DOI: 10.1111/j.1570-7458.2008.00785.x

Penetration into rice tissues by brown planthopper and fine structure of the salivary sheaths

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Accepted: 11 August 2008

Key words: *Nilaparvata lugens*, morphology, preference, leaf sheath, vascular cells, probing, Homoptera, Delphacidae, feeding behaviour

Abstract

The fine structure of the salivary sheaths in plant tissues can provide important information on homopteran probing and ingestion behaviors. Salivary sheaths secreted by the brown planthopper (BPH), *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae), and their tissue pathway were investigated using light, scanning electron, and transmission electron microscopy. About half of the salivary flanges on the surface of the food substrate were connected with internal salivary sheaths. Only 43% of the salivary sheaths showed side branches. Many sculpture-like protuberances and small cavities had been formed on the outer surface of the salivary sheath, but the sheath lumen circumferences were sealed. Brown planthoppers showed a preference for probing and leaving salivary sheaths in the susceptible rice variety TN1 rather than in the resistant variety B5 during the first 2 days of the experiments. The salivary sheaths in rice tissues reached the inner tissue layer of the leaf sheaths and stems, but were mostly observed to end in the first and second layer of the leaf sheaths. Brown planthoppers also preferred to probe into the thick segment of the outer leaf sheath. After ingestion by the insect, the cytoplasm in both phloem and companion cells degraded and the main organelles were lost. Numerous small vesicles were found in most of the phloem cells, but cell walls remained intact. Large numbers of symbiont-like structures were observed inside the salivary sheath lumen. These results indicated that BPH has complicated feeding behaviors, which warrants further investigation.

Introduction

The brown planthopper (BPH), *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae), is a ubiquitous rice pest in Asia, the Pacific Islands, and Australia, which causes extensive damage primarily by ingestion of phloem sap (Wilson & Claridge, 1991; Heinrichs, 1994; Settle et al., 1996). Recent research has investigated the causes of BPH damage by studying the species' population dynamics, host plant morphology and physiology, and molecular profiles in both rice and BPH (Sogawa, 1982; Backus et al., 2005a; Wang et al., 2005; Yang et al., 2006; Zheng et al., 2007). The feeding behavior of BPH can give an important clue for identifying the susceptibility or resistance mechanism in rice plants under insect infestation. It has been shown that feeding behaviors of BPH, particularly saliva secretions, are related to the degree of rice resistance to this pest (Sogawa & Pathak, 1970; Sogawa, 1982; Spiller, 1990).

In BPH, the salivary sheath is made of solid saliva that is secreted during probing. Naito (1964) and Sogawa (1977) first reported the structure of the BPH salivary flanges on rice leaf epidermis and their associated salivary sheaths in rice tissues or on the membrane surface of artificial diets. It was observed that the associated sheaths formed in rice tissues or in the diet are single or branched tubes, and the sheaths' surface is irregularly beaded (Naito, 1964; Sogawa, 1977). This suggests that the insect secretes sheath material intermittently and repeatedly, by partly withdrawing its stylets and reinserting them into new tissue areas while probing for ingestion sites, thus making sheaths branches. The stylet tips are inserted into the phloem to ingest plant sap, resulting in blockage of vessels (Sogawa, 1970). Little more is known about the salivary sheaths' fine structure. Scanning electron microscopy (SEM) and transmission electron microscopy have shown that the salivary and

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food canals of the brown planthopper are separated for their entire length (Aljunid & Anderson, 1983; Foster et al., 1983a,b). The food canal lies in the centre of the interlocked maxillae, but the salivary duct is actually located in a ridge of one of the maxillae. Using amputated stylets (stylectomy), which remained embedded in the plant tissue, Spiller (1990) described the ultrastructure of the stylets and their sheaths and found that only two maxillae were inserted in rice tissues. He also noted an intracellular penetration pathway. These features match the 'maxillary stylets ahead' method of homopteran probing described by Backus (1988). However, the morphology of the tissue-embedded sheaths and their ducts remain unknown.

In rice, blockage of assimilate translocation may represent an important factor in BPH damage (Kenmore, 1980). Necrotic lesions and occlusion in the vascular tissues were also reported in rice (Sogawa, 1982). However, Watanabe & Kitagawa (2000) demonstrated that removal of assimilates and reduction in photosynthesis by *N. lugens* has a greater effect on growth and yield of rice plants than disruption of assimilate translocation. Senescence and death of lower leaves may result from plant respiration and *N. lugens* ingestion, leading to a deficit of carbohydrates, which are revealed earlier in cells of the lower leaf sheath than in mesophyll cells. Notwithstanding these observations, ultrastructural changes in these cells have not been reported. Previous studies revealed that intracellular penetration is accompanied by disruption and degeneration of the organelles, presumably resulting in cell death (Spiller, 1990). But few cytological studies of the peripheral cells at the piercing site or along the stylet pathway have been conducted. Furthermore, as a typical vascular feeder, BPH primarily ingests phloem sap. The depth of stylet penetration by BPH is likely to have noticeable effects on assimilate translocation and the plant's compensation for the insect's injury. However, the penetration depths of BPH have yet to be described. Leaf sheaths of rice plants were shown to be annular and encircled each other from the opposite direction. Furthermore, single leaf sheaths showed variability in thickness in different regions. Salivary sheath distribution along the circumference of leaf sheath cross-sections may also be a clue for understanding feeding behavior of the insect. It is unknown whether a penetration preference exists for the outer epidermis around the leaf sheaths.

The objectives of this study were to examine the predominantly branched forms, fine structure of BPH salivary sheaths, and morphology of leaf sheaths and stems of rice where probed, using light microscopy and SEM. The distribution of salivary sheaths on the outer epidermis was investigated, to establish which tissue layers *N. lugens* can penetrate and whether a preference is evident. In addition, we described the peripheral cell ultrastructure at the puncture site in rice.

