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Role of hydroperoxide lyase in white-backed planthopper (*Sogatella furcifera* Horváth)-induced resistance to bacterial blight in rice, *Oryza sativa* L.

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SUMMARY

A pre-infestation of the white-backed planthopper (WBPH), *Sogatella furcifera* Horváth, conferred resistance to bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in rice (*Oryza sativa* L.) under both laboratory and field conditions. The infestation of another planthopper species, the brown planthopper (BPH) *Nilaparvata lugens* Stål, did not significantly reduce the incidence of bacterial blight symptoms. A large-scale screening using a rice DNA microarray and quantitative RT-PCR revealed that WBPH infestation caused the upregulation of more defence-related genes than did BPH infestation. *Hydroperoxide lyase 2* (*OsHPL2*), an enzyme for producing C_6 volatiles, was upregulated by WBPH infestation, but not by BPH infestation. One C_6 volatile, (*E*)-2-hexenal, accumulated in rice after WBPH infestation, but not after BPH infestation. A direct application of (*E*)-2-hexenal to a liquid culture of *Xoo* inhibited the growth of the bacterium. Furthermore, a vapour treatment of rice plants with (*E*)-2-hexenal induced resistance to bacterial blight. *OsHPL2*-overexpressing transgenic rice plants exhibited increased resistance to bacterial blight. Based on these data, we conclude that *OsHPL2* and its derived (*E*)-2-hexenal play some role in WBPH-induced resistance in rice.

Keywords: induced resistance, hydroperoxide lyase, (*E*)-2-hexenal, (2*E*,6*Z*)-nonadienal, C_6 volatiles.

INTRODUCTION

Interspecific interactions between organisms utilizing the same plant have been one of the important subjects studied in community ecology. Damage made by herbivorous insects induces chemical and physiological changes in plants (Green and Ryan, 1972; Tallamy and Raupp, 1991; Baldwin, 1994; Dicke, 1994; Schoonhoven *et al.*, 1998). In several systems, such physiological changes cause deleterious effects on subsequent attacks by other herbivores (Karban and Myers, 1989; Denno *et al.*, 1995). A negative effect between herbivore and fungus has also been observed. For example, in an interaction between a spider mite, *Tetranychus urticae*, and a vascular wilt fungus, *Verticillium dahliae*, physiological changes in cotton seedlings caused by prior exposure to spider mites reduced the probability of infection and severity of the symptoms caused by the fungus (Karban *et al.*, 1987). However, to our knowledge, information about the molecular mechanisms involved in such herbivore-induced pathogen resistance in plants is limited (Walling, 2000; Thompson and Goggin, 2006). Here, we report on such mechanisms in a system

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consisting of rice plants (*Oryza sativa* L.), the white-backed planthopper (WBPH) *Sogatella furcifera* Horváth and the pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*).

The WBPH is an economically important pest of rice throughout South-East and Far-East Asia, including Japan. WBPH feeds on phloem and causes serious damage, called hopperburn, to rice-plant cultivars, particularly in the tropics (Khan and Saxena, 1985). *Xoo* causes a vascular wilt disease, known as 'bacterial blight', that is one of the most serious rice-plant diseases in rice-growing countries.

Kanno and Fujita (2003) found that resistance to rice blast caused by *Magnaporthe grisea* in rice is induced by WBPH infestation, and that such resistance is systemic. It has also been demonstrated that there is no significant sex-specific difference in infestation by WBPH, and between mechanically wounded and untreated rice plants, on the incidence of rice blast symptoms (Kanno and Fujita, 2003; Kanno *et al*., 2005). These results indicate that feeding by WBPH specifically induces resistance to rice blast, and that mechanical wound signalling alone is not enough for the induction of resistance. This phenomenon was also observed under field conditions in Japan (Satoh *et al*., 2005). Moreover, Matsumura and Suzuki (2003) reported that infestation with WBPH induced resistance in rice to subsequent infestations of WBPH and the brown planthopper (BPH), *Nilaparvata lugens* Stål. These facts suggest that WBPH infestation can induce resistance against various pests in rice.

There is a dearth of research on herbivore-induced bacterial disease resistance. The working hypothesis of this study is that WBPH induces resistance to bacterial blight in rice. To test this hypothesis, we conducted a laboratory bioassay using WBPH and BPH to test whether another planthopper induced resistance to bacterial blight in rice. We also conducted a field test to see whether the resistance observed under laboratory conditions also applies under field conditions. Based on the laboratory and field data, we then investigated the molecular mechanisms involved in the resistance by monitoring the gene expression profiles. Finally, we show that *hydroperoxide lyase 2* (*OsHPL2*), an enzyme for producing C_6 volatiles, and its derived (*E*)-2-hexenal have an important role in WBPH-induced resistance in rice.

RESULTS

WBPH infestation confers induced resistance to bacterial blight in rice

Before performing the following experiments, we confirmed that hopperburn damage was not observed on rice plants infested with WBPH for 24 or 48 h. We first performed a WBPH-induced resistance test against *Xoo* using the experimental conditions reported by Kanno and Fujita (2003) and Kanno *et al.* (2005). After infestation with WBPH for 48 h, rice plants were inoculated with virulent *Xoo*. Two weeks after inoculation, the lengths of the blight lesions on the fourth and fifth leaf blades of the WBPH-infested plants were significantly shorter than those of the control plants (Figure 1a,b). Furthermore, there was no significant difference in mean lesion length between plants infested with male and female WBPH (Figure 2a), suggesting that gender was not responsible for the resistance. There was an inverse relationship between the number of WBPH-infested rice plants and length of the blight lesions (Figure 2b). Simple mechanical wounding of rice plants did not induce resistance (Figure 2c). When WBPH infestation was restricted to the leaf sheaths (Figure 2e), the lengths of the lesions on both the fourth and fifth leaves of the WBPH-infested plants were significantly shorter than those of the control plants. Taken together, these results were very similar to those of

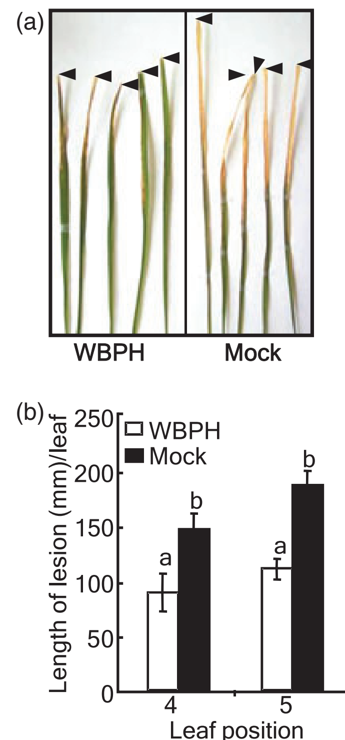

Figure 1. White-backed planthopper (WBPH)-induced resistance to bacterial blight in rice.
The fourth and fifth leaves of the plants were inoculated with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) after WBPH infestation for 48 h, and the length of lesions made by *Xoo* was measured 2 weeks after inoculation.
(a) Disease symptoms of bacterial blight exhibited by pre-infestation with or without (mock) WBPH. The fifth leaves were photographed 2 weeks after inoculation with *Xoo*. Arrowheads indicate the sites of clipping inoculation.
(b) The length of lesions on the fourth and fifth leaves 2 weeks after inoculation with *Xoo*. Values are means ± SEs. Means accompanied by different letters are significantly different at $P < 0.05$ (Student's *t*-test; $n = 10$ per treatment).

