the 21 extremely susceptible RI lines were mixed to form a protein sample of susceptible bulk. The two protein sample bulks were analyzed for protein patterns with two-dimensional electrophoresis. In theory, R bulk and S bulk should differ in their allelic content only at loci in the chromosomal region close to the BPH resistance genes.

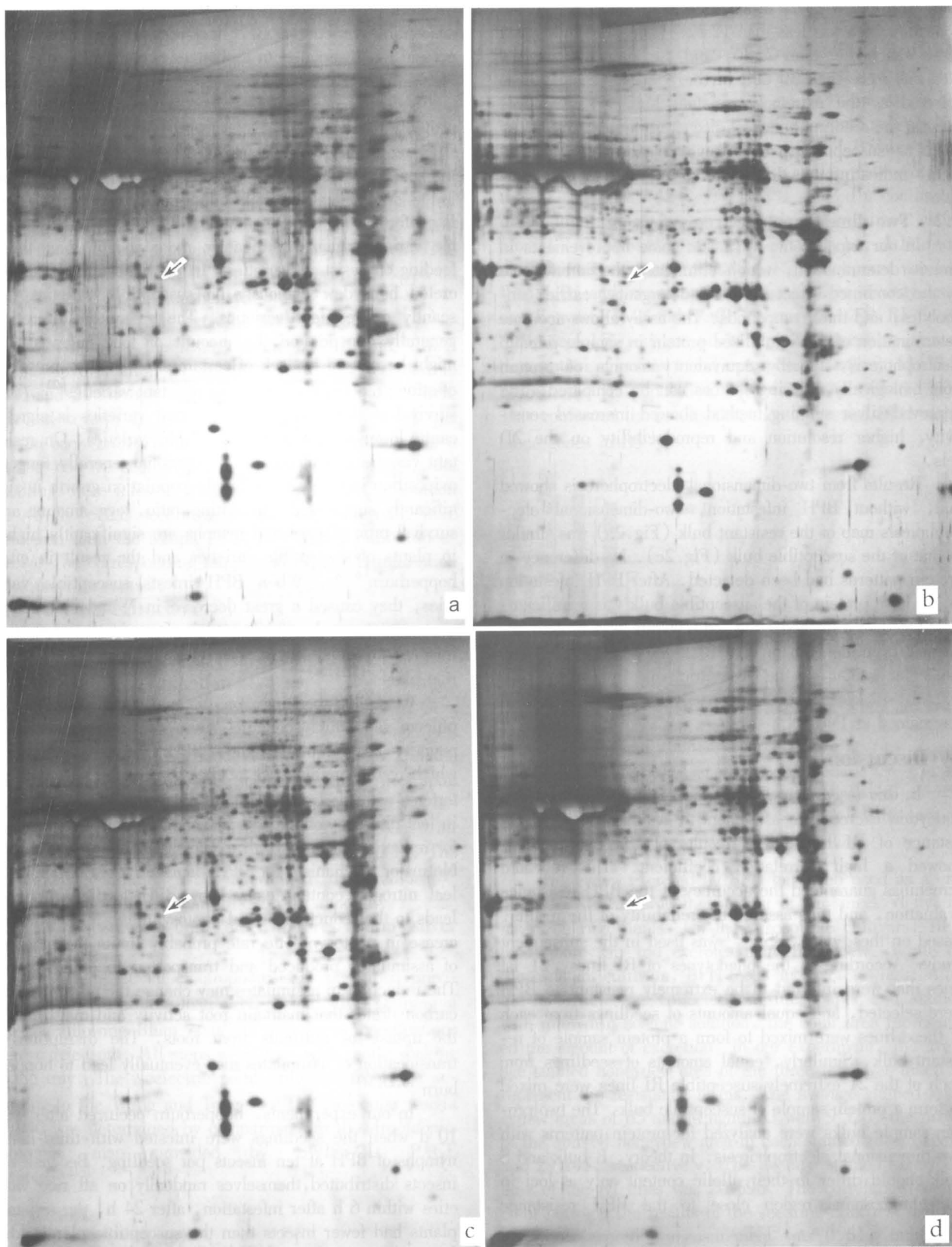
By two-dimensional electrophoresis, it was found that without BPH infestation, the similar expression of P40 was detected in both the R bulk and the bulk, but with BPH infestation of 48 h, the amount of P40 was significantly decreased in the S bulk, while the abnormal expression of P40 was not detected in the R bulk. From the

different physiological responses of resistant varieties and susceptible varieties to BPH sucking, we suppose that the protein P40 is related to the interaction responses of rice plants to BPH infestation.