Materials and methods

Insects and plants

Nilaparvata lugens (biotype 1) was reared on the susceptible rice variety Taichung Native 1 (TN1) at 26/22 °C (day/night), 60 ± 8% r.h., and L13:D11 photoperiod. Early fourth instars were collected and starved for 2 h before initiating feeding experiments. Varieties TN1 and B5, a line highly resistant to BPH, were used in the experiments. Sterilized seeds of TN1 and B5 were individually sown in 1/2 MS (Murashige & Skoog, 1962) culture medium in glass tubes (2.5 × 20 cm). Rice seedlings were grown in a controlled environment under 26/22 °C (day/night), 60 ± 8% r.h., and L13:D11 photoperiod. The 15-day-old seedlings at 2–3 leaf stage were used for feeding. All experiments were carried out at the Genetics Institute of Wuhan University, Wuhan, China.

Feeding and sampling

Two methods were employed to obtain salivary sheath samples of BPH. The first method was to use an artificial diet contained in a parafilm sachet (Sogawa, 1967; Begum & Wilkins, 1998). Fifty nymphs were enclosed in a small feeding chamber covered by the stretched parafilm sachet, which was made by injecting a 2.5% sucrose solution onto one parafilm layer and then covering the solution with a second layer. After 4 days, the sucrose solution was removed and the membrane was used for SEM or stained for light microscopy. The artificial-diet experiment was repeated five times using five separate chambers. In the second method used to obtain sheaths, the insects were reared on rice in a glass tube. Twenty nymphs, starved for 2 h, were allowed to probe and feed on individual TN1 or B5 seedlings. After 2 and 4 days, 1.5 cm portions of the seedling stems were sampled for light microscopy. Sections containing sheaths were selected for scanning and transmission microscopy. The experiments with TN1 and B5 plants were repeated eight times.

Light microscopy

The parafilm pieces from diet sachets on which the insects fed were stained with oil-red O, haematoxylin, Coomassie Brilliant Blue R-250, or I2-KI and observed under a light microscope (Esen, 1978; Yin & Tsutsumi, 2003; Akin et al., 2004; Kamenetsky et al., 2004). Twelve sites (each site is actually the visual field under the microscope and is about 2 mm in diameter) were selected from each piece of parafilm, and in total 60 sites from five pieces of parafilm were examined. All salivary flanges and sheaths were

counted. The salivary sheaths were subsequently classified by the number of branches. The salivary flanges with sheaths on parafilm were also examined under excitation wavelengths of 520–550, 460–490, and 330–385 nm using Olympus AX80 (Olympus, Osaka, Japan) fluorescence microscopy.

The 1.5 cm portions of rice stems on which BPH probed were fixed, dehydrated, infiltrated, and embedded in wax (paraffin) according to standard procedures (Zhu et al., 2006). Each portion was then divided into 0.5-cm long blocks, and each block was serially cross-sectioned at 8 μm. The sectioned blocks of TN1 and B5 came from the same locations on seedlings. The sections were then dewaxed, dried, stained with oil-red, and observed under the light microscope. Proportions of the sections with salivary sheaths in total sections were compared between TN1 and B5. In order to determine probing site preference in outer leaf sheath by BPH, the salivary sheaths left in the thin and thick segments of leaf sheaths were also counted.

Scanning electron microscopy

The salivary sheaths from parafilm were attached to a stub, coated with gold in a Hitachi IB-5 sputter coater (Eiko, Tokyo, Japan), and observed with a Hitachi S-450 SEM. To investigate the distribution and pathway of salivary sheaths in rice tissues by SEM, the paraffin sections for light microscopy were temporarily mounted on glass cover slips and examined under a light microscope (Olympus AX80). Those sections with intact sheaths were dewaxed, coated with gold, and observed via SEM.

Transmission electron microscopy

After being fed upon for 3, 5, and 8 days by BPH, 0.5 cm portions of leaf sheath at the base of each TN1 plant were fixed for 3 h in 2.5% glutaraldehyde at pH 7.2, prepared in 0.025 M phosphate buffer, post-fixed in buffered 1% osmium tetroxide, and then dehydrated through an ethanol series. The samples were then embedded in Epon 812 resin. Ultrathin sections were placed on copper grids and stained with uranyl acetate followed by lead citrate and examined in a JEX-100CX transmission electron microscopy (JEOL, Tokyo, Japan).

Data analysis

Analysis of variance (Proc MIXED; SAS Institute, 1999) was employed for data analysis. The means for percentages of salivary sheaths with different branch types or salivary sheaths located in different positions were compared using the test of least significant differences (LSD), with differences considered significant at $\alpha = 0.05$.

Results

Fine morphology of salivary sheaths on parafilm

The brown planthopper secreted various forms of branched sheaths seemingly randomly in the 2.5% sucrose solution (Figure 1B–G). The insects produced full salivary sheaths for 281 (i.e., 47%) of 598 salivary flanges at 60 sites (Table 1). The more branches were observed in the saliva sheaths, the lower was the percentage occurrence (Table 1). One of the sheaths had 24 branches and most of them originated from the base of the sheath (Figure 1H). The length of salivary sheaths varied from 0 to 750 μm and the average length was 300 μm.

The hollow lumen of the sheaths was visible extending to the tapered tip when the sheaths were stained with haematoxylin (Figure 2A,B). The width of this lumen averaged 8–10 μm. Some sheath tips appeared open (Figure 2B), but others seemed to be sealed (Figure 3E). Other than at the tip, the sheaths were usually hollow, indicating that this species dose not typically fill a sheath with saliva upon withdrawal of the stylets.

Several staining methods were compared to examine salivary sheaths via light microscopy. Oil-red O was best for visualizing rice tissue sheaths (Figures 2C and 4A,C). After staining with oil-red, I2-KI, and Coomassie Brilliant Blue R-250, salivary sheaths showed up light red, yellow, and blue, respectively (Figure 2C–E). The sheath stained purple after haematoxylin and blue-black with toluidine blue, suggesting that the sheath materials were alkaline (Figure 2A,E). Under excitation wavelengths of 520–550, 460–490, and 330–385 nm, the sheath emitted red, blue, and green fluorescence, respectively. The salivary flanges presented a more intense fluorescence than the sheath proper (Figure 2F–H).

Table 1 Mean (± SE; n = 3) percentage of full salivary sheaths of brown planthopper, *Nilaparvata lugens*, and their branch numbers on parafilm

Total salivary flanges	Full salivary sheath (%)	Sheaths with different number of branches (% of full)				
		0 branch	1 branch	2 branches	3 branches	>3 branches
598	47 ± 4.82	57 ± 2.73a	22 ± 0.66b	11.5 ± 1.10b	5 ± 0.94c	5 ± 0.77c

Different letters following means indicate a significant difference (LSD: $P<0.05$).