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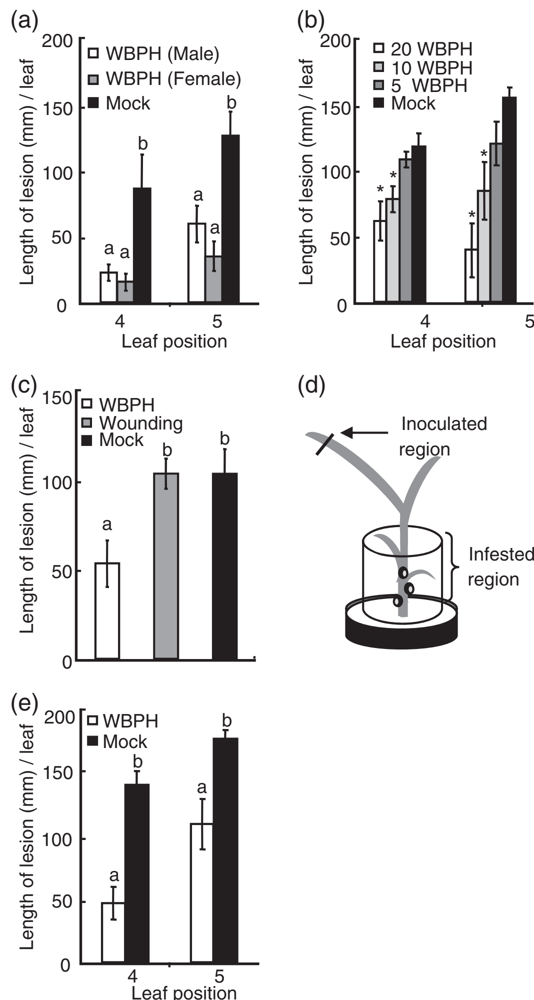

Figure 2. Effect of gender, number of white-backed planthopper (WBPH) individuals, whole-plant or restricted infestation of WBPH, and mechanical wounding on the development of bacterial blight.
The infestation time of WBPH was 48 h and the length of lesions made by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) was measured 2 weeks after the inoculation for all experiments. Values are means ± SEs.
(a) Effect of gender. The fourth and fifth leaves of rice plants were inoculated with *Xoo* after infestation with male or female WBPHs. Data were analysed with a Tukey–Kramer test (n = 12 per treatment).
(b) Effect of various numbers of WBPH individuals. Data were analysed by a Williams test (n = 10 per treatment).
(c) Effect of mechanical wounding. Leaves were punctured with 20 extra-fine insect pins that were left in place for 48 h, and the plants were placed in a cage without planthoppers. Data were analysed with a Tukey–Kramer test (n = 14 for WBPH, 13 for wounding and 11 for mock).
(d) Illustration of WBPH infestation restricted to the leaf sheath.
(e) Effect of infestation by WBPH restricted to the leaf sheath. Data were analysed with a Student's t-test (n = 20 per treatment). Means accompanied by different letters are significantly different at $P < 0.05$, and those accompanied by * are significantly different from the control at $P < 0.05$.

WBPH-induced resistance to rice blast, as confirmed by Kanno and Fujita (2003) and Kanno *et al.* (2005), indicating that WBPH infestation confers induced resistance to both rice blast and bacterial blight in rice.

WBPH release induces resistance to bacterial blight under field conditions

To extend the laboratory characterization of WBPH-induced resistance to bacterial blight, we evaluated the induced resistance under field conditions over the summers of 2004 and 2005. We used imidacloprid, a pesticide of planthoppers, to suppress WBPH. We confirmed that imidacloprid had no effect on the incidence of bacterial blight (Figure S1). The population density of WBPH was lower on plants treated with imidacloprid than on those that were untreated (Table S1). When the experimental plants were inoculated with *Xoo*, the lesions of rice plants treated with imidacloprid were significantly longer than those on plants not treated with imidacloprid ($P < 0.001$; Student's t-test; Table S2).

We also conducted WBPH release experiments in the field. When WBPH was released and allowed to feed on rice plants, the blight lesions on plants in the WBPH-released field were significantly shorter than those on plants in the unreleased field ($P < 0.05$; Student's t-test; Table S3).

Comparison of induced resistance to bacterial blight on rice infested with WBPH and BPH

We confirmed that the resistance to bacterial blight was induced by feeding behaviour rather than the oviposition behaviour of females (Figure 2a), and found that 20 planthoppers gives the greatest reduction in the length of lesions caused by *Xoo* (Figure 2b). We found that induced resistance also occurred when the WBPH-infestation time was reduced from 48 to 24 h (Figure 3), indicating that 24 h of WBPH infestation is sufficient to induce resistance to *Xoo*. Thus, in the following experiments, we used 20 adult male planthoppers and an infestation time of 24 h.

To determine whether induced resistance is a phenomenon specific to WBPH, we tested for possible induced resistance by infestation with another planthopper, BPH, which is also a phloem feeder of rice. Before performing the following experiments, we confirmed that hopperburn damage was not observed on rice plants infested with BPH for 24 or 48 h, as with WBPH infestation. Rubia-Sanchez *et al.* (2003) reported that WBPH and BPH infestation negatively affected the growth of rice, and that the reduction in plant height caused by WBPH feeding was greater than that of BPH when rice plants were continuously infested for 7 days with 32 or 64 planthoppers per plant. However, under our experimental conditions there was no difference in plant height between WBPH and BPH infestation at 2 or 7 days infestation with 20 planthoppers per plant (Figure S2), indicating that the effect of planthoppers on rice differs with study conditions.

When plants infested with WBPH or BPH for 24 h were inoculated with *Xoo*, the mean length of lesions was significantly shorter in the WBPH-infested plants than in

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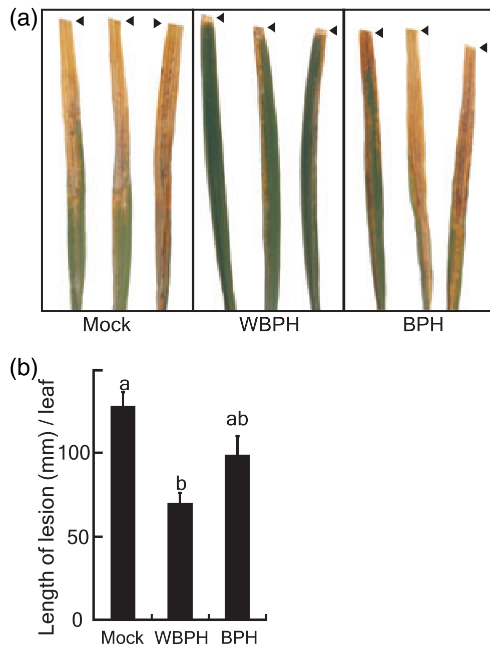

Figure 3. Comparison of induced resistance to bacterial blight by infestation with white-backed planthopper (WBPH) or brown planthopper (BPH).
Leaves were inoculated with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) after WBPH infestation for 24 h. Two weeks after the inoculation, leaf blades were photographed (a), and the length of lesions made by *Xoo* was measured (b). Arrowheads indicate inoculated sites of *Xoo*. Values are means ± SEs. Means accompanied by different letters are significantly different at $P < 0.05$ (Tukey–Kramer test; $n = 20$ for mock, 19 for WBPH and 18 for BPH).

the control plants, and there was no significant difference between the BPH-infested and control plants, or between the WBPH- and BPH-infested plants (Figure 3a,b).

