Resistance of Rice to Insect Pests Mediated by Suppression of Serotonin Biosynthesis

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Abstract

Rice is one of the world's most important foods, but its production suffers from insect pests, causing losses of billions of dollars, and extensive use of environmentally-damaging pesticides for their control[1,2]. Breeding resistant cultivars is a major priority. However, the molecular mechanisms of insect-resistance remain elusive; although a few resistance genes for planthopper have been cloned, no rice germplasm is resistant to stem borers. We report that biosynthesis of serotonin, a neurotransmitter in mammals[3], is induced by insect infestation in rice, and its suppression confers resistance to planthoppers and stem borers, the two most destructive pests of rice[2]. Serotonin and salicylic acid (SA) derive from chorismate[4]. In rice, the cytochrome P450 gene *CYP71A1* encodes tryptamine 5-hydroxylase, which catalyzes conversion of tryptamine to serotonin[5]. In susceptible wild type rice, planthopper feeding induces biosynthesis of serotonin and SA, whereas in mutants with an inactivated *CYP71A1* gene, no serotonin is produced, SA levels are higher and plants are more insect-resistant. Addition of serotonin to the resistant rice mutant and other BPH-resistant genotypes results in a loss of insect resistance. Similarly, serotonin supplementation in artificial diet enhances performance of both insects. Furthermore, SA depresses *CYP71A1* expression and thus serotonin production, and serotonin represses expression of SA biosynthesis genes and thus SA synthesis, suggesting a mutual negative feedback mechanism regulating differential accumulation of these two hormones. These insights demonstrate that regulation of serotonin biosynthesis plays an important role in defence, and may prove valuable for breeding insect-resistant cultivars of rice and other cereal crops.

Rice brown planthopper (BPH; *Nilaparvata lugens* Stål) and striped stem borer (SSB, *Chilo suppressalis*) are the two most serious pests in rice production. BPH not only causes direct damage to the plant, through abstraction of nutrients and blocking of sieve-elements, but also indirect damage by the transmission of plant viruses. Under heavy infestation levels BPH causes complete desiccation of the crop known as "hopperburn", resulting in serious economic loss[6]. SSB, which is a chewing insect,

feeds on newly formed tillers and stems, causing “dead hearts” and “white heads”, resulting in significant yield losses[1, 2]. Both BPH and SSB are difficult to control using chemical pesticides, indiscriminate use of which has resulted in these two pests becoming primary pests in rice[2]. The development of insect-resistant rice varieties is seen as a viable and ecologically sustainable approach for controlling these devastating insect pests[2]. While more than 20 genetic loci that confer BPH resistance have been identified and a few of genes cloned, e.g. *Bph14*[7], *Bph3*[8], and *Bph9/1*[9], the mechanism of action is known only for *Bph14*[7]. In contrast, no SSB resistance genes have been identified in rice. For both pest species there remains an urgent need to identify new resistance genes and elucidate the underling mechanism(s) for developing efficient approaches to breed insect-resistant rice cultivars.

Molecular responses of plants to herbivores are strongly correlated with the mode of feeding and the degree of tissue damage at the feeding site. In sucking insects (such as BPH), which produce little tissue damage, the salicylic acid (SA) signaling pathway plays a major role in response to BPH infestation[10]. SA is derived from a common precursor, chorismate, as is serotonin (5-hydroxytryptamine or 5HT) (Supplementary Fig. 1). Serotonin is ubiquitous across all forms of life and in mammals it is well known as a neurotransmitter[3] and in insects it is thought to be involved in behavior and immunity[11,12]. Serotonin also is involved in plant growth, development, and response to biotic and abiotic stresses, but the mechanisms of its various functions remain largely elusive[13].

In rice, the gene *CYP71A1* encodes a cytochrome P450 monooxygenase, which exhibits tryptamine 5-hydroxylase enzyme activity, catalyzing the conversion of tryptamine to serotonin[5] (Supplementary Fig. 1). In *CYP71A1* knockout mutants, prevention of serotonin synthesis increases resistance to rice blast *Magnaporthe grisea*[14] but increases susceptibility to rice brown spot disease *Bipolaris oryzae*[15]. Furthermore, SSB could induce serotonin synthesis in rice plants[16], suggesting a potential role of serotonin in the regulation of insect resistance.