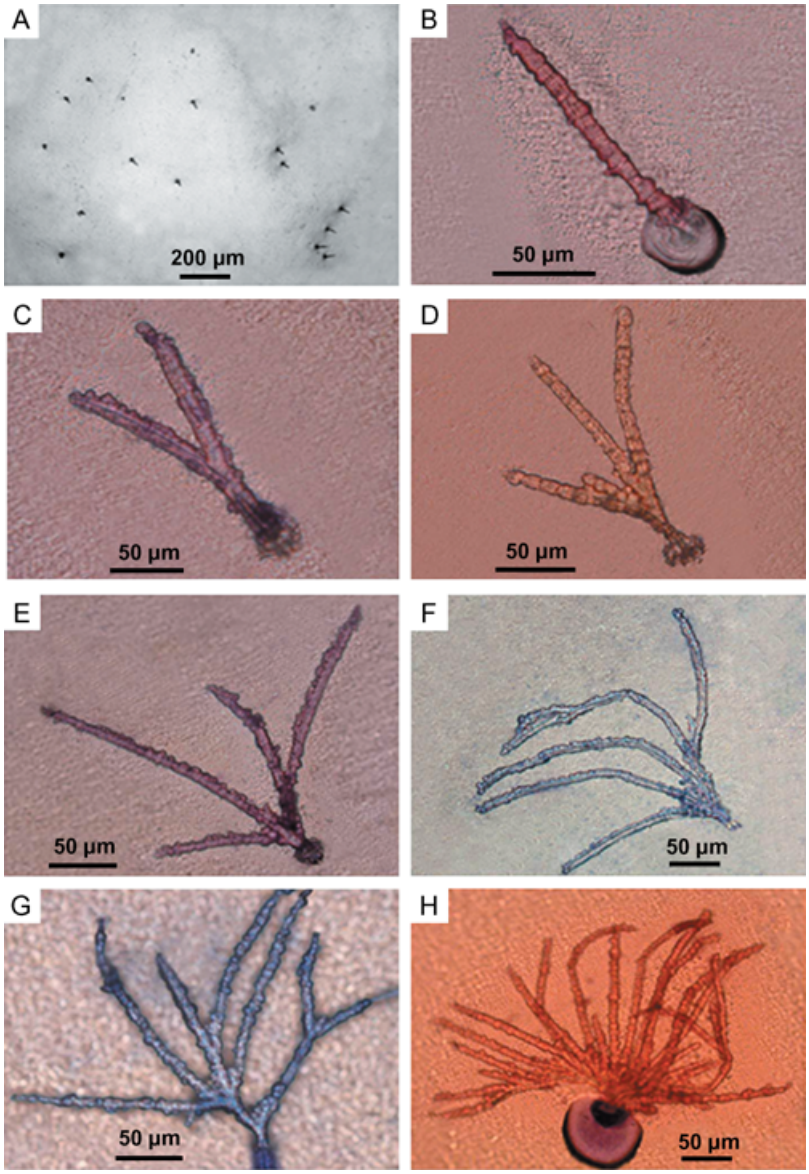

Figure 1 Branch morphology of freely formed salivary sheaths, left on parafilm by the brown planthopper, *Nilaparvata lugens*, fed on a sucrose solution. (A) Unstained, (B, C, D, E, H) stained with haematoxylin, and (F, G) stained with Coomassie Brilliant Blue R-250.

The outer diameter of the sheath varied, with an average of 9 ± 0.8 µm (Figure 3). Protuberances and pit-like structures were observed on the outer surface of the salivary sheath (Figures 2A and 3A,C,D). The saliva protuberances seemed to be randomly distributed on the outer surface of sheaths. Due to the depth of pits in some sheaths, it was difficult to determine by SEM whether pits were connected with the internal sheath lumen.

Most secretions on the outside of the sheath wall (protuberances) were released during free sheath formation, but the bud-like saliva seems characteristic of a new sheath branch (Figure 3B). The cylindrical salivary flange was wider than the sheath and approximately 12 µm in diameter (Figure 3F). The flange was hollow after withdrawal of the mouthparts and the distal sheath tip was tapered and sealed.

Distribution of salivary sheaths in rice leaf sheaths

The proportions of sections with salivary sheaths left by brown planthoppers in the leaf sheath of the two rice varieties were investigated (Table 2). During the first 2 days, most sections had either no or only one salivary sheath. Also, only very few sections had two salivary sheaths (salivary flanges). The number of sheaths was significantly

