Herbivore-Induced Callose Deposition on the Sieve Plates of Rice: An Important Mechanism for Host Resistance\textsuperscript{1}[W][OA]

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The brown planthopper (\textit{Nilaparvata lugens} Stål; BPH) is a specialist herbivore on rice (\textit{Oryza sativa}) that ingests phloem sap from the plant through its stylet mouthparts. Electronic penetration graphs revealed that BPH insects spent more time wandering over plants carrying the resistance genes \textit{Bph14} and \textit{Bph15}, but less time ingesting phloem than they did on susceptible plants. They also showed that their feeding was frequently interrupted. Tests with \textsuperscript{14}C)sucrose showed that insects ingested much less phloem sap from the resistant than the susceptible plants. BPH feeding up-regulated callose synthase genes and induced callose deposition in the sieve tubes at the point where the stylet was inserted. The compact callose remained intact in the resistant plants, but genes encoding \beta-1,3-glucanases were activated, causing unplugging of the sieve tube occlusions in susceptible plants. Continuing ingestion led to a remarkable reduction in the susceptible plants’ sucrose content and induced callose deposition in the sieve tubes at the point where the stylet was inserted. The compact callose remained intact in the resistant plants, but genes encoding \beta-1,3-glucanases were activated, causing unplugging of the sieve tube occlusions in susceptible plants. Our results demonstrate that BPH feeding induces the deposition of callose on sieve plates in rice and that this is an important defense mechanism that prevents insects from ingesting phloem sap. In response, however, the BPH can unplug sieve tube occlusions by activating \beta-1,3-glucanase genes in rice plants.

Globally there is an enormous diversity of herbivorous insects that attack and damage plants. However, long-term coevolution of herbivorous insects and plants has led to the development of an array of constitutive and induced defenses that enable plants to protect themselves from herbivore attack (Rauscher, 2001; Becerra, 2007). Constitutive defense mechanisms include physical barriers (such as cuticles, thorns, trichomes, and cell walls) and preexisting metabolites that are harmful or even toxic to insects (Mithöfer et al., 2005). Induced plant defenses involve the activation of mechanisms that directly or indirectly deter herbivores (Walling, 2000). Some secondary metabolites (Baldwin et al., 2001; Benderoth et al., 2006) and induced proteins, such as lectins, chitinases, proteinase inhibitors, and enzymes (Zavala et al., 2004; Chen et al., 2005; Harfouche et al., 2006), often play direct defense roles by, for example, repelling insects, inhibiting their feeding, or damaging their digestive or neural systems. Indirect defenses include the release of volatiles that signal the location of insects on infested plants to parasitoids and predators (De Moraes et al., 2001; Schnee et al., 2006; Kempema et al., 2007). When attacked by insects, plants can also produce endogenous signal molecules, such as stress hormones, including jasmonic acid (JA), ethylene, abscisic acid (ABA), and salicylic acid (SA) that regulate signal transduction cascades in plant cells, leading to the activation and modulation of defense-related genes (Li et al., 2002; Schmelz et al., 2006).

Plants show varied responses to herbivores that are strongly correlated with the mode of herbivore feeding. Chewing insects and cell-content feeders, such as caterpillars and beetles, cause extensive tissue damage and activate wound-signaling pathways in which JA plays a central role (Kandoth et al., 2007). In contrast, attacks by phloem feeders, such as aphids, planthoppers, and whiteflies, elicit only weak wound responses, but induce the transcription of SA and pathogen-signaling pathways, which may mirror responses to virus vectors or insect-associated bacterial endosymbionts. Alternatively, this transcription signature may be due to similarities between intercellular fungal hyphae growth and stylet penetration, and hence may produce similar responses (Baldwin et al., 2001; Li et al., 2006). Limitations associated with JA-mediated defense responses may arise from antagonistic cross talk with SA and ethylene signaling or be due to...
stealthy feeding behavior, which minimizes the amount of tissue damage (Voelckel et al., 2004).

The brown planthopper (*Nilaparvata lugens* Stål; BPH) is an insect that feeds on the leaf sheath of rice (*Oryza sativa*) plants, ingesting nutrients specifically from the rice phloem using its piercing mouthparts (stylet), forming a stylet sheath during the feeding process. Feeding by numerous BPHs on a single plant generally results in the susceptible plants yellowing, browning, and drying. In the last decade, the BPH has frequently caused widespread destruction of rice crops and heavy losses of yields (Shi et al., 2003; Park et al., 2007).