Identification of WBPH-responsive genes in rice

The above results indicate that an uncharacterized WBPH-induced defence system(s) occurs in rice. Thus, we performed a DNA microarray using the Agilent rice 22K custom oligo DNA microarray to identify WBPH-responsive genes in rice. We extracted RNAs from the leaves of WBPH- and BPH-infested and control rice plants at 24 h post-infestation, and compared the gene expression patterns between WBPH- or BPH-infested and control plants. We performed three biological replicates for each treatment. Statistical analysis was performed using the ANOVA-false discovery rate (ANOVA-FDR, *q*-value < 0.05; Benjamini and Hochberg, 1995; Sharov *et al.*, 2005), and 4428 and 6356 spots were extracted for WBPH and BPH, respectively. We next extracted spots with changes in expression based on the criterion of a twofold increase or decrease in the average levels of fold change (Table S4). Based on this criterion, WBPH infestation upregulated the expression of 382 genes, and downregulated the expression of 167 genes, whereas BPH infestation upregulated the expression of 144 genes, and downregulated the expression of 76 genes. The expression of 33 genes was upregulated, and that of five genes was downregulated in both treatments. Among the upregulated genes, 349 were unique to WBPH and 111 were unique to BPH. Among the downregulated genes, 162 were unique to WBPH and 71 were unique to BPH (Figure S3). To determine whether WBPH-infested rice regulates the expression of a particular class of genes, we classified all WBPH-responsive genes (unique to WBPH) by their putative functions. As a result, about 13% of the upregulated WBPH-responsive genes were defence-related genes (Tables S5 and S6). However, none of the WBPH-downregulated genes were defence related (Tables S5 and S6). We then focused on WBPH-upregulated defence-related genes.

Tsuji *et al.* (2006) reported that characterized marker genes were overlooked when the threshold was set at $P < 0.05$ using Bonferroni's multiple-comparison correction in this microarray system. This could have been because the gene expression levels were too low to detect significant differences from the control. Thus, to prevent overlooking important genes, we extracted spots in which the changes in signal intensity were induced more than threefold by WBPH, but not by BPH, disregarding *q*-values, and classified these genes by their putative functions. From this extraction, we found four defence-related genes in which levels of induction were more than threefold, but where the *q*-values were more than 0.05 (Table 1). To validate that our selected defence-related genes were upregulated at higher levels by WBPH than by BPH, we performed quantitative reverse transcription-PCR (qRT-PCR) using same RNA samples that were used for the microarray. With the exception of a qRT-PCR unsuccessful gene [*AK109480* (15)], all 36 genes were reproducibly upregulated at higher levels by WBPH than by BPH (Table 1). We performed the following experiments using these confirmed WBPH-upregulated defence-related genes.

The WBPH-upregulated defence-related genes were further categorized into four groups: 'pathogenesis-related (PR) protein', 'oxylipin pathway-related', 'response to biotic stress' and 'response to abiotic stress' (Table 1). It has been shown that PR proteins are involved in a resistance response to fungal and bacterial pathogens in many plant species (van Loon *et al.*, 2006). Among PR proteins, *β-1,3-glucanase* [*AK104862* (1)] was confirmed as a WBPH-upregulated gene in our previous study (Kanno *et al.*, 2005). *PBZ1* [*AK071613* (2)], *POX22.3* [*AK073202* (10)], *POX8.1* [*AK101772* (12)], class-III *chitinase 1* (*OsChib1*) [*AK059767* (13)] and *PRb1* [*AK060057* (17)] are reported as upregulated genes following inoculation with avirulent *Xoo* or *M. grisea* (Midoh and Iwata, 1996; Chittoor *et al.*, 1997; Park *et al.*, 2004). Among the oxylipin pathway-related genes, *RCI-1* [*AK066737* (18)] is identified as a lipoxygenase induced by a chemical-resistant activator in rice (Schaffrath *et al.*, 2000).

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Table 1 A list of defence-related genes upregulated more than threefold by white-backed planthopper (WBPH) but not by brown planthopper (BPH) infestation, and induction folds of the genes from microarray or qRT-PCR analyses