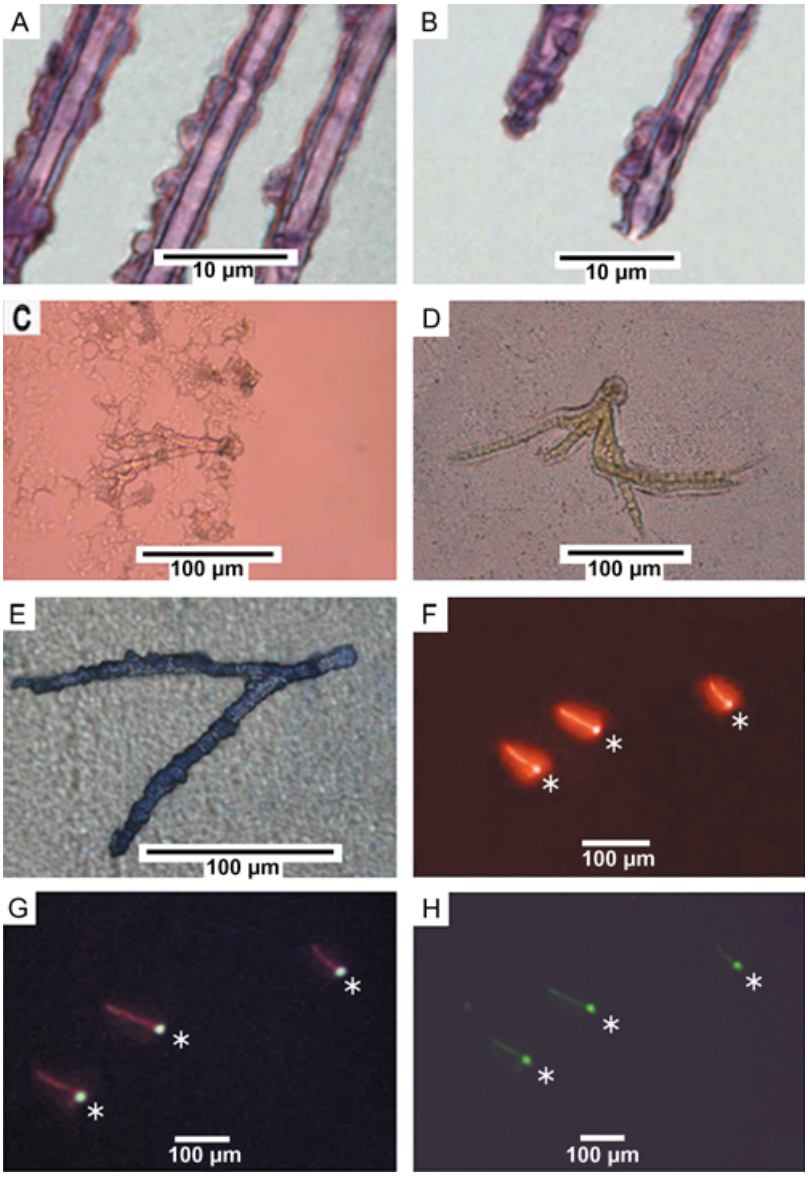

Figure 2 Salivary sheaths produced by the brown planthopper, *Nilaparvata lugens*, stained with (A, B) hematoxylin, (C) oil-red, (D) I_2-KI, (E) Coomassie Brilliant Blue R-250, or excited with light of different wavelengths: (F) 520–550 nm, (G) 460–490 nm, and (H) 330–385 nm. The asterisks in F, G, and H indicate the salivary flanges, which emitted strongly under laser excitation. The salivary sheath in C is in rice tissue, the others are on parafilm.

lower on B5 (Table 2). Therefore, it was concluded that brown planthoppers probe more often in susceptible TN1 than in B5 during the first two feeding days. After prolonged exposure, the proportion of sections with salivary sheaths left in the resistant B5 increased rapidly (Table 2).

In the experiment, BPH aggregated and probed on the leaf sheaths at the basal portion of the TN1 plants. In addition, BPH penetrated the inner leaf sheaths as well (Figure 4A–C). Most salivary sheaths were found in the first leaf sheath, but BPH occasionally penetrated as deeply as the second and third leaf sheath. Among the 189 sections of the basal portion in TN1, the percentages of salivary sheaths that reached the first, second, and third leaf sheath were 62, 28, and 4%, respectively (Figure 4D). Moreover, no signs of directional pathway changes were evident when BPH penetrated from one leaf sheath to the next or across an air space in the tissue.

Figure 5A–H illustrates the distribution of salivary sheaths in different segments of the outer leaf sheath. Analysis of 215 TN1 sections with salivary sheaths in tissues showed that 71% of the salivary sheaths were located in the thick segment of the outer leaf sheath (Figure 5I,J). In addition, we found some salivary sheaths in the adaxial and abaxial

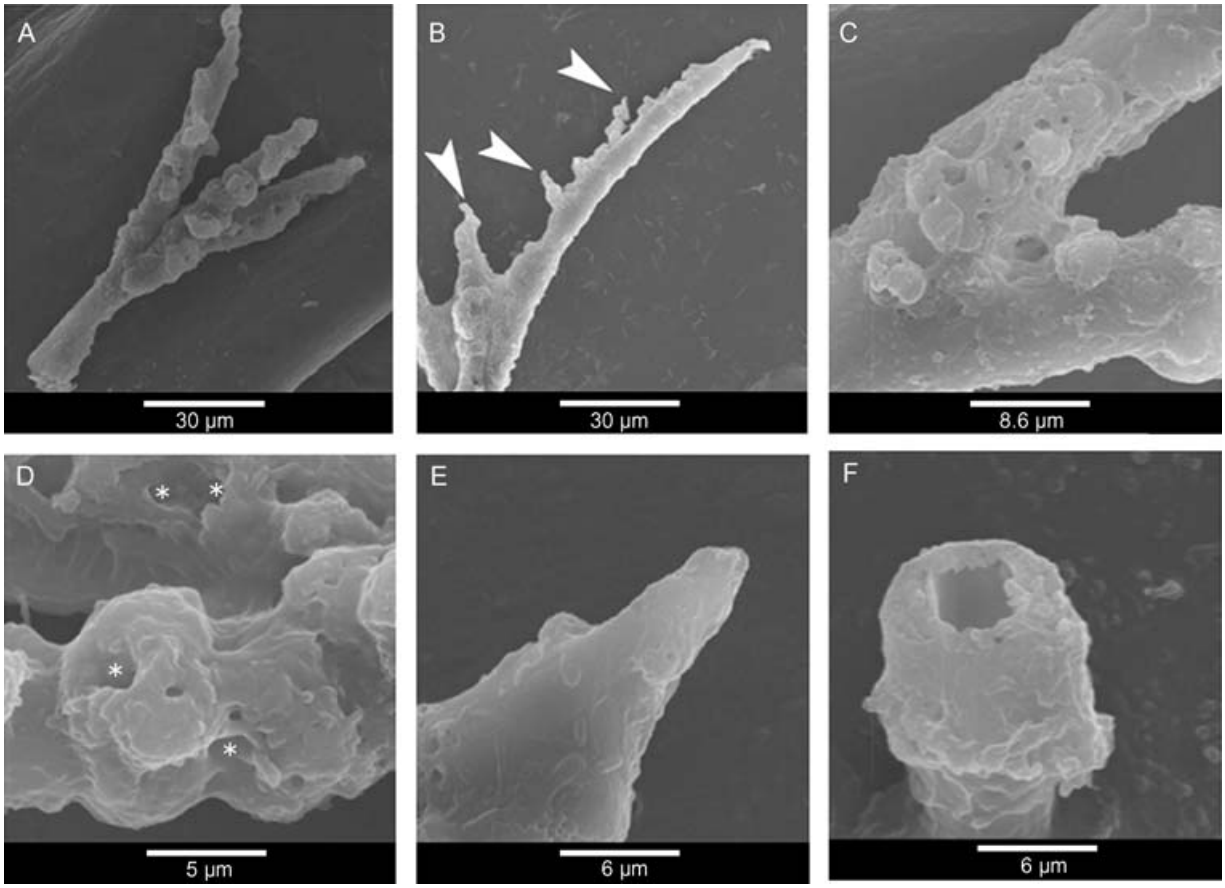

Figure 3 Surface of salivary sheaths produced by Brown planthopper, *Nilaparvata lugens*, observed by scanning electron microscopy (SEM). (A) Complete salivary sheath, (B) a branch with several buds, (C) topography of one fork, (D) protuberances and caves (asterisks) on the outside wall of the salivary sheath, (E) normal topography of the sheath top, and (F) flange of a salivary sheath. The arrowheads in B indicate the bud-like saliva sheath.

rice leaf epidermis, indicating the insects tried to probe and ingest from rice leaves (data not shown).