The main methods used to control BPH pests are to apply chemical insecticides and/or develop and grow resistant varieties in an integrated pest management strategy. However, the cost of chemical control is often very high and the chemicals can destroy the natural balance of BPH predators that help to keep the BPH population in check. The misuse of chemical pesticides may also cause a resurgence of the insect. Therefore, the most economic and efficient method for controlling the BPH is to exploit the host resistance to attack (Renganayaki et al., 2002). To date, 19 BPH resistance genes in rice have been reported and several have been used in rice breeding programs (Yang et al., 2004; Jena et al., 2006; Zhang, 2007). Various molecular techniques, including suppression subtractive hybridization, northern blotting, and cDNA array analysis, have been used to study rice responses to BPH feeding (Zhang et al., 2004; Wang et al., 2005, 2008; Yuan et al., 2005; Park et al., 2007). BPH feeding is thought to result in a reorganization of the gene expression profile of rice and most of the strongly regulated genes are associated with metabolism, cell defense, cellular transport, cellular communication, or signal transduction and the biogenesis of cellular components. In contrast, the expression of genes related to the flavonoid pathway, aromatic metabolism, and the octadecanoid pathway are mostly unchanged or down-regulated. This indicates that BPH feeding induces plant responses associated with a JA-independent pathway and cross talk with responses related to abiotic stress, pathogen invasion, and phytohormone signaling pathways (Zhang et al., 2004; Wang et al., 2005, 2008; Yuan et al., 2005). Many studies have reported the effects of BPH feeding on physiological properties and metabolic changes in rice plants (Cagampang et al., 1974; Qiu et al., 2004). However, the mechanism of rice resistance to BPH attack still remains largely unknown.

The aim of this study was to further explore the interactions between the BPH insects and rice plants in an attempt to elucidate the mechanisms involved in rice resistance to the BPH. We used a susceptible rice plant variety (TN1) as a control and first studied the feeding behavior of the BPH on rice plants carrying the BPH resistance genes *Bph14* and *Bph15* using the electronic penetration graph (EPG) technique. We then examined the anatomical features of the punctured phloem cells, especially the induced callose, by observing and counting the number of sieve plates with callose deposition. Real-time PCR was performed to examine the expression of genes coding for callose synthases and degrading enzymes. To our knowledge, this is the first targeted callose analysis of rice resistance to BPH feeding. Our results suggest that the induced callose sealing in sieve tubes plays an important role in the inhibition of BPH feeding. However, the BPH can unplug the sieve tube occlusions by activating β-1,3-glucanase genes in rice plants.

**RESULTS**

**EPG Monitoring the Feeding Behavior of BPH**

BPHs ingest phloem sap from rice plants through their narrow piercing-sucking mouthparts, which are called stylets. During the feeding process, the stylet transiently punctures the epidermis, making the first probing, and then penetrates the plant cell walls; the insect subsequently salivates into the cells and ingests the phloem sap. In this study, the feeding activities of the BPH on different varieties of rice, with varying levels of resistance, were monitored electrically using a real-time EPG technique (Tjallingii, 2006). Five types of waveform were identified, representing different types of insect feeding behavior: nonprobing (type 1), pathway (type 2), phloem puncture (type 3), xylem ingestion (type 4), and phloem sap ingestion (type 5; Fig. 1; Supplemental Fig. S1).

Waveform type 1 appeared when the stylet was outside the plant tissue and the electronic circuit remained open. Type 1 behavior can be divided into two subtypes: (1) BPH insects resting on plants; and (2) BPH insects walking or searching for feeding sites (Supplemental Fig. S1A). In an 8-h recording period, the total duration of type 1 behavior on the resistant variety B5 (carrying *Bph14* and *Bph15* genes) was 168.2 min, much longer than that on the susceptible rice variety TN1 (19.7 min, carrying no BPH-resistance gene). Two moderately resistant varieties, RI35 (carrying *Bph14*) and YHY15 (carrying *Bph15*), showed similar results to B5, with total durations of type 1 behavior of 110.4 min and 120.5 min, respectively (Table I, total durations). Type 1 behavior occurred 35 times on a resistant B5 plant and only 14 times on a susceptible TN1 plant (Table I, no. of times). The mean duration for type 1 behavior per feeding event on the three resistant varieties (5.5–8.7 min) was significantly longer than on the susceptible TN1 variety (1.5 min; Table I, mean durations). These findings indicate that BPHs spent more time resting or moving about on resistant plants than on susceptible ones, probing more frequently on resistant plants; the data also show that feeding was often interrupted on resistant plants.