		Microarray[a]		qRT-PCR[b]	
		WBPH		WBPH	BPH
Accession number	Gene name	Fold change	q-value	Fold change	
PR protein					
AK104862 (1)	*O. sativa* β-1,3-glucanase	36.06 ± 20.69	<0.001	108.92 ± 57.70	1.40 ± 0.16
AK071613 (2)	Rice probenazole-inducible protein PBZ1	19.50 ± 14.42	<0.001	75.57 ± 50.70	1.37 ± 0.43
AK108037 (3)	*Gossypium hirsutum* peroxidase	17.52 ± 10.09	<0.001	47.98 ± 20.52	2.81 ± 0.76
AK064050 (4)	*O. sativa* trypsin inhibitor	11.16 ± 2.96	<0.001	27.64 ± 4.80	3.91 ± 0.25
AK104472 (5)	*O. sativa* β-1,3-glucanase	7.92 ± 1.79	<0.001	14.77 ± 3.12	1.08 ± 0.29
AK059239 (6)	*O. sativa* IAI2	7.85 ± 0.87	<0.001	35.49 ± 10.51	4.16 ± 1.25
AK062495 (7)	*H. vulgare* subtilisin-chymotrypsin inhibitor	6.91 ± 7.06	<0.001	37.41 ± 21.97	3.22 ± 1.78
AK065090 (8)	*Zea mays* peroxidase	6.40 ± 1.82	<0.001	13.38 ± 1.51	1.06 ± 0.16
AK068247 (9)	*O. sativa* β-1,3-glucanase	5.87 ± 1.43	<0.001	7.47 ± 3.60	0.73 ± 0.03
AK073202 (10)	*O. sativa* peroxidase (POX22.3)	5.35 ± 1.12	<0.001	19.10 ± 4.75	1.24 ± 0.18
AK061042 (11)	*O. sativa* endochitinase	4.60 ± 3.06	0.005	13.05 ± 4.82	1.23 ± 0.08
AK101772 (12)	*O. sativa* peroxidase (POX8.1)	4.52 ± 2.33	0.002	22.40 ± 9.95	2.86 ± 0.14
AK059767 (13)	*O. sativa* class III chitinase (OsChib1)	4.31 ± 1.85	<0.001	7.81 ± 1.94	0.77 ± 0.02
AK069182 (14)	*O. sativa* thaumatin-like protein	4.18 ± 1.32	<0.001	2.10 ± 0.81	0.55 ± 0.09
AK109480 (15)	*Hordeum vulgare* peroxidase (Prx5)	3.61 ± 1.45	<0.001	n.a	n.a
AK065846 (16)	*O. sativa* proteinase inhibitor	3.57 ± 0.43	<0.001	36.86 ± 8.06	4.09 ± 0.15
AK060057 (17)	*O. sativa* pathogenesis-related protein 1 (PRb1)	3.16 ± 0.93	<0.001	6.62 ± 0.79	1.02 ± 0.09
Oxylipin pathway-related					
AK066737 (18)	*O. sativa* lipoxygenase (rci-1 gene)	82.87 ± 29.56	<0.001	363.47 ± 247.71	1.74 ± 0.21
AK069082 (19)	*O. sativa* Myb transcription factor JAMyb	11.02 ± 8.32	0.197	246.36 ± 21.92	3.12 ± 0.72
AK107161 (20)	*O. sativa* hydroperoxide lyase (OsHPL2)	3.87 ± 3.52	0.572	25.25 ± 14.98	1.43 ± 0.52
AK066825 (21)	*O. sativa* lipoxygenase (CM-LOX2)	3.48 ± 1.67	<0.001	9.51 ± 4.56	1.06 ± 0.14
AK068620 (22)	*Solanum tuberosum* allene oxide synthase	3.10 ± 0.82	<0.001	5.51 ± 1.29	0.98 ± 0.15
Response to biotic stress					
AK067801 (23)	*H. vulgare* phenylalanine ammonia-lyase	13.37 ± 14.05	<0.001	68.78 ± 15.60	1.52 ± 0.23
AK070415 (24)	*Atropa belladonna* salicylic acid carboxyl methyltransferase	11.21 ± 2.72	<0.001	723.36 ± 371.50	1.61 ± 0.57
AK100778 (25)	*O. sativa* PibH8 like protein	6.53 ± 0.49	<0.001	14.48 ± 5.18	1.77 ± 0.22
AK100592 (26)	*O. sativa* fatty acid alpha-oxidase	5.56 ± 0.92	<0.001	27.40 ± 5.43	1.87 ± 0.60
AK100234 (27)	*Zea mays* cinnamoyl-CoA reductase	4.54 ± 1.90	<0.001	19.02 ± 6.45	1.94 ± 0.33
AK109390 (28)	*Nicotiana tabacum* DNA binding protein	4.20 ± 2.19	0.265	5.98 ± 2.82	1.36 ± 0.32
AK106022 (29)	*Arabidopsis thaliana* putative disease resistance response protein (At4g23690)	3.10 ± 1.05	<0.001	22.74 ± 13.78	1.80 ± 1.39
Response to abiotic stress					
AK059839 (30)	*Triticum aestivum* zinc-finger protein	6.34 ± 1.62	<0.001	50.95 ± 25.74	5.84 ± 2.44
AK109382 (31)	*Nicotiana tabacum* alcohol dehydrogenase	5.12 ± 2.20	<0.001	9.25 ± 3.73	1.81 ± 0.15
AK073848 (32)	*O. sativa* OsNAC4	4.88 ± 1.97	<0.001	27.23 ± 7.82	1.84 ± 0.19
AK072460 (33)	*Arabidopsis thaliana* clone 36488	4.82 ± 1.47	<0.001	8.82 ± 2.07	0.79 ± 0.08
AK100389 (34)	Similar to *O. sativa* mitogen-activated	4.10 ± 1.64	0.167	8.96 ± 3.39	2.20 ± 0.25
AK060563 (35)	Protein kinase wjumk1	3.41 ± 1.52	0.002	3.64 ± 1.28	0.97 ± 0.11
AK111782 (36)	*Ananas comosus* epoxide hydrolase	3.38 ± 1.28	<0.001	14.10 ± 7.21	1.64 ± 0.17
AK061645 (37)	*H. vulgare* myb3 Similar to *O. sativa* mitogen-activated protein kinase wjumk1	3.20 ± 1.10	<0.001	3.98 ± 1.08	1.52 ± 0.21

n.a., qRT-PCR unsuccessful.

Fold inductions (relative to mock-treated plants) and false discovery rate (q-values) of defence-related genes on WBPH- or BPH-infested plants from two independent microarray[a] or qRT-PCR[b] analyses. Values are means ± SDs (microarray) or ± SEs (qRT-PCR) of three independent biological replications.

To ensure the reliability of the results obtained from the microarray analysis, we validated six genes {*RCI-1* [*AK066737* (18)], *anthocyanidin reductase* (*AK072654*) and four genes of unknown function (*AK0062493*, *AK064848*, *AK107273* and *AK108536*)} that are upregulated by WBPH infestation, but not by BPH infestation, using qRT-PCR. We used two independent RNA samples for each planthopper infestation that were not used for the microarray analysis. Results from this qRT-PCR experiment (Figure S4; Table S7) reproducibly concurred with the microarray results

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(Table S4). Moreover, we validated the upregulation of *AK072654*, one of the foregoing six genes, by qRT-PCR using more RNA samples. Ten independent biological replicates were conducted for each planthopper infestation, and a high upregulation of *AK072654* was confirmed in WBPH infestation but not in BPH infestation (Figure S5).

Systemic induction of WBPH-induced defence-related genes

We demonstrated that the incidence of bacterial blight was suppressed by infestation of WBPH restricted to the leaf sheath (Figure 2e). This result indicates that the physiological changes in rice plants might be systemic. To test for the possible systemic upregulation of genes, we performed a qRT-PCR analysis of the 36 defence-related genes using RNAs extracted from uninfested leaf blades of plants, where WBPH infestation had been restricted to the leaf sheaths. As a result, two genes encoding a trypsin inhibitor [AK064050 (4)] and hydroperoxide lyase 2 (OsHPL2) [AK107161 (20)], were significantly upregulated more than threefold in uninfested leaf blades compared with the control plants (Figure 4).

HPL-derived (*E*)-2-hexenal accumulation in WBPH-infested rice

Interestingly, *OsHPL2* [*AK107161* (20)] was upregulated in both whole-plant and restricted-plant infestations of WBPH (Figure 4; Table 1). HPL is an enzyme that catalyzes the cleavage of fatty acid hydroperoxides produced by lipoxygenase into aldehydes and oxoacids. HPLs are grouped into two subfamilies, 13-HPLs and 9-/13-HPLs, depending on their substrate specificities. Recently, it was reported that OsHPL2 had an activity as a 9-/13-HPL, and could produce C_6 and C_9 volatiles (Kuroda *et al.*, 2005; Chehab *et al.*, 2006). It appears that the C_6 volatiles have antibacterial (Croft *et al.*, 1993), antifungal (Hamilton-Kemp *et al.*, 1992; Gomi *et al.*, 2003) and insect repellant or attractant properties (Vancanneyt *et al.*, 2001; Shiojiri *et al.*, 2006). Thus, using a semi-quantitative analysis with gas chromatography/mass spectrometry (GC/MS), we measured the level of C_6 volatiles in the leaf blades after infestation with WBPH to confirm whether *OsHPL2* upregulation caused an accumulation of these volatiles. First, GC/MS revealed that only (*E*)-2-hexenal was detectable in leaf blades under all experimental conditions, and that other 9-/13-HPL-derived C_6 and C_9 volatiles, such as *n*-hexanal and (2*E*,6*Z*)-nonadienal, were undetectable (Figure S6). After whole-plant infestation with WBPH or BPH for 24 h, the level of (*E*)-2-hexenal in leaf blades was higher than the control in WBPH treatments, but not in BPH treatments (Figure 5a). After infestation restricted to leaf sheaths for 24 h, the level of (*E*)-2-hexenal in leaf blades did not differ between treatments (Figure 5b). However, the level of (*E*)-2-hexenal at 2 h after *Xoo* inoculation in leaf blades pre-infested with WBPH for 24 h on the leaf sheaths was significantly higher than that of the control plants that were inoculated with *Xoo* without WBPH pre-infestation (Figure 5c). This suggests that *OsHPL2* upregulation by WPBH correlates with (*E*)-2-hexenal release.