Morphology of stylet pathway in rice tissues

Scanning electron microscopy observations indicated that the stylet penetration pathway is partially intracellular (Figure 6D,E), or probably partially intercellular (Figure 7G,H). The salivary sheath diameter is larger than most phloem cells, but smaller than the diameter of both the xylem and parenchyma cells (Figure 6B,C). Some differences were observed in the salivary flange morphology between the artificial sucrose solution and rice tissue (Figures 3F and 6D). The salivary flanges in rice tissue were bead-shaped with a thin collar patch of congealed secretion on the epidermis. Longitudinal sections of the tip of the sheath showed that the lumen was completely sealed with saliva.

Table 2 Mean (± SE; n = 4) percentage of sections with salivary sheaths left in B5 (resistant rice variety) and TN1 (susceptible rice variety) tissues of basal plant parts after brown planthopper, *Nilaparvata lugens*, stylet penetration

	Variety/feeding duration			
	TN1/2 days	TN1/4 days	B5/2 days	B5/4 days
Total sections examined	265	322	382	513
Section with sheaths left	148	159	29	134
Percentage	55.5 ± 3.7a	49.4 ± 7.4a	7.6 ± 0.53c	32.4 ± 5.6b

Different letters following means indicate a significant difference (LSD: $P<0.05$).

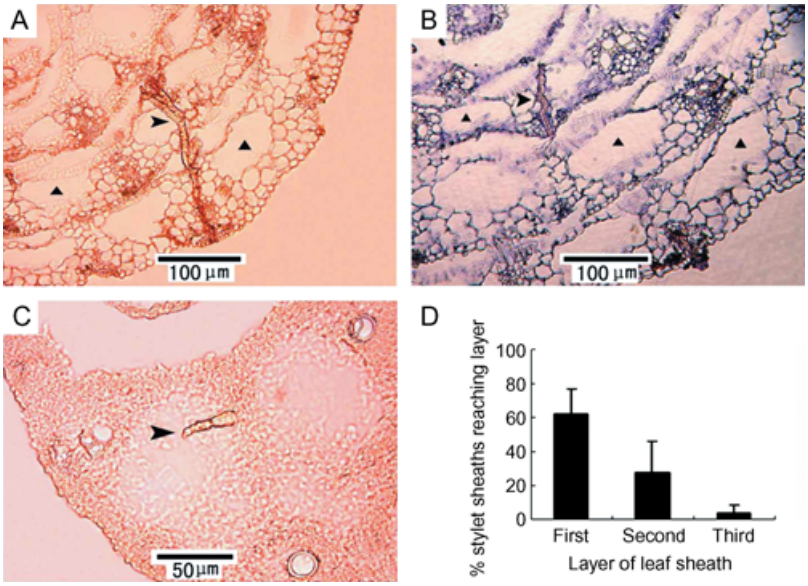

Figure 4 Depth of stylet penetration of brown planthopper, *Nilaparvata lugens*, in rice leaf sheath. Salivary sheaths left in the (A) first and second, (B) third, and (C) fourth leaf sheath. Sections of A and C were stained with oil-red. Section B was stained with haematoxylin. (D) indicates the percentage (mean + SE) of salivary sheaths left in various rice leaf sheaths. The arrowheads in A, B, and C indicate the salivary sheaths left in rice leaf sheaths. The triangles in A and B indicate the air space in rice leaf sheaths.

The inner diameter of the salivary sheath was 4–5 μm and the sheath wall was approximately 2–2.5 μm (Figure 6E).

Effects of brown planthopper ingestion on vascular cells in rice leaf sheath

After BPH fed on the leaf sheath, the vascular cells of TN1 changed (Figure 7A–D). For example, following the third feeding day, the cytoplasm in the phloem companion cells began to condense (Figure 7B). At the fifth feeding day, the continuity of cytoplasm in both the phloem and companion cells was severely degraded. Numerous deeply stained linear structures were observed in the phloem cells, but the cell wall was intact (Figure 7C,D). Moreover, some foreign materials in the cells adjacent to the salivary sheath stained homogeneously and were probably secreted by the insect and filled in the cells (Figure 7E,F). Alternatively, these materials could have been coagulated plant metabolites from a localized plant response to saliva. Examination of the salivary sheath tips in rice tissues revealed many bacteria-like structures in the tube (Figure 7G,H).

Discussion

The morphology of salivary sheaths left by BPH in plants or artificial diets provides useful information on certain probing and ingestion behaviors. A number of investigators have described the morphology of salivary sheaths produced by various species of Homoptera using microscopy or microcinematography (Naito, 1964; Sogawa & Pathak, 1970; Sogawa, 1970; Miles, 1972, 1999; Spiller et al., 1985; Brennan & Weinbaum, 2001; Freeman et al., 2001; Lett et al., 2001; Hardie & Powell, 2002; Leopold et al., 2003; Backus et al., 2005b; Joost et al., 2006). In this study, two types of salivary sheaths were found on parafilm by light microscopy. As reported by Sogawa (1977, 1982), one is a bead-like salivary sheath (Figure 1D) formed from saliva that appeared to be intermittently secreted by the insect. In our laboratory, the insect was observed by microscopy to secrete individual drops of saliva into a sucrose solution, thereby forming these beads (Peiying Hao & Ming Tang, unpubl.). This confirms that brown planthoppers secrete saliva in an intermittent manner, similar to aphids (McLean & Kinsey, 1965; Miles, 1999). Moreover, we described another type of salivary sheath that possesses a more irregular structure on the outer surface and has no visible subsections. It is likely that continuous secretions at variable speeds, and not intermittent secretions, formed this kind of salivary sheath.