Host-searching behavior is an important process by which insects seek resources to acquire food, oviposit and establish nesting sites. We observed in host-choice studies that the *CYP71A1* mutant rice line Jiazhe LM was not visibly damaged whereas BPH infestation caused complete destruction of the parental wild type rice (WT; Jiazhe B) (Fig. 1a). BPH showed a clear preference for the WT, with the greatest differences in

the rate of settling being 1.55-fold greater 12 h post infestation (Fig. 1b), although significant differences already occurred 8 h post infestation ($p = 0.0022$). This avoidance resulted in a significant decrease in the number of eggs on the mutant line (Fig. 1c), although, there was no difference in subsequent viability (Supplementary Fig 2a). Honeydew production is a good indicator of feeding and hence host suitability, thus the approx. 30% reduction in honeydew produced per adult (Supplementary Fig. 2b) again supports the finding that this mutant is not a suitable host for BPH. Not only was a deterrent effect observed on the Jiazhe LM mutant, but when plants were infested with neonates in no-choice assays, there was a small, but significant reduction in survival (Fig. 1d). Not surprisingly this mutant was also resistant to the closely related white-backed planthopper (WBPH; *Sogatella furcifera*), another major pest of rice, in host-choice assays, resulting in lower oviposition (Supplementary Fig. 2c-d).

The results with the resistant Jiazhe LM mutant rice line suggest that regulation of serotonin biosynthesis plays an important role in plant defence against insect herbivores, hitherto not previously described. Rice with resistance to BPH has previously been described and enables us to test our hypothesis that the observed resistance in the Jiazhe LM mutant is due to the absence of serotonin. Firstly, we show that serotonin accumulation is induced in the susceptible WT in response to BPH feeding, being 5.4-fold and 1.5-fold greater than the background level in the sheath and leaf, respectively (Fig. 1f). In contrast, it is absent in the resistant mutant (Supplementary Fig. 3a-e). The finding that accumulation is greatest in the sheath is consistent with the feeding and oviposition behavior of this insect (Fig. 1f). Secondly, we demonstrate that whilst expression of *CYP71A1* is significantly induced in the two susceptible genotypes (Jiazhe B and TN1) in response to BPH, there was little corresponding induction of expression of this gene in four other BPH-resistant rice genotypes (Fig. 1e, 1g). Furthermore, the basal levels of *CYP71A1* expression in these two susceptible genotypes was 2.7 to 14.6-fold greater compared to the resistant genotypes (Fig. 1e, 1g). Consequently, BPH-resistant rice genotypes not only had lower basal levels of serotonin than these two susceptible genotypes, but also showed no significant increase after BPH challenge, in marked contrast to the two susceptible genotypes (Fig. 1i). Thirdly, we show that serotonin supplementation of the Jiazhe LM mutant reduces BPH-resistance in a dose dependent manner, with treated plants

becoming susceptible to the hoppers at the higher serotonin concentrations (Fig. 2a-d). Irrespective of resistant genotype, serotonin supplementation resulted in increased susceptibility, with increased levels of BPH infestation (Fig. 2h-k), again demonstrating a negative role for serotonin in defence. Our hypothesis is further supported by changes in serotonin levels in rice plants and hoppers. After 12 h of supplementation, serotonin levels in the mutant had increased to that of the WT (Fig. 2e), and both the mutant and the WT were equally susceptible to BPH (Fig. 2f-g). BPH is primarily dependent on the rice plant for provision of tryptophan, although endosymbionts may also play a role [17], which it is then able to convert to serotonin via tryptophan 5-hydroxylase and decarboxylase. The basal levels of serotonin in female adults starved for 12 h was only 5.3 ng/g. However, in those transferred to the susceptible WT, the levels gradually increased with time, reaching a maximum of 28.54 ng/g tissue after 1 day compared to only 11.19 ng/g for those transferred to the resistant mutant rice line (Supplementary Fig. 3f). These results show that the involvement of serotonin in BPH resistance is not unique to the Jiazhe LM mutant, but can also be extended to other lines carrying different BPH resistance genes and hence, may represent a more general mechanism of hopper resistance in rice.