Waveform type 2 occurred when the BPH insects used their stylets to search for the target cells in plant tissues in a series of activities, including penetrating plant cells, salivating, tasting, and forming branches of
the styllet sheath (Supplemental Fig. S1A). The total duration of this waveform type on the resistant B5 plant was 114.6 min, significantly longer than that on the susceptible TN1 plant (33.3 min). This suggests that the insects spend more time searching for suitable target feeding cells in the resistant plant tissue (Table I, total durations). The frequency of this behavior over the 8-h recording period showed a general tendency to increase with higher levels of plant resistance (Table I, no. of times).

Waveform type 3 occurred when the styllet penetrated the vascular bundle of the rice plant (Supplemental Fig. S1B). Overall, the total duration of waveform type 3 was correlated with the plants’ level of resistance, but there was a significant difference between the two moderately resistant varieties (RI35 and YHY15) and the difference between TN1 and YHY15 was insignificant (Table I, total durations). Such differences in type 3 behavior of BPHs on these moderately resistant varieties might be attributable to the differences in resistance genes of the plants.

Waveform type 4 represented xylem ingestion by the BPHs (Supplemental Fig. S1C). We assumed that the xylem was not the resistant element within the plant because of the irregular duration of this behavior on the B5 (75.5 min), YHY15 (125.9 min), RI35 (123.9 min), and TN1 (58.2 min) varieties. There was no clear relationship between resistance level and total duration of this type of behavior (Table I, total durations).

Waveform type 5, representing phloem ingestion, gave a better indication of resistance because it reflected the relative quantity of phloem sap ingested by the BPHs (Supplemental Fig. S1D). During the 8-h recording period, the total duration of type 5 behavior on the resistant variety B5 was 33 min, approximately one-tenth of that on the susceptible variety TN1 (340.2 min; Table I, total durations). Moreover, the mean duration of each period of phloem sap ingestion was much

Table I. Feeding behaviors of BPH recorded by EPG

<table>
<thead>
<tr>
<th>Rice Variety</th>
<th>Waveform Type</th>
<th>Total duration (min) of each EPG waveform type on different host plants over an 8-h recording period</th>
<th>No. of times each EPG waveform type occurred on different host plants over an 8-h recording period</th>
<th>Mean duration (min) of each EPG waveform type on different host plants over an 8-h recording period</th>
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<td></td>
<td></td>
<td>Waveform Type</td>
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<tr>
<td></td>
<td></td>
<td>Nonprobing (Type 1)</td>
<td>Pathway (Type 2)</td>
<td>Ph³ Puncture (Type 3)</td>
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<tr>
<td>B5</td>
<td></td>
<td>114.6 ± 34.2 a</td>
<td>88.8 ± 16.7 a</td>
<td>75.5 ± 21.6 ab</td>
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<td>RI35</td>
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<td>97.1 ± 38.3 a</td>
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<tr>
<td>YHY15</td>
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<td>33.1 ± 13.9 b</td>
<td>125.9 ± 46.9 a</td>
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<td>TN1</td>
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<td>33.3 ± 7.2 b</td>
<td>28.6 ± 9.4 b</td>
<td>58.2 ± 23.3 b</td>
</tr>
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<td></td>
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<td>17.7 P &lt; 0.001</td>
<td>15.2 P &lt; 0.002</td>
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<tr>
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<td>114.6 ± 34.2 a</td>
<td>88.8 ± 16.7 a</td>
<td>75.5 ± 21.6 ab</td>
</tr>
<tr>
<td>B5</td>
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<td>38.4 ± 14.0 a</td>
<td>32.9 ± 7.8 a</td>
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<td>8.4 ± 2.2 a</td>
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<tr>
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<td>13.5 P &lt; 0.004</td>
<td>11.6 P &lt; 0.009</td>
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<td></td>
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<td>2.0 ± 0.7 a</td>
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<td>7.6 ± 3.2 ab</td>
</tr>
<tr>
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</tr>
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<td>10.3 ± 4.6 c</td>
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<tr>
<td>TN1</td>
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<td>17.6 P &lt; 0.001</td>
<td>11.3 P &lt; 0.01</td>
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³Ph, Phloem; Xy, xylem. ⁴Values (means ± so) followed by the same letter within a column are not significantly different; Scheffe’s post-hoc pairwise comparison (P < 0.05). ⁵Kruskal-Wallis one-way ANOVA by ranks. Rice variety B5 carries both BPH resistance genes Bph14 and Bph15; RI35 and YHY15 carry genes Bph14 and Bph15, respectively; TN1 has no BPH resistance gene.
shorter on the resistant varieties than on the susceptible control TN1 (Table I). These findings suggest that the BPHs spend less time ingesting phloem sap from resistant plants than from susceptible plants.