Effect of (*E*)-2-hexenal on *Xoo*

We analysed the antimicrobial activity of (*E*)-2-hexenal on *Xoo* because there is no information regarding the properties of this compound on rice pathogens. We performed the experiment according to the method described by Croft *et al.* (1993), who performed the antibacterial activity test against *Pseudomonas syringae* pv. *phaseolicola* with 5.8–173 μM (*E*)-2-hexenal. There was a significant negative effect on the growth of the bacterium at concentrations greater than 28.8 μM (*E*)-2-hexenal. Thus, we used concentrations of (*E*)-2-hexenal starting at 25 μM. When (*E*)-2-hexenal was added to liquid cultures of *Xoo* at concentrations of 25, 50 and 100 μM, there appeared to be a direct relationship between the concentration of (*E*)-2-hexenal and the retardation of bacterial growth (Figure S7). However, the concentrations of (*E*)-2-hexenal required to inhibit the growth of *Xoo* would be higher than that found in the plant (Figure 5a,c).

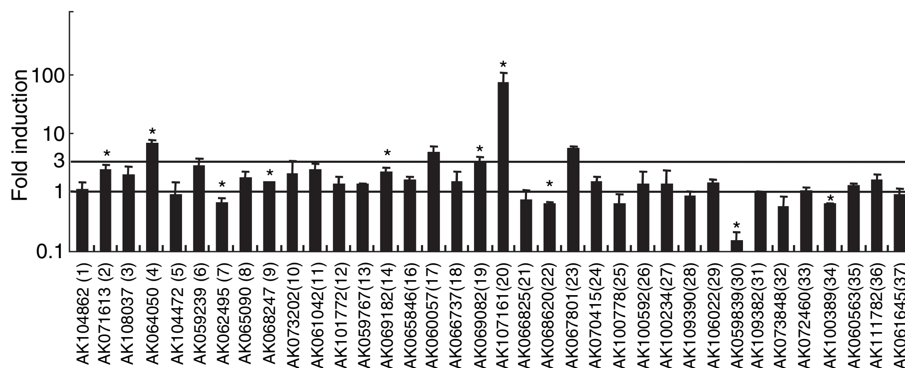

Figure 4. Expression levels of white-backed planthopper (WBPH)-upregulated defence-related genes in leaf blades (uninfested region) at 24 h after WBPH infestation restricted to the leaf sheaths.
Values are means ± SEs of three replicates. Four leaf blades were used per replicate. The numbers in parentheses are equivalent to those in Table 1. Data were analysed with a Student's *t*-test after Box–Cox transformation. *Means significantly different from the control at $P < 0.05$.

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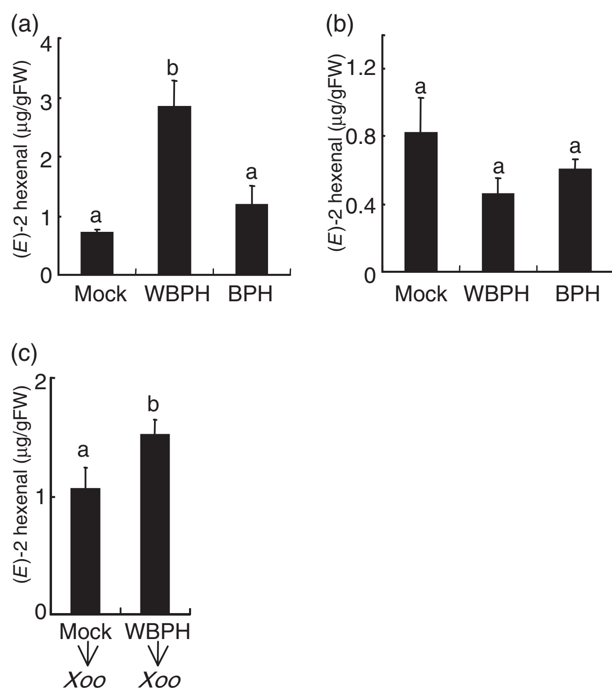

Figure 5. Accumulation of (*E*)-2-hexenal after infestation with white-backed planthopper (WBPH) in rice.
Levels of (*E*)-2-hexenal in leaf blades after infestation with WBPH or brown planthopper (BPH) for 24 h that occurred on the whole plant (a), or was restricted to the leaf sheath (b). Values are means ± SEs of three (Mock) or five (WBPH and BPH) replicates. Five leaf blades were used per replicate. Data were analysed with a Tukey–Kramer test.
(c) The levels of (*E*)-2-hexenal at 2 h after the inoculation with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in leaf blades that were pre-infested with or without WBPH on the leaf sheath for 24 h. Values are means ± SEs of three replicates. Three leaf blades were used per replicate. Data were analysed with a Student's *t*-test. Means accompanied by different letters are significantly different at $P < 0.05$.

Effect of (*E*)-2-hexenal vapour treatment on rice

We performed a resistance test of rice to *Xoo* after vapour treatment with (*E*)-2-hexenal for 24 h at concentrations of 1, 2, 5 and 10 μM. After vapour treatment, the chemical was removed and plants were inoculated with *Xoo*. Two weeks after inoculation, the length of lesions on each the plant was measured. The mean lesion length arising from *Xoo* infestation was significantly shorter with a treatment of 10 μM (*E*)-2-hexenal for 24 h than with the control treatment (Figure 6a).

We next performed qRT-PCR analysis of the defence-related genes after vapour treatment with 1 or 10 μM (*E*)-2-hexenal for 24 h to test whether these genes respond to (*E*)-2-hexenal. Three genes encoding protease inhibitors [AK064050 (4), AK059239 (6) and AK062495 (7)] were significantly upregulated by more than threefold after vapour treatment with 10 μM (*E*)-2-hexenal, compared with the control plants. With vapour treatment of 1 μM (*E*)-2-hexenal, only *thaumatin-like protein* [*AK069182* (14)] was significantly upregulated by more than threefold compared with the control plants (Figure 6b).

The above results indicate that the treatment of rice plants with 10 μM (*E*)-2-hexenal was needed to cause a clear induction of defence responses, and one-tenth of this concentration failed to induce such a response. However, this high concentration of (*E*)-2-hexenal could not be found in nature. To solve this problem, we measured the levels of trapped (*E*)-2-hexenal in (or on) leaf blades by semi-quantitative analysis with GC/MS after vapour treatment with (*E*)-2-hexenal. After vapour treatment with 10 μM (*E*)-2-hexenal for 24 h, the level of trapped (*E*)-2-hexenal was almost same as after WBPH treatment [3.3 and 2.8 $\mu g\ g^{-1}$ fresh weight (FW) for the vapour and WBPH treatments, respectively; Figures 5a and 6c). Conversely, after vapour treatment with 1 μM (*E*)-2-hexenal for 24 h, the level of (*E*)-2-hexenal in leaf blades was almost the same as that of the mock treatment (Figure 6c). These results indicate that vapour treatment with 10 μM (*E*)-2-hexenal was needed to accumulate the same level of (*E*)-2-hexenal as accumulated by WBPH infestation in the leaf blades.