Previous studies have shown that SA plays a role in the resistance response of rice to BPH, while two other signaling molecules involved in the induced-herbivore response, jasmonic acid (JA) and H_2O_2, have only a limited role[7,10]. Consistent with these reports, the *CYP71A1* mutant and the WT line had similar levels of JA and H_2O_2 (Supplementary Fig. 4). To further elucidate the mechanisms of insect resistance in Jiazhe LM, we studied components of induced defence pathways and the potential interactions between SA and serotonin, both of which derive from a common precursor, chorismate (Supplementary Fig. 1). We show that, unlike with serotonin, accumulation of SA increased in response to BPH challenge in both genotypes, but to a greater extent in the resistant mutant than the susceptible WT, these differences becoming significant 8 h post infestation (Fig. 3a). These changes were reflected in the transcript levels of genes involved upstream of, or in, SA biosynthesis: Transcription of genes encoding the enzymes OsICS1 (isochorismate synthase 1), OsPAL (phenylalanine ammonia-lyase), OsPAD4 (phytoalexin deficient 4), and regulator factors OsEDS1 (enhanced disease susceptibility 1) induced by BPH as part of the early defence response was significantly higher in Jiazhe LM compared to the WT (Fig. 3b-d; Supplementary Fig. 5a). Similar to Jiazhe LM, all BPH-resistant

genotypes accumulated significantly greater levels of SA 8 h post infestation, in contrast to the two susceptible genotypes (Fig. 3e).

The rice genome harbours four genes encoding anthranilate synthase, responsible for metabolism of chorismate in the pathway leading to the biosynthesis of tryptophan and serotonin (*OsASα1; OsASα2; OsASβ1*; *OsASβ2*; Supplementary Fig. S1). We reveal that in both the WT and Jiazhe LM mutant expression of *OsASα2, OsASβ1* and *OsASβ2* is upregulated in response to BPH, but significantly more so in the WT, with 3.15-fold increase in expression of *OsASβ1* after 3 h (Supplementary Fig. 5c-e). In contrast, there was no difference in expression of *OsASα1*, either between genotype or in response to BPH (Supplementary Fig. 5b). Expression of tryptophan synthase, another essential gene in this pathway, was also induced by BPH and again, to greater levels in the WT (Supplementary Fig. 5f). Although expression of *CYP71A1*, involved in the final step of serotonin biosynthesis, is induced by BPH in both genotypes (Fig. 1e), the hormone is only accumulated in the WT (Supplementary Fig. 3e).Thus we show that genes involved in SA biosynthesis are more highly expressed in the resistant mutant, whereas genes involved in serotonin biosynthesis are more highly expressed in the susceptible WT.

We also show that BPH induces greater levels of the serotonin precursor tryptophan in the susceptible WT and conversely greater levels of the SA precursor phenyalanine in the resistant mutant (Supplementary Fig. 6). This suggests a closely regulated feedback between SA and serotonin via the Trp and Phe/Tyr pathways, where the absence of *CYP71A1* expression, and hence serotonin production, causes a reprogramming resulting in greater SA accumulation. In agreement with this hypothesis, treatment of plants with SA significantly decreases *CYP71A1* expression (Fig. 3f) and serotonin levels (Figure 3g). Conversely, treatment of plants with serotonin represses expression of two SA biosynthesis related genes, the *OsPAL* gene and a regulator gene *OsPAD4* (Fig. 3h-i), and consequently significantly lowers SA levels (Figure 3j). In contrast, expression of *OsICS1* and *OsESD1* was not depressed by serotonin (Supplementary Fig. 5g-h), suggesting that the *OsICS1* SA synthesis pathway is not negatively regulated by serotonin (Supplementary Fig. 1). The more pronounced increases in serotonin level in susceptible genotypes (340-541%, Fig. 1i)

8 h post BPH infestation, than increases in SA level in BPH resistant genotypes (139-146%, Fig. 3e) over this same duration, suggests a greater role for serotonin in the observed BPH resistance.