**Use of $^{14}$C Suc to Quantify Phloem Sap Ingestion by BPH**

Suc is the main carbohydrate that is transported long distances through the phloem and ingested by the BPH. By culturing rice plants in a $^{14}$C Suc solution, $^{14}$C can easily be introduced into the phloem. The quantity of phloem sap ingested by the BPHs can then be estimated by monitoring the radioactivity of $^{14}$C in the insects. We used the ratio of radioactivity ($^{14}$C) in the insect to that in the plant, designated the I:P index, as an indicator of the relative quantity of phloem sap ingested by BPHs. The results showed that the I:P ratio for BPHs that had fed on resistant B5 plants was very low (0.02, compared with 0.85 for insects feeding on susceptible TN1 plants) over a 20-h period (Fig. 2). The I:P ratios for BPHs that fed on the moderately resistant varieties YHY15 and RI35 were 0.54 and 0.28, respectively. These results strongly indicate that the BPH insects ingested less phloem sap from the resistant rice plants than they did from the susceptible control (TN1).

**Callose Deposition on the Sieve Plates of BPH-Infested Plants**

To investigate the mechanisms that prevent BPHs from continuously ingesting phloem sap from resistant rice plants, the leaf sheaths of BPH-infested and BPH-free resistant and susceptible plants were sectioned and examined histopathologically. The sections were stained with 0.1% aniline blue and examined under a fluorescence microscope. In the BPH-free untreated rice plants, there was little or no callose deposition on the sieve plates in the leaf sheaths (Fig. 3, E and F). When the plants were infested with the BPH, more callose was deposited on the sieve plates of the target sieve tubes, where the stylets had been inserted—the sieve plates were obviously thickened and emitted strong fluorescence (Fig. 3, A–D). Counts of the bright callose plugs revealed that callose deposition increased during the first 3 d of infestation in both B5 and TN1 plants, but there were more callosic sieve plates in the former (13.7 callosic sieve plates in 50 sections) than in the latter (5.8). Moreover, with prolonged BPH feeding, the callose deposition decreased quickly in TN1 plants to only 2.4 callosic sieve plates in 50 sections after 4 d, but a high level of callose deposition remained in the B5 plants (12.7; Fig. 3G). Further analysis revealed that there were strong fluorescence signals from callose in almost all the sieve tubes penetrated by stylet sheaths in the resistant B5 plants, but a large proportion of the target sieve tubes did not have bright callose depictions in the susceptible TN1 plants (Fig. 4, D and H).

**Expression of Callose Synthase and $\beta$-1,3-Glucanase in Rice Plants**

Callose deposition is a dynamic process coordinated through the activities of callose synthase and the callose-hydrolyzing enzyme $\beta$-1,3-glucanase. To investigate the mechanisms responsible for the differential callose deposition in the resistant and susceptible rice plants, the expression of 10 callose synthase-encoding genes were investigated using semiquantitative reverse transcription (RT)-PCR. We detected transcripts of four of these genes, namely, OsGSL1, OsGSL3, OsGSL5, and OsGSL7 (Supplemental Fig. S2). Three of the detected genes, OsGSL1, OsGSL3, and OSGS55, were further analyzed by real-time PCR. These genes were clearly up-regulated after the B5 and TN1 plants were treated with BPH for 6 h, reaching high levels after 12 h (2- to 4-fold of uninfested plants). The expression levels remained high in the following 72 to 96 h, generally over 3-fold higher than those in uninjured plants (Fig. 3, H–J). These observations suggest that the callose synthase genes were up-regulated and consequently callose synthesis was enhanced in both the resistant and susceptible plants attacked by the BPH.

The expression patterns of six $\beta$-1,3-glucanase genes were also investigated. The patterns of four of them were found to differ between BPH-infested B5 and TN1 plants. Osg1 was up-regulated by 1.2-fold within 3 h in susceptible TN1 plants and kept increasing from 6 to 48 h, with a decline after 72 h. The highest expression level occurred after treatment for 48 h, about 5-fold increased in comparison to the untreated control (Fig. 4J). Gns5 showed an increase both in TN1 and B5 plants, but the increase in TN1 was much higher than in B5 plants. The greatest increase in TN1 was >9-fold relative to the untreated control (Fig. 4J). Gns4 was constitutively expressed in B5, but was induced in TN1 plants. Gns6 shared similar expression patterns with Gns5. Little or no expression of either Gns2 or Gns3 was detected in the leaf sheath of the rice plants (Supplemental Fig. S2). The expression of $\beta$-1,3-glucanase
genes, such as OsG1 and Gns5, was clearly up-regulated in the susceptible rice plants; in contrast, the expression level of these genes was up-regulated much less (Gns5) or even absent at a detectable level (OsG1) in resistant ones.