Increased resistance of *OsHPL2*-overexpressing rice plants to bacterial blight

To strengthen our analysis of the biological function of OsHPL2, we generated *OsHPL2*-overexpressing rice plants. We confirmed the expression of the transgene by RT-PCR (Figure 7a). The second generation of two independent lines (lines 18 and 26) was used for further experiments. First, to confirm whether overexpressed *OsHPL2* genes work actively in these transgenic rice plants, we measured the level of (*E*)-2-hexenal by semi-quantitative analysis with GC/MS. Although there was no statistical difference between the untreated wild-type (WT) plants and untreated *OsHPL2*-overexpressing plants, the levels of (*E*)-2-hexenal tended to accumulate more in both *OsHPL2*-overexpressing lines than in the WT (Figure 7b). In addition, a peak of (2*E*,6*Z*)-nonadienal was detected in both *OsHPL2*-overexpressing lines, but not in WT (Figure 7c). It has been reported that this compound was produced by 9-HPL using 9-hydroperoxide of linolenic acid (9-HPOT) as a substrate, and OsHPL2 harnessed 9-HPL activity to produce this compound (Kuroda *et al*., 2005). From these results, we conclude that overexpressed *OsHPL2* genes in both transgenic rice plants were active. When these rice plants were inoculated with *Xoo*, the length of the blight lesions of the *OsHPL2*-overexpressing plants was significantly shorter than that of the WT (Figure 7d).

DISCUSSION

In this study, we demonstrated that WBPH infestation confers resistance to *Xoo* in rice plants under both laboratory and field conditions, whereas there was no statistical

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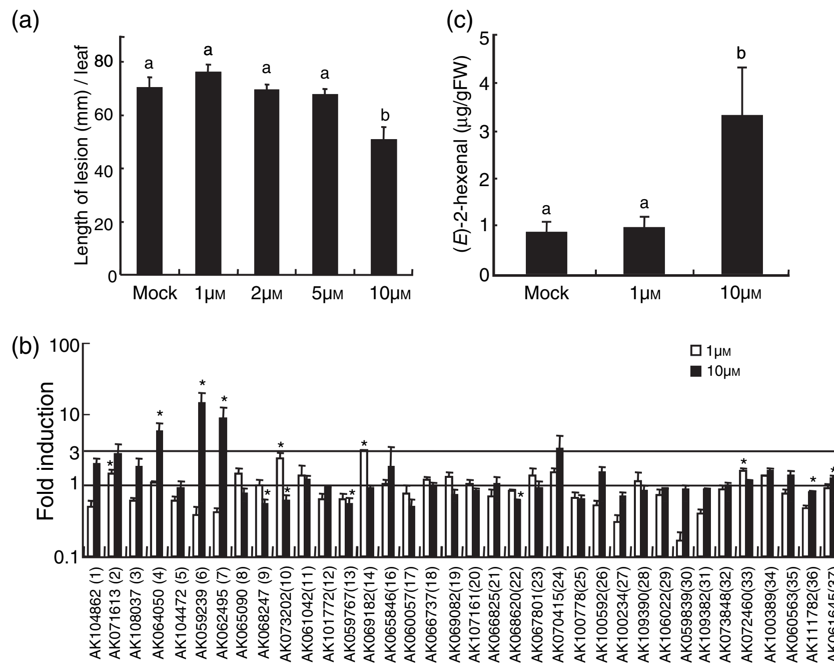

Figure 6. Effect of (*E*)-2-hexenal on rice.
(a) Effect of (*E*)-2-hexenal on disease resistance against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Rice plants were exposed to 0 (mock), 1, 2, 5 or 10 μM (*E*)-2-hexenal for 24 h at 25°C, and were then inoculated with *Xoo*. The length of lesions was measured 2 weeks after inoculation with *Xoo*. Values are the means ± SEs. Data were analysed with a Williams test [n = 19 for mock and 1 μM (*E*)-2-hexenal; 20 for 2, 5 and 10 μM (*E*)-2-hexenal].
(b) Expression levels of white-backed planthopper (WBPH)-upregulated defence-related genes after vapour treatment with 1 or 10 μM (*E*)-2-hexenal for 24 h. Values are means ± SEs of three (1 μM) or four (10 μM) replicates. Three leaf blades were used per replicate. The numbers in parentheses are equivalent to those in Table 1. Data were analysed with a Student's *t*-test after Box–Cox transformation.
(c) The levels of (*E*)-2-hexenal in leaf blades after vapour treatment with 1 and 10 μM (*E*)-2-hexenal. Rice plants were exposed in the sealed box with 1 or 10 μM (*E*)-2-hexenal for 24 h at 25°C. Values are means ± SEs. Data were analysed with a Williams test after Box–Cox transformation (n = 4 per treatment). Means accompanied by different letters are significantly different at $P < 0.05$; *means significantly different from the control at $P < 0.05$.

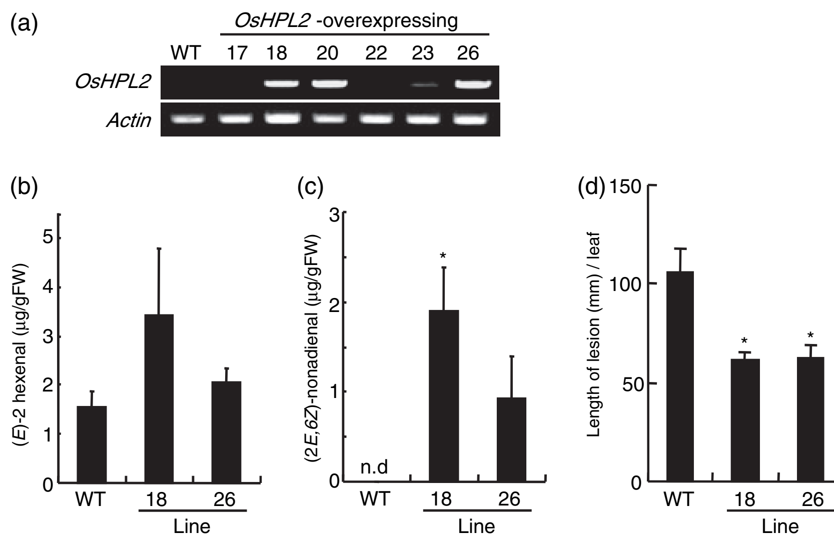

Figure 7. Increased tolerance of *OsHPL2*-overexpressing rice plants to bacterial blight.
(a) RT-PCR analysis of leaf blades from wild-type (WT) and *OsHPL2*-overexpressing rice plants for expression of *OsHPL2* and *actin*.
(b) The levels of (*E*)-2-hexenal in leaf blades of WT and *OsHPL2*-overexpressing rice plants (n = 4 for WT and line 18; n = 3 for line 26).
(c) Levels of (2*E*,6*Z*)-nonadienal in leaf blades of WT and *OsHPL2*-overexpressing rice plants (n = 4 for WT and line 18; n = 3 for line 26). n.d., not detected.
(d) The length of lesions of WT and *OsHPL2*-overexpressing rice plants (n = 6 for WT; n = 3 for line 18; n = 4 for line 26). The length of lesions was measured 2 weeks after inoculation with *Xoo*. For (b–d), values are means ± SEs. *Significant difference from WT at $P < 0.05$ (Dunnett's test).