As a proof of concept and to test our model, which states that the observed resistance exhibited by the mutant Jiazhe LM is specifically due to the absence of serotonin, knockout mutants (*CYP71A1*–KO) were created using CRISPR /Cas9 (Supplementary Fig. 7a). As seen with the Jiazhe mutant LM, the level of BPH infestation on the *CYP71A1*–KO was significantly lower (Fig. 1j), as was the number of eggs produced (Supplementary Fig. 7b). This important finding supports our hypothesis that BPH-resistance exhibited by the mutant Jiazhe LM is due to the absence of serotonin caused by loss of tryptamine 5-hydroxylase activity, as a direct result of mutation in the *CYP71A1* gene. However, since serotonin is synthesized from tryptamine and converted to melatonin in plants (Supplementary Fig 1), and since other *CYP71A1* KO mutants have been shown to have elevated levels of tryptamine[14, 18] and decreased levels of melatonin[19], (also confirmed in our mutant; Supplementary Fig. 8), we investigated whether these two compounds were also involved in resistance to BPH in our mutants. First, we investigated the expression of genes involved in both the biosynthesis of serotonin from tryptamine, and its subsequent conversion to melatonin. We show that whilst BPH infestation induced transcription of the tryptophan decarboxylase gene (*TDC*) in both the mutant and WT (Supplementary Fig. 9a), infestation had no effect on the two genes, *AANAT* and *ASMT*, responsible for conversion of serotonin to melatonin. Second, we assessed the effects of tryptamine and melatonin supplementation in artificial diets on the survival of BPH nymphs, but did not observe any consistent effects within the concentration ranges tested (Supplementary Fig. 9d-e). For melatonin, we further tested resistance of the mutant grown on medium supplemented with melatonin, and again no dose-dependent effects were observed on the rate of nymph survival (Supplementary Fig. 9f-h). Indeed, the melatonin levels were not affected by BPH infestation, irrespective of rice genotype (resistant or susceptible to BPH) (Supplementary Fig. 8b). These data again suggest that it is serotonin, not its precursor tryptamine or metabolite melatonin, that regulates resistance to insect pests in rice.

In addition to resistance to BPH, the Jiazhe LM mutant was also resistant to SSB larvae, resulting in developmental retardation and reduced number of larvae in the subsequent generation (Fig. 4a-c; Supplementary Table 1). By day 14, 66.7% of the larvae had reached the third instar on the WT plants, compared to 10 % on the mutant (Fig. 4b; p=0.0177); by day 21, 77.5% of larvae had reached the 5th instar on the WT compared to only 46.7% on the mutant (p=0.0072), with 5.3% still in the 3rd instar (Fig. 4b). This increase in the developmental period was reflected in the time to pupation, which was approx. 22% greater on the mutant (Fig. 4c), and in significant effects on fecundity and egg viability, with > 72% reduction in oviposition (Supplementary Table 1). Furthermore, the cumulative effects at different development stages resulted in a significant reduction of viable larvae in the following generation (> 4-fold fewer on the mutant compared to WT, Supplementary Table 1). As with BPH, SSB significantly induced expression of *CYP71A1* 2 h post feeding both in Jiazhe B and the mutant (50-fold increase 4 h post infestation; Fig. 4d). However in contrast to BPH the levels of two SA biosynthesis genes, *OsICS1* and *OsPAL*, were decreased after SSB challenge (Fig. 4e-f).

In further support of the role of serotonin in BPH and SSB resistance in rice, the beneficial effect of this hormone was demonstrated in artificial diets, with increased performance of both BPH (Fig. 2l) and SSB (Fig. 4h). This finding is consistent with the known role of serotonin in SSB immunity and behaviour[3]. However, to date no studies have suggested any link between serotonin and host plant resistance. Importantly, the resistance of Jiazhe LM to these devastating insect pests was also observed in the field. Resistance to BPH was demonstrated in two different geographic regions, with significantly reduced levels of infestation (Fig. 1h). Field resistance to SSB was shown by a significant reduction in the number of dead tillers (Fig. 4h**)**.

Host-plant resistance represents an effective and environmentally sustainable approach to reduce insect pest damage and increase yield potential of rice cultivars. However, resistance to insect pests is multifaceted involving highly sophisticated regulation in both the insect and the host plant itself. To our knowledge, no previous study has demonstrated the role of serotonin regulation as part of the plant's defence armoury against insect pests. Since there is only one single highly conserved homologue of *CYP71A1* in each genome of cereal crops (Supplementary Fig. 10) and

since the shikimic pathways (Supplementary Fig. 1) are conserved, this defence mechanism may exist across plant species. Our highly novel data suggest that exploiting this discovery is likely to provide a valuable resource for molecular breeding of insect-resistant rice cultivars. A field trial of the Jiazhe mutant showed that the *CYP71A1* knockout had negative effects on grain yield (Supplementary Fig. 11), although no negative effects on vegetative growth were observed under controlled environment conditions (Supplementary Fig. 12). The broad-spectrum resistance to insect pests of rice produced by the *CYP71A* knockout is of major potential agronomic importance, but its exploitation in future breeding programmes as an alternative or complementary approach to resistance produced by transgenes expressing Bt toxins[20] will require further engineering to link *CYP71A1* expression to BPH/SSB infestation, minimising any negative effects on yield.