Decomposition of Starch in Compensation for Sugar Losses in Susceptible Plants

To investigate the anatomical effects of BPH feeding on rice plants, leaf sheaths of plants representing the most resistant and most susceptible varieties (B5 and TN1, respectively) were sectioned, stained in 3% KI-1% I₂ solution, and examined under a microscope. We found abundant starch granules in the leaf sheaths of the uninfested plants and there appeared to be more in the B5 than in the TN1 plants (Fig. 5A, top two sections). In the BPH-infested plants, starch granules were rapidly consumed after 1 d in the susceptible TN1 variety and most were exhausted after 3 d of infestation (Fig. 5A, right). In contrast, the starch granules disappeared much more slowly in the resistant variety (B5; Fig. 5A, left).

The starch content of leaf sheaths was also determined. Results showed that the starch content decreased much more quickly in TN1 than in B5 under the stress of BPH infestation. However, it should be noted that there was much more starch in B5 (22.7 mg g⁻¹ fresh weight) than in TN1 (16.7 mg g⁻¹ fresh weight) plants that were not infested by BPHs (Fig. 5B, bottom). The Suc content varied in a similar way to that of the starch content (Fig. 5B, top). In the BPH-infested TN1 plants, Suc content fell to approximately 56% and 30% of the untreated control plant levels after 1 and 4 d, respectively (Fig. 5B), showing that susceptible TN1 plants can be rapidly and seriously deprived of carbohydrates during infestation by these insects.

The expression of nine α-amylase and three β-amylase genes in the leaf sheaths of rice plants was investigated using semiquantitative RT-PCR. We found that RAmy3D (Os08g0473900), one of the genes examined, was strongly induced in the BPH-infested TN1 plants, but was only weakly expressed in the B5 plants (Supplemental Fig. S2). This indicates that RAmy3D plays a role in the response of susceptible rice plants to BPH feeding by breaking down the starch.
DISCUSSION

Enhancing host resistance is an important component of integrated pest management. However, the mechanism of rice’s resistance to BPH is still uncertain. In the past, researchers considered that it might be governed by the presence of chemicals confined to the phloem (Sogawa and Pathak, 1970). Bph1 was the first BPH resistance gene identified and is associated with the production of flavonoids, including SA; amino acids, such as Asp, Glu, Ala, Ser, Leu, Asn, and Val; and organic acids, such as succinic acid, malic acid, oxalic acid, and transaconitic acid (Sogawa and Pathak, 1970; Sogawa, 1976). There is now considerable evidence to suggest that induced defenses are effective and have low fitness costs. Several studies have revealed that gene expression profiles in rice are reorganized in response to BPH feeding (Zhang et al., 2004; Yuan et al., 2005; Park et al., 2007; Wang et al., 2008). Such reorganization has been well documented in other plant-herbivore systems, supporting the hypothesis that inducible defenses contribute to rice resistance to the BPH. The data gathered in this experiment show that BPH feeding affects the expression of genes associated with the synthesis and hydrolysis of callose and starch decomposition in rice plants, resulting in the deposition of callose plugs on the sieve plates. This prevents BPH insects from continuously ingesting phloem sap and allows normal carbohydrate levels to be maintained in resistant plants, as described below.

Phloem, the target of BPH feeding, mainly consists of sieve tubes and companion cells. The functional units of sieve tubes are series of sieve elements that have porous sieve plates at their abutting ends, allowing the phloem sap to flow continuously (Will et al., 2007). The sieve element/companion cell modules are highly sensitive to biotic and abiotic disturbance, and elaborate sealing mechanisms, such as protein plugging and callose formation, have evolved (McNairn and Currier, 1967; Will and Bel, 2006). Callose has several functions in the normal development of plants (Chen et al., 2007), and its formation and deposition can be induced by either biotic or abiotic stress (Jacob et al., 2003; Ueki and Citovsky, 2005). We found more callose deposits on sieve plates in both the resistant and susceptible rice plants infested by BPH than in the uninfested controls (Fig. 3). However, in the resistant B5 plants, almost all the target sieve tubes showed strong fluorescence, indicating that abundant, compact callose had been deposited within them. In contrast, the callose signals were fainter in the susceptible TN1 plants, and there were no compact callose deposits in many of their sieve tubes where BPH insects had fed.