The outer surface of the sheath was studded with protuberances and contained deep cavities. The lumen of the sheath had a smooth inner surface. In rice tissues, the protuberances could be an adaptive mechanism for the insect to reduce friction and smooth the stylet pathway through plant cells to build an ingestion channel. However, when sucrose was the food source, the protuberances seem redundant. The bud-like saliva may be the initiation of a new branch. It is unknown why an insect stops building a new branch. Sogawa (1970) suggested that an insect builds the sheath branches in different directions to choose the best food source by gustation. Many branches were formed in a sucrose solution, in which the insect found similar taste irrespective of the direction. It is probable that BPH is

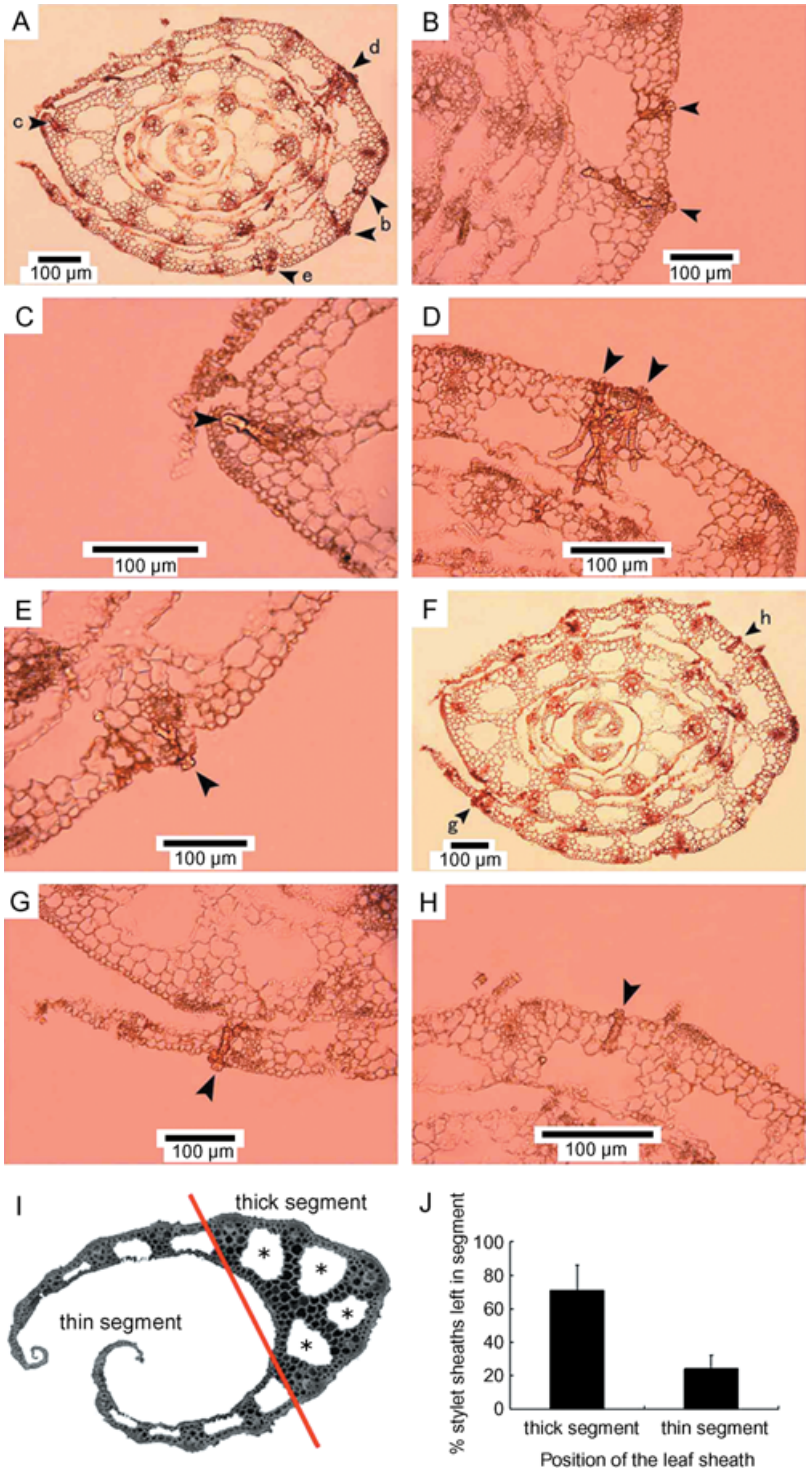

Figure 5 Brown planthopper, *Nilaparvata lugens*, probing sites around the periphery of the leaf sheath. Areas indicated with arrowheads at b, c, d and e in (A) are magnified in (B), (C), (D), and (E), respectively. Likewise, g and h in (F) are magnified in (G) and (H). (I) Outermost leaf sheath, partitioned into two segments. (J) Percentages of probing sites in these two segments of the leaf sheath. Arrowheads in B, C, D, E, G, and H indicate salivary sheaths left around the periphery of the leaf sheath.

inclined to produce a new branch because of unsatisfactory chemical factors, unsuitable physically factors, no pressure of the liquid food (sucrose) solution, or no target found during a maximal extension of the last branch, allowing no further stylet extension into the food. Observations indicated that most sheath lumens were sealed at their distal tips. The details of sealing behavior and its functions need to be investigated further.