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difference in mean lesion length between the BPH-infested and control plants. This result indicates that rice plants are affected differently by each planthopper species. It is known that WBPH and BPH feed in different locations on plants: BPH tends to feed near the plant base, whereas WBPH tends to feed on the upper region of the leaf (Kuno, 1968; Rubia-Sanchez *et al.*, 2003). This leads us to speculate on the possibility that the conferred resistance to *Xoo* by WBPH, but not BPH, was caused by a physical interaction between the planthopper and host as a result of the feeding location on the host. However, resistance to *Xoo* was also induced when WBPH infestation was restricted to the leaf sheaths (Figure 2e), indicating that the induced resistance depends on a biological interaction between the rice plant and WBPH.

We found that the upregulation of many defence-related genes in rice is caused by WBPH but not by BPH. Our results suggest that the WBPH-upregulated defence-related genes not upregulated by BPH infestation play an important role in WBPH-induced resistance to rice pathogens. The upregulation of PR genes is one of the best characterized plant defence responses against pathogen attack, and several PR proteins, such as β-1,3-glucanase and chitinase, have well-recognized antifungal activity as a result of their hydrolytic action (van Loon *et al.*, 2006). Thus, we hypothesize that WBPH-induced resistance is at least partly caused by the coordinated expression of PR genes. It has been reported that transgenic rice plants overexpressing a *trypsin inhibitor* exhibit strong resistance to *M. grisea* (Qu *et al.*, 2003). It has also been reported that populations of protease-defective mutants of *Xoo* are between 10- and 100-fold smaller than those for a wild-type strain in rice plants (Xu and Gonzales, 1987), indicating that proteases of *Xoo* are important in growth, and that protease inhibitor(s) of rice might prevent the growth of *Xoo*. Among the WBPH-upregulated *protease inhibitors*, *AK064050* (4) was upregulated by both the whole and restricted infestation of WBPH (Figure 4; Table 1). Furthermore, this gene was upregulated by vapour treatment with 10 μM (*E*)-2-hexenal (Figure 6b). Further studies are needed to determine the efficacy of the protease inhibitor(s) in the induced resistance to *Xoo.*

OsHPL2 [*AK107161* (20)] was upregulated by WBPH infestation (Figure 4; Table 1). Three functional *HPLs* (*OsHPL1*–*OsHPL3*) have been identified in the rice genome (Chehab *et al.*, 2006). Interestingly, *OsHPL2* is not upregulated by wounding (Chehab *et al.*, 2006), suggesting that the upregulation of *OsHPL2* might be a specific response to WBPH feeding behaviour. We further demonstrated that *OsHPL2*-overexpressing rice plants exhibited increased resistance to *Xoo* (Figure 7d), suggesting that OsHPL2 plays some role in WBPH-induced resistance to *Xoo* in rice. It is noteworthy that *HPL* was also upregulated by cucumber–*Trichoderma*–*Pseudomonas* interactions (Yedidia *et al.*, 2003). Thus, the upregulation of *HPL* might play an important role in such induced resistance in some plant species.

We recorded (*E*)-2-hexenal, which is one of the OsHPL2-derived metabolites (Kuroda *et al.*, 2005; Chehab *et al.*, 2006), as a WBPH-induced C_6 volatile (Figure 5), and demonstrated that (*E*)-2-hexenal had a negative effect on the growth of *Xoo* (Figure S7). We also revealed that vapour treatment with (*E*)-2-hexenal could make rice plants more resistant to *Xoo* with the upregulation of some defence-related genes (Figure 6a,b). This is consistent with findings that vapour treatment with (*E*)-2-hexenal upregulates the expression of some defence genes in *Arabidopsis thaliana* (Kishimoto *et al.*, 2005) and *Citrus jambhili* (Gomi *et al.*, 2003).

In *OsHPL2*-overexpressing rice plants, we detected a peak of (2*E*,6*Z*)-nonadienal (Figures S6 and 7c). We also revealed that (2*E*,6*Z*)-nonadienal had antibacterial activity against *Xoo* (Figure S8), suggesting that the increased resistance of *OsHPL2*-overexpressing rice plants to *Xoo* might be caused by the synergistic activity of (*E*)-2-hexenal and (2*E*,6*Z*)-nonadienal. However, in WBPH-infested rice under natural conditions, (2*E*,6*Z*)-nonadienal was not detected (Figure S6), whereas *OsHPL2* was upregulated. Chehab *et al.* (2006) suggested the existence of additional mechanism(s) for regulating the levels of OsHPL2-derived metabolites at the post-transcriptional level. The regulation of the levels of OsHPL2-derived metabolites in WBPH-infested rice under natural conditions might be controlled by such mechanism(s).

Factors of WBPH origin that trigger specific resistance in rice remain unknown. Signal compound(s) triggering induced resistance would be products specifically associated with WBPH but not BPH infestation, as the gene response to WBPH in rice was different to that of BPH. One possibility is an interspecific difference in the salivary composition secreted into rice tissues, as it has been reported that salivary composition is highly variable among species (Miles, 1972). Thus, the different compositions of saliva between WBPH and other species might trigger a different response in rice. We are now characterizing the WBPH-specific component(s) in saliva.

EXPERIMENTAL PROCEDURES

Plant and planthopper materials

Rice plants (*O. sativa* L. cv. Hinohikari and Nipponbare) were grown from seed under glasshouse conditions [25 ± 1°C, 60–80% relative humidity (RH)]. The susceptible *O. sativa* ssp. *japonica* (cv. Reiho) was used for planthopper rearing. The rearing cages were put in a controlled-environment chamber (25°C, 50–70% RH) until adults emerged 20 days after oviposition. These newly emerged adults were used for all experiments. Details for experimental conditions on planthopper infestation are provided in the Results and in Appendix S1. Photographs of WBPH and BPH are shown in Figure S9. Details of the behaviour of WBPH and BPH are given in Appendix S1.

Planthopper infestation and mechanical wounding

Rice plants (cv. Hinohikari) at the five-leaf stage were covered with transparent plastic cylinders (70 × 15 cm) and infested with adult

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planthoppers. Control plants were put into transparent plastic cylinders without planthoppers. After 24 or 48 h, all plants were removed from the cylinders, and all planthoppers were removed from the plants. These rice plants were inoculated with virulent *X. oryzae* pv. *oryzae* (*Xoo*), as described below. For WBPH infestation restricted to the leaf sheaths, rice plants were encased in transparent plastic cylinders (5 × 15 cm), covering only the leaf sheath region, and 20 adult WBPH males per plant were released into each cylinder (Figure 2d). Control plants were put into transparent plastic cylinders without planthoppers. For mechanical wounding, leaves were punctured with 20 extra-fine insect pins (16.5 mm in length, 0.16 mm in diameter; Shiga Konchu Fukyu, http://www.shigakon.com) that were left in place during the treatment, and the plants were placed into a cage without planthoppers.

Bacterial inoculation

The bacterial strain was cultured on a nutrient agar (Becton, Dickinson & Co., http://www.bd.com) slant containing 0.5% sucrose at 25°C for 48 h. *Xoo* strain T7174 (race I) was used in this study. This strain is virulent to Hinohikari and Nipponbare. A bacterial suspension was prepared by washing bacterial cells from the cultured slant, and adjusted to a concentration of approximately 1×10^6 colony-forming units (cfu) ml^{-1} in sterilized distilled water. The fully-opened fourth and fifth leaf blades of the rice plants were inoculated by the clipping inoculation technique (Kauffman *et al.*, 1973). The length of blight lesions was measured for each leaf at 2 weeks after inoculation.