It has been reported that callose synthesis is Ca^{2+}-dependent (King and Zeevaart, 1974), and phloem-feeding insects seem to induce the release of Ca^{2+} stored in the reticulum or the apoplasm, thereby activating callose synthesis (Arsanto, 1986; Volk and Franceschi, 2000). It has also been demonstrated that large amounts of callose on sieve plates reduce the rate of phloem translocation and can even block it completely (McNairn and Currier, 1967). Our results strongly suggest that the BPHs had little chance to ingest phloem sap continuously from the B5 plants. EPG data confirmed that the BPHs spent more time searching for, and tasting, cells for feeding on resistant plants than on susceptible plants, and that their feeding was often interrupted on the former (Table I, waveform types 1 and 2). The total duration of phloem sap ingestion on resistant plants was approximately one-tenth that on susceptible plants (Table I, waveform type 5). The ^{14}C-labeling data also support the conclusions that phloem feeding was inhibited on B5 plants and that the BPHs sucked only small amounts of sap from them (Fig. 2). Therefore, we conclude that callose...
deposition plays an important role in preventing BPHs from ingesting phloem sap and thus contributes to the resistance of rice to these insects. Sieve tube occlusion, in response to biotic and abiotic stress, has been previously reported (Will and Bel, 2006). Cagampang et al. (1974) showed that the rate of upward sap transport in rice plants infested by BPHs was only 60% of the rate in their uninfested counterparts, and Nielson et al. (1990) found evidence that the acropetal movement of photo-synthates in alfalfa (Medicago sativa) was seriously disrupted by potato leafhopper (Empoasca fabae) feeding. In cotton (Gossypium hirsutum), basipetal phloem translocation was completely inhibited by callose deposition in a $^{14}$CO$_2$ labeling trial (McNairn and Currier, 1967). Furthermore, forisomes (spindle-like protein bodies in the sieve tubes) have been shown to inhibit aphid feeding in broad bean (Vicia faba; Will et al., 2007).

Because callose synthase genes were up-regulated and callose deposition occurred in both the resistant and susceptible rice plants a short time after BPH feeding commenced (Fig. 3), the insects had to overcome the physical barriers imposed by callose to obtain sufficient food even from susceptible varieties. In the aphid/broad bean system, aphid saliva can prevent sieve tube plugging by forisomes (Will et al., 2007), which have only been found in the Fabaceae. Our results, in contrast, indicate that BPH feeding induced the expression of genes encoding $\beta$-1,3-glucanases, causing decomposition of the callose barriers in the susceptible rice plants (Fig. 4, A–H). These enzymes play important roles in plant defense and development. $\beta$-Glucanase-encoding genes have been classified into four subfamilies, based on their structure and function. Two tandem gene clusters, Gns2–Gns3–Gns4 and Gns5–Gns6, have been identified in the defense-related subfamily A (Romero et al., 1998). In addition, the novel Osg1 gene of rice has also been assigned to this subfamily (Tomoya et al., 2002). Isozymes belonging to the Gns subfamily A differ widely in their requirements, needing 1,6-$\beta$-glucan branch linkages nearby in the polymer chain for activity. Polymers of $\beta$-1,3-glucan are found in both plants and fungi, but polymers of 1,3; 1,6-$\beta$-glucans are found only in fungi. Thus, GNS4 (which specifically hydrolyzes 1,3; 1,6-$\beta$-glucans) is likely to play an important role in antifungal defense rather than callose decomposition (Akiyama et al., 1997; Tomoya et al., 2002); thus, high levels of $\beta$-1,3-glucanase may not be directly correlated with reductions in callose levels. However, isozymes that mainly hydrolyze 1,3-$\beta$-glucans, such as OSG1 and GNS5, have been presumed to play important functions in the breakdown of callose, as well as in defending against pathogen attack (Akiyama et al., 1997). The Osg1 and Gns5 genes were clearly induced by BPH attack and are likely to play important roles in callose decomposition, which ultimately facilitates ingestion of phloem sap by BPHs from susceptible rice plants. Therefore, the absence of expression (or limited expression) of these genes allows the sieve tube occlusions to be maintained in the resistant plants, and this is probably the key reason for their resistance. This conclusion is further supported by the findings that the expression of Osg1 is up-regulated in Hejiang19, another variety that is susceptible to the
BPH, but cannot be detected in the moderately resistant varieties RI35 and YHY15, either in the control or BPH-treated plants (Supplemental Fig. S3).