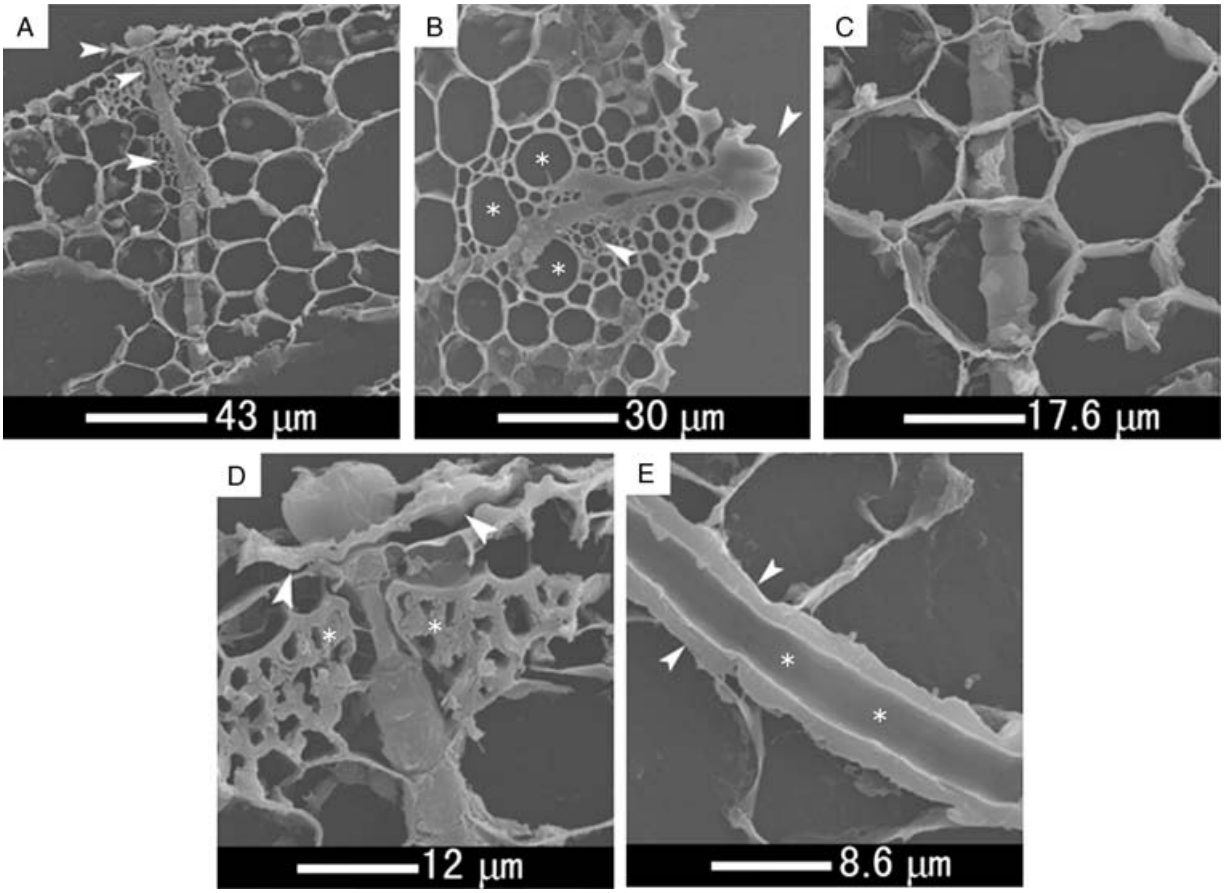

Figure 6 Penetration pathway of brown planthopper (BPH), *Nilaparvata lugens*, in rice tissues observed by scanning electron microscopy (SEM). (A) Salivary sheath (arrowheads) traversing vascular and parenchyma cells, (B) salivary sheath reaching and showing branch tips in the vascular tissues and passing further into deeper tissues; asterisks and arrowhead indicate the xylem elements and phloem tissue, respectively, (C) salivary sheath traversing the parenchyma (detail of A), (D) salivary flange seems to be formed out of two parts (detail of A), a flat collar-like base (arrowheads) on the leaf surface and a ball-shaped bead of saliva protruding from the collar center; the asterisks indicate that BPH can penetrate the thick-wall cells under the epidermis, and (E) longitudinal section of a salivary sheath; the asterisks indicate the smooth inner surface of the sheath lumen. Arrowheads in E indicate the sheath wall left in rice tissues by BPH.

Rice defense against BPH is initiated at three BPH behavioral stages, namely, distance orientation to the host, surface exploration, and probing (Sogawa, 1982). In addition, ingestion is probably a fourth behavioral stage for rice defense. It has been reported that BPH showed no preference for resistant varieties and significantly more individuals settled on susceptible individuals (Saxena & Okech, 1985; Velusamy, 1988; Velusamy et al., 1995). However, the literature provides evidence that the insects perform more probes in resistant than in susceptible varieties (Sogawa & Pathak, 1970; Velusamy & Heinrichs, 1986; Cook et al., 1987; Kimmins, 1989; Zhu et al., 2002). The probing frequency on different rice varieties was described as the number of flanges on the plant surface stained with erythrocin (Sogawa & Pathak, 1970), separate AC-I waveforms in an electrical penetration graph (EPG) study (Velusamy & Heinrichs, 1986), and stationary contact of the labium with the plant surface for more than 2 min (Cook et al., 1987). However, during the first 2 days of this study, more salivary flanges with sheaths were actually left by BPH in susceptible TN1 than in B5. The discrepancy in probing frequency may arise from the differences in investigation methods or the particular resistance of different rice varieties. Alternatively, more stained flanges or longer settling times and fewer sheaths may be a common response of BPH to resistant rice varieties (Sogawa, 1977; Yoshihara et al., 1979a; Velusamy, 1986; Cook et al., 1987; Woodhead & Padgham, 1987; Huang et al., 2005). Brown planthoppers that landed on resistant varieties moved around frequently, secreted saliva or honeydew, and eventually abandoned the