A series of researches indicate that BPH probes repeatedly, salivates for a long time, and ingests for a very short period on the resistant varieties when compared with the susceptible cultivars. Quantity of honeydew excretion is low on resistant and high on susceptible cultivars. This is conformed by the fact that insects feeding on susceptible varieties gain significantly more weight than those feeding on resistant varieties. In our research, BPH excreted honeydew copiously on susceptible varieties but scanty on resistant varieties. The amount of excretion generally depends on the amount of food ingested. It might be caused by lack of certain stimuli or by presence of strong repellent substance in resistant varieties<sup>[22]</sup>. The survival of BPH nymphs on resistant varieties is significantly lower than that on susceptible varieties. On resistant varieties, nymphal development is generally longer, oviposition is severely inhibited, population growth is significantly suppressed. Inhibiting ratio, egg amount and survival ratio of eggs and nymphs are significantly higher in plants of susceptible varieties and the result in oteohopperburn<sup>[6-8]</sup>. When BPH infests susceptible varieties, they caused a great decrease in N and chlorophyll contents, organ dry weight and increase in the content of free amino acids, sucrose and leaf ferric ion content in rice plants<sup>[11, 23, 24]</sup>.

Generally speaking, on the one hand, BPH sucks phloem sap which contains sucrose and nitrogen compounds, on the other hand, BPH injects its secretion which results in blockage of the vascular bundle and interferes with the translocation of nutrients<sup>[4, 10]</sup>. Reduction in leaf nitrogen content, which might be partly responsible for reduction in the growth of the main shoot, is a possible major mechanism for BPH damage. The reduction in leaf nitrogen content caused by BPH feeding probably leads to the reduction in leaf photosynthetic rate. The decrease in photosynthetic rate probably limits the amounts of assimilates produced and transported to other tillers. The reduction in assimilates may change the amount of the carbohydrates that maintain root activity and may impede the uptake of nutrients from roots. The disruption of translocation of assimilates may eventually lead to hopperburn<sup>[10, 23, 24]</sup>.

In our experiments, hopperburn occurred after 7 - 10 d when the seedlings were infested with third-instar nymphs of BPH at ten insects per seedling. Because the insects distributed themselves randomly on all rice varieties within 6 h after infestation, after 24 h, the resistant plants had fewer insects than the susceptible plants, differences became more pronounced after 48 h of infestation. As honeydew excretion measurement proved, BPH ingested more on susceptible varieties, so P40 was significantly decreased after BPH feeding 48 h. On resistant varieties, BPH ingested less, and no obvious protein change was observed after BPH feeding. This work provided



**Fig.2.** Two-dimensional electrophoresis profiles of rice seedling protein. Arrowheads indicate P40. a. Resistant bulk without BPH infestation, showing the normal amounts of P40. b. Resistant bulk with BPH infestation for 48 h, showing P40 remained uninfluenced. c. Susceptible bulk without BPH infestation, showing the normal amounts of P40. d. Susceptible bulk with BPH infestation for 48 h, showing P40 significantly reduced or vanished.

the possible molecular evidence for the mechanism of rice responding to BPH. In the course of BPH ingesting, the rice plant must alter its gene expression and hence the protein pattern. The fact that BPH induced changes in gene expression was also verified in mRNA differential hybridization experiments in our laboratory. P40 detected in this experiment may be an early protein that is related to the interaction responses of rice plants to BPH infestation. Further work is needed to obtain more information about P40 to illuminate its practical function in rice-BPH interaction.

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# 水稻对褐飞虱抗性相关蛋白的双向电泳分析

陈荣智 翁清妹 黄臻 祝莉莉 何光存\*

(武汉大学生命科学院, 植物发育生物学教育部重点实验室, 武汉 430072)

摘要: 以药用野生稻(*Oryza officinalis*)的转育后代 B5(高抗褐飞虱(*Nilaparvata lugens* Stål))与感虫品种明恢 63(*Oryza sativa* L.)为亲本, 构建了一个重组自交系群体。通过抗褐飞虱鉴定, 筛选出极端抗虫株系和极端感虫株系, 运用分群分析法(bulked segregant analysis, BSA)分别建成了极端抗虫集团(resistant bulk)和极端感虫集团(susceptible bulk)的蛋白质池。利用双向电泳技术, 分别分析了极端抗虫集团和极端感虫集团受虫害与未受虫害的秧苗蛋白质的变化。结果发现, 虫害 48 h 后, 感虫集团的一个分子量为 40 kD 的蛋白质 P40 (pI = 6.3)的表达明显减弱甚至消失, 而在抗虫集团中, P40 的表达未受影响。与褐飞虱为害后抗虫株系和感虫株系不同的生理反应相联系, 推测 P40 与水稻受褐飞虱虫害后引起的应答反应相关。

关键词: 水稻; 重组自交系; 褐飞虱抗性; 双向电泳

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\* 通讯作者。E-mail: <gche@whu.edu.cn>.

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