Starch is a major end product of photosynthesis; it is produced in chloroplasts and is the main energy storage substance in cereal grains and leaf sheaths. In the chloroplast and amyloplast, starch metabolism is closely related to other metabolic processes in the cytosol, such as Suc metabolism, glycolysis, and glycogenogenesis, so starch content is indicative of the dynamic transformation of carbohydrates. In treated susceptible TN1 plants, a large amount of phloem sap was ingested by BPHs and plant Suc content rapidly declined. In contrast, photosynthesis was suppressed in the BPH-infested susceptible plants (Yuan et al., 2005). Consequently, these plants had to up-regulate genes such as RAMy3D to decompose the starch stored in leaf sheaths to meet their carbohydrate and energy requirements (Supplemental Fig. S2). This decomposition process may continue until the stored starch has been exhausted. In this aspect, starch is also important in resisting (or tolerating) the BPH, by providing sugars for the plants. Greater starch storage would allow the plant to tolerate BPH feeding for a longer period of time. In this study, we found there was more starch in the uninfested plants of resistant variety B5 than in the uninfested susceptible TN1 plants, indicating that B5 plants were capable of surviving feeding stress for a longer period than TN1. Under the stress imposed by BPH, the resistant B5 plants also faced the threat of losing phloem sap, but ingestion was inhibited by the synthesis of callose, which effectively blocked the target sieve tubes. In such cases, the plant only needs to provide carbohydrate for itself and, consequently, less Suc loss and less starch decomposition will occur. Therefore, the resistant plants can survive a long period of stress from BPH, even when the phloem transportation is affected to some extent; eventually, the BPH will die of starvation. Competition for sugar, as well as other nutrients, plays an important role in the interaction between the herbivore and the plant. However, mechanisms allowing plants to resist (or tolerate) herbivore attack may differ widely between phloem-sucking insects and chewing insects. For example, in response to foliar herbivores, the allocation of sugars to roots increased in the annual *Nicotiana attenuata* so that plants better tolerate herbivores (Schwachtje et al., 2006).

Our understanding of the resistance mechanism can be encapsulated in the following model of interactions between the BPH and the rice plant (Fig. 6). First, the BPH acts on the plant by penetrating its tissues, ejecting saliva into its cells, and sucking up phloem sap. In response to BPH feeding, the plant up-regulates expression of its callose synthase and β-1,3-glucanase genes. Consequently, callose deposition occludes the sieve tubes and prevents the BPH from ingesting the phloem sap. However, β-1,3-glucanases that decompose the deposited callose and thereby facilitate the BPH’s continued feeding from the phloem are strongly induced in susceptible plants, but much more weakly induced in resistant plants. Thus, differential expression of β-1,3-glucanases can account for between-plant differences in resistance levels.

**CONCLUSION**

We have demonstrated that feeding by the BPH can induce callose synthesis and deposition on the sieve plates of rice plants. Callose deposition affects phloem transportation and plays an important role in preventing the BPH from ingesting the phloem sap. Our results show not only that callose deposition is sufficient for resistant plants to defend themselves against the BPH, but also that some specific β-1,3-glucanases are active callose-decomposing enzymes, induced by BPH activity and responsible for the susceptibility of TN1 plants. The differential expression of these enzymes may result in different resistance levels in rice plants.

**Figure 6.** Model of the interaction between the BPH and rice plants. The BPH first acts on a plant by penetrating its tissues, ejecting saliva into its cells, and sucking up phloem sap. In response to feeding by the BPH, the plant up-regulates genes encoding callose synthases and β-1,3-glucanases. Consequently, callose deposition occludes the sieve tubes and prevents the BPH from ingesting the phloem sap. Then, specific β-1,3-glucanases decompose the deposited callose in susceptible plants (but little in resistant plants), allowing the BPH to resume feeding from the phloem. Thus, differential expression of β-1,3-glucanases accounts for the differences in their resistance levels. Arrows indicate promotion or positive modulation of the process; vertical bars indicate inhibition or negative modulation of the process.
heterous females (2 d after the final molt) were collected at 9 AM, and attached to
were able to categorize the waveform types (Supplemental Fig. S1).

in real time. By relating the feeding behavior of BPH insects under the
(diameter 0.1 mm) immersed in the food were linked to a Giga 4 model DC-
through the parafilm. The gold wire from each insect and a copper wire
dorsum by conductive silver glue were then allowed to probe the food
parafilm to serve as an artificial food source and mounted under a coverslip.
were carried out on rice plants at the three- to five-leaf stage.