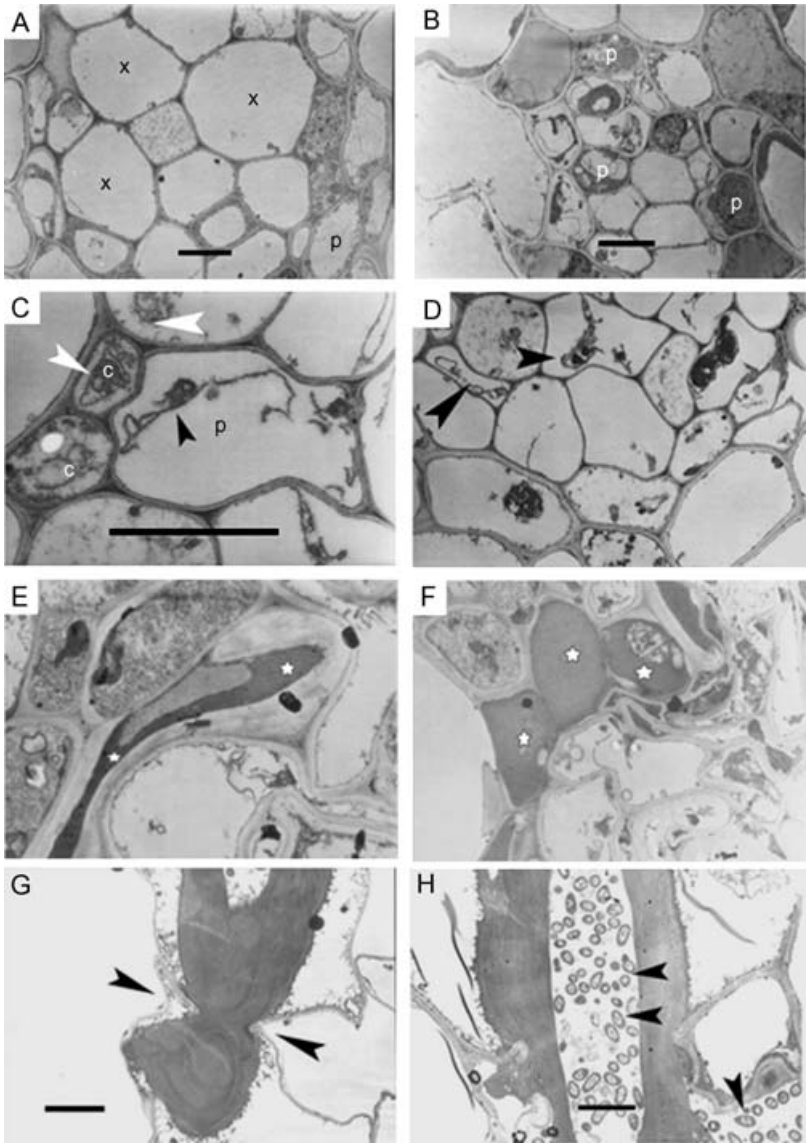

Figure 7 Ultrastructure of cells adjacent to the salivary sheath of brown planthopper (BPH), *Nilaparvata lugens*, in a leaf sheath of TN1. (A) Normal vascular cells (control) in a leaf sheath of rice, (B, C, D) phloem cells in leaf sheath of rice after probing and ingesting for 3, 5, and 8 days, respectively, (E, F) foreign materials filling rice cells, and (G, H) longitudinal salivary sheath sections in rice tissues. x, xylem vessel; p, phloem sieve element; and c, companion cell. Arrowheads in C and D indicate the deeply stained linear structures in phloem cells. Stars in E and F are the foreign materials filled in plant cells. Arrowheads in G indicate the position of the cell wall puncture by BPH stylets. Bacteria-like structures inside the sheath lumen are indicated by arrowheads in H. Bar = 1.5 μm.

plant. It is likely that resistant varieties elicit defensive mechanisms that inhibit sheath building via compounds, such as oxalic acid, phenols, and apigenin-C-glycosides (Yoshihara et al., 1979b, 1980; Stevenson et al., 1996; Zhao et al., 2004, 2005). Furthermore, some defensive pathways such as proteinase inhibitors induced by BPH could be involved in inhibiting sheath building (Yoshihara et al., 1980; Zhou et al., 2003; Zhang et al., 2004; Cho et al., 2005; Wang et al., 2005).

Probing is defined as all behaviors between stylet insertion and withdrawal (Backus, 2000). In the present experiment, the density of insects and the duration of infestation were probably not enough to allow more probes than one sheath per cross section. The present study confirmed that, what an insect on the plant surface does includes walking, stopping, labial dabbing, flange depositing, inserting, sensing, saliva secreting, sucking, branch building, and honeydew excreting (Sogawa, 1982; Backus, 1988). Some of these behaviors may be indistinguishable (i.e., the stained marks of flange deposition or honeydew excretion) (Habibi et al., 2008). However, the number of sheath branches is an appropriate approximation for probing duration and frequency. Furthermore, a combination of EPG recording and microscopy would be the best and most vigorous method for investigation of BPH probing behavior (Backus et al., 2005b). Most of the rice varieties studied should be treated synchronously, to compare probing frequency statistically.

Few studies have determined salivary sheath depth and peripheral distribution of *N. lugens* in rice plants. Our results revealed that the insect prefers to probe the two outer leaf sheaths, and the third and fourth leaf sheaths can occasionally be reached (Table 1). This is in contrast to previous results in which it was found that *N. lugens* usually do not feed on inner leaf sheaths and internodes (Watanabe & Kitagawa, 2000). To evaluate the effects on rice by BPH probing, leaf sheaths should be treated separately. *Nilaparvata lugens* prefers the thick leaf segments for probing and ingestion. Thick-walled cells of the vascular bundles in B5 did not appear to provide mechanical difficulties for stylet penetration, as far as this can be determined from observations on salivary sheaths. Therefore, the toughness of this tissue was not an effective defense in B5 rice. Modified photosynthesis and translocation of assimilates (source-sink relations) seem an important factor in rice to compensate for BPH injury (Rubia-Sanchez et al., 1999).

Based on our ultrastructural observations, probed rice phloem cells began to lose their cytoplasmic continuity. This is different from the effects of other species, such as potato leafhopper, *Empoasca fabae* (Harris), on alfalfa, *Medicago sativa* (Ecale & Backus, 1995). We suggest that in cells neighboring the stylet pathway programed cell-death-like changes may be induced by BPH probing and ingestion. Research on this hypothesis is underway.

Endosymbionts have been reported in both BPH and rice (Chen et al., 1981a,b, 2006; Cheng & Hou, 1996; Hongoh et al., 2000; Lü et al., 2004). However, this is the first visual evidence that suggests a relationship between rice, BPH, and bacteria-like structures in relation to insect probing. More molecular and histochemical studies are warranted, because we have not observed the same structures in other regions of the insect body or the rice plant.

Acknowledgements

The authors thank Dr Jie Zhao for providing the experimental convenience in the study, Mr Mingsheng Zu for help in transmission electron microscope observation, and Dr Mengxiang Sun for advice on statistics. We also thank Dr Elaine Backus and Prof Paul Sutherland for valuable comments and suggestions. Financial support was provided by The National Natural Science Foundation of China (30570140).

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