Oligo DNA microarray analysis

A rice 22K custom oligo DNA microarray kit (Agilent Technologies, http://www.agilent.com) was used for the microarray analysis, which contains 21 938 oligonucleotides, based on the sequence data of the rice full-length cDNA project (Kikuchi *et al.*, 2003). Total RNA was extracted from leaf blades that had been infested with WBPH or BPH for 24 h, and from mock-treated leaf blades. For each replicate, material from three plants was pooled to provide a single sample for RNA extraction ($n = 3$ replicates per treatment). All microarray procedures and data analyses were performed according to the manufacturer's instructions. Details for procedures of the microarray are provided in Appendix S1. To identify genes up- or downregulated by WBPH infestation, data were statistically analysed using the National Institutes of Aging array analysis tool (http://lgsun.grc.nia.nih.gov/ANOVA; Sharov *et al.*, 2005). We used the most conservative error model, 'Maximum of intensity-specific average error variance and actual error variance', to reduce false positives. Analysis was performed using the ANOVA-false discovery rate (ANOVA-FDR) *q*-value < 0.05. We extracted spots with changes in expression based on the criterion of a twofold increase or decrease at average levels of fold change. Fold changes in expression level in each treatment were compared with those of the respective mock-treated controls. Finally, we removed all spots with BPH-regulated changes in expression based on the criterion of a twofold increase or decrease at average levels of fold change, and obtained 466 spots as WBPH-responsive genes. The microarray data files are deposited in the Gene Expression Omnibus Database (accession no. GSE8811). Details for sequence homology analysis and functional classification of genes are provided in Appendix S1.

Quantitative RT-PCR

Quantitative RT-PCR was performed using iQ SYBR Green Supermix (BioRad, http://www.bio-rad.com) in an iCycler (BioRad), according to the manufacturer's instructions. Analysis of the data obtained was performed according to the method described by De Vos *et al.* (2005), with minor modifications. We performed at least three replicates for each treatment. The RNAs used for qRT-PCR were the same as those used for the microarray analysis, because we wanted to know whether the genes with *q*-values greater than 0.05 in the microarray analysis were upregulated on the same RNAs as by qRT-PCR. We also conducted qRT-PCR to experimentally validate the upregulation of some genes using plant materials distinct from the ones used for the microarray experiments. We used two independent RNA samples for each planthopper infestation to validate six genes (*AK066737*, *AK072654*, *AK0062493*, *AK064848*, *AK107273* and *AK108536*). Moreover, we validated the upregulation of *AK072654* using 10 independent RNA samples for each planthopper infestation. The transcript level of each gene was normalized by comparison with *actin* (*AK060893*). Normalized transcript levels of the analysed genes in each treatment were compared with those of the respective mock-treated controls, and the fold change in expression level was calculated. Sequences of gene-specific primers used for qRT-PCR are shown in Table S8.

Analysis of volatiles in rice

Volatiles in the leaf blades of rice were measured according to the method of Chehab *et al.* (2006), with some modifications. Leaf blades were ground to a powder in liquid nitrogen with a pestle. The powder was introduced into a 50-ml glass vial containing 10 ml of 1% NaCl, and then weighed immediately. The headspace of the vial was collected in a water bath at 50°C for 1 h with a Twister [polydimethylsiloxane (PDMS)-coated stir bar, film thickness 0.5 mm, 10 mm in length; Gerstel GmbH & Co. KG, http://www.gerstel.com]. We collected between three and five samples for each odour source, and analysed them individually by GC/MS with an HP-5MS capillary column (Agilent Technologies) equipped with a thermo-desorption system, a cooled injection system and a cold trap system (Gerstel GmbH & Co. KG). The oven temperature of the GC was programmed to rise from 40°C (9-min hold) to 280°C at 10°C min^{-1}. The compounds were identified by comparing the GC retention times and mass spectra with those of authentic compounds.

Vapour treatment of rice with (*E*)-2-hexenal

To test for resistance to *Xoo*, a vapour treatment with (*E*)-2-hexenal was performed according to the method of Gomi *et al.* (2003), with some modifications. Four-leaf-stage rice plants were used. (*E*)-2-Hexenal solutions were prepared by dilution with cold ethanol, and 230 μl of the diluted compound was added to a cotton swab hanging from the lid of a 23-L sealed box in which the plants had been placed. The box was incubated for 24 h at 25°C. For the control, the same quantity of ethanol was added.

Construction of *OsHPL2*-overexpressing vector and rice transformation

The open reading frame of *OsHPL2* was subcloned into the binary vector pMLH7133 containing a partial *β-glucuronidase* (*GUS*) sequence fragment (200 bp) at the 3′ end of the cloning site. This vector has a 35S promoter containing seven enhancer sequences upstream of the 35S promoter (Mitsuhara *et al.*, 1996). This vector was introduced into *Agrobacterium tumefaciens* EHA101 by electroporation (Wen-jun and Forde, 1989). Rice (cv. Nipponbare) transformation was performed as described by Hiei *et al.* (1994). Transgenic plants were selected on the medium containing 50 mg L^{-1} hygromycin. Second generation plants that had not been treated with planthoppers or *Xoo* were used for the experiments. Details for RT-PCR analysis, to check for the expression of the transgene, are provided in Appendix S1.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effect of imidacloprid application on the development of bacterial blight.

Figure S2. Effect of planthopper feeding on plant growth.

Figure S3. Venn diagrams showing the numbers of overlapping and unique genes that were upregulated or downregulated by infestation with white-backed planthopper (WBPH) or brown planthopper (BPH).

Figure S4. Validation of the results of microarray analysis by qRT-PCR.

Figure S5. Confirmation of the upregulation of *AK072654* using a number of RNA samples.

Figure S6. GC profiles showing the levels of (*E*)-2-hexenal and (2*E*,6*Z*)-nonadienal in the leaf blades of rice plants.

Figure S7. Dose-dependent effect of (*E*)-2-hexenal on *Xoo* growth in liquid culture.

Figure S8. Dose-dependent effect of (2*E*,6*Z*)-nonadienal on *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) growth in liquid culture.

Figure S9. Photographs of white-backed planthopper (WBPH) and brown planthopper (BPH).

Table S1. Effect of imidacloprid on the population density of white-backed planthopper (WBPH).

Table S2. Incidence of bacterial blight in paddy fields where the occurrence of white-backed planthopper (WBPH) was regulated by imidacloprid.

Table S3. Incidence of bacterial blight in paddy fields where white-backed planthopper (WBPH) was released.

Table S4. Data set for all white-backed planthopper (WBPH)- or brown planthopper (BPH)-responsive genes with *q*-values < 0.05.

Table S5. Classification of white-backed planthopper (WBPH)-responsive genes.

Table S6. List of white-backed planthopper (WBPH)-responsive genes.

Table S7. Reference table for Figure S4; excerpt from Table S4.

Table S8. Sequences of gene-specific primers used for qRT-PCR.

Appendix S1. Detailed information of planthoppers and experimental procedures that are not presented in the printed version of this article.

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Data deposition: the data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, http://www.ncbi.nlm.nih.gov/geo/ (accession no. GSE8811).

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