For EPG recordings of BPH insects feeding on rice plants, adult brachyp-
link the EPG waveforms with the feeding behavior patterns of the BPH,
EPG Waveform Characterization and Quantification

To link the EPG waveforms with the feeding behavior patterns of the BPH, a
microscope was coupled to EPG equipment, as follows. Special plastic
slides, each with a 1-cm-diameter hole in the center, were prepared and
covered with stretched parafilm. Suc solution or tap water (each with a small
amount of active carbon powder to trace the water flow) was dropped onto the
2.3. The supernatants containing the extracted soluble fractions were
collected and concentrated to 500 µL, 100 µL of which was used to determine their
14C content, using a Beckman LS6500 liquid scintillation spectrometer (Beckman). The E/P index was used to evaluate the distribution of
14C between the insects and plants, reflecting the proportion of soluble 14C ingested by the insects from the phloem.

Histochemistry and Microscopy

Rice plants were each infested with 10 BPHs. Leaf sheaths were collected,
fixed in FAE (formaldehyde:acetic acid:70% ethanol, 5:5:90 [v/v/v]), dehy-
drated, embedded in paraffin, and cut into 10-µm-thick sections using a
microtome. The sections were mounted on microscope slides, dewaxed, and
rehydrated for staining at room temperature.

To highlight starch and saliva sheaths, the rehydrated sections were
stained in 3% (w/v) KI-1% I2 solution for 1 min, then examined under a light
microscope. For callose observations, 10-µm-thick sections were mounted on
glass slides (50 sections/slide). Callose staining was performed as described by
Dietrich et al. (1994) with some modifications. Rehydrated sections were
stained with 0.1% (w/v) aniline blue in 0.15 M K2HPO4 for 5 min and
examined under a UV epifluorescence microscope (Olympus BX51). Callose
on individual sieve plates was classified as either faint or bright. Faint types
included clearly visible plates with a thin, green-yellow appearance, whereas
bright was used to describe all thickly callosed sieve plates with bright blue
fluorescence (McNairn and Currier, 1967). The amount of callose deposition in
each plant examined was evaluated by counting the number of sieve plates that
had bright callose. At least 400 sections were examined for each treatment.

Determination of Suc and Starch Content

Fresh leaf sheaths (approximately 1 g) were powdered in liquid nitrogen,
homogenized in 4 mL of 80% (v/v) ethanol, heated in a water bath at 80°C for
40 min, and centrifuged at 4,000g for 10 min. The supernatant fraction was
collected and the solid fraction was washed with 80% ethanol and centrifuged;
this procedure was repeated twice. The supernatants collected from each
sample were combined and then active carbon was added and filtered for Suc
analysis, whereas the pellet was dried for starch determination.
Suc was measured using the anthrone-sulfuric acid method (Trevelyan and Harrison, 1952) with modifications. For colorimetric determination, 0.5 mL of the 80% ethanol extract was added to 1 mL of water and digested with 1 mL 10% (w/v) aqueous KOH in a water bath at 100°C for 3 min. The cooled reaction mixture was placed immediately in an ice-water bath, and then 4 mL (original, 5-, 25-, 125-diluted) were included. The slopes of Ct and Δ2

m

28 s. After the amplification steps, the melting curve was determined for each sample. The amplification program consisted of an initial denaturation step at 94°C for 5 min. This procedure was repeated at least three times.

Real-Time PCR Analysis

Genes for real-time PCR analysis were screened based on the results of semiquantitative RT-PCR: the genes that showed obvious variation were chosen for real-time PCR, whereas the genes that could not be detected or showed no obvious variation were not chosen for further study. The primers (Table 1D) for real-time PCR were redesigned using primer premier 5.0 software according to cDNA sequences obtained from NCBI GenBank (see above). Reactions were carried out on the ABI PRISM 7300 real-time PCR system (Applied Biosystems) using three-step cycling conditions of 95°C for 15 s, 55°C for 1 s, and 72°C for 1 s, and a final extension at 72°C for 5 min. This procedure was repeated at least three times.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Details of the EPG waveforms shown in Figure 1.

Supplemental Figure S2. Results of semiquantitative RT-PCR for callose synthases, β-1,3-glucanases, and amylases.

LITERATURE CITED


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Supplemental Figure S3. Results of RT-PCR analysis of Oog1.

Supplemental Table S1. Sequences of primers for semiquantitative RT-PCR.

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