Methods

Plant materials. Jiazhe LM is a lesion mimic mutant, selected from the M_2 population following irradiation of Jiazhe B (the wild type) seeds with 350 Gy ^{60}Co gamma rays. A “G” deletion in the gene *CYP71A1* is responsible for the mutant phenotype[21]. Four other BPH resistant genotypes were either available ‘in house’ or provided by other labs: Mudgo, carrying the *BPH9/1* gene[9]; RHT and IR56, both carrying the *BPH3* gene[8]; B5, carrying the *Bph14* and *Bph15* genes[7].
The *CYP71A1* knockout (*CYP71A1*-KO) rice was created by CRISPR/Cas9 technology[22] inducing an "A" insertion at 83 site (from "ATG") in the commercial cultivar Xidao No.1, originally developed by the group.

Insect rearing, maintenance, behaviour and performance studies. All insect colonies were originally collected from rice fields in Hangzhou, China. Colonies of brown planthopper *Nilaparvata lugens* (BPH) and white-backed planthopper *Sogatella furcifera* (WBPH) were maintained on Xiushui 110 seedlings (a susceptible variety to BPH) under controlled conditions (as for plants). Rice striped stem borer *Chilo suppressalis* (SSB) were reared on artificial diet and maintained at 25 ±1C, 80% RH, L : D = 14:10[23].

BPH host choice behaviour and fecundity studies were carried out as follows: each pair of Jiazhe B and Jiazhe LM seedlings (30 days old) was confined in ventilated

glass cylinders (diam 4 cm, ht 8 cm) and infested with 15 gravid females. The numbers of BPH settling on each plant were counted at 1, 2, 4, 8, 12, 24 and 48 h post release. Ten replications were carried out.

BPH fecundity was monitored by harvesting 10 cm leaf sheath (region where eggs are deposited) sections, and counting egg numbers under a microscope. Six days post BPH release, the numbers of hatched BPH nymphs on each plant were recorded and removed daily until no insects were detected. Feeding rates were determined based on honeydew production[24] but using a folded parafilm sachet closely attached to the tillers of plants, each containing one female adult. After 24 h, the honeydew in each sachet was weighed using a 0.1 mg sensitivity balance. Thirty replications were carried out for both wild type and mutants. BPH survival and development on Jiazhe B and Jiazhe LM seedlings (30 day-old) was determined. Each plant was infested with 15 second-instar nymphs, and the number of surviving BPH recorded over a 15-day period. Ten replications were carried out for both wild type and mutants. Host choice bioasssays for WBPH were carried out as for BPH. For SSB, each rep (of 4 plants) was infested with 10 neonates and life history parameters recorded up to pupation. Twenty replicates were carried out for both Jiazhe B and Jiazhe LM; plants were changed every 7 days.

Quantification of SA, JA, and H_2O_2 levels in plants in response to infestation. Individual plants were infested with 15 female adult BPH. The sheaths (feeding site) were harvested at 0, 3, 8, 24, 48 h post treatment, and JA and SA levels were analyzed by gas chromatography-mass spectrometry using labeled internal standards[25]. The H_2O_2 concentrations were determined according to Amplex® Red hydrogen Peroxide/peroxidase Assay Kit (Invitrogen) as described previously. Each treatment at each time point was replicated six times.

Quantification of serotonin levels in plants and BPH. Basal levels (0 h) of serotonin in both the WT and mutant plants and levels 8 h post BPH infestation were quantified by HPLC according to Kang *et al*[26], with some modifications. Extraction and quantification of serotonin in BPH was carried out according to Ma *et al.*[27]. Female adults were starved for 12 h and then placed on individual plants (15/plant) and allowed to feed. After a series of different time points (0.5, 1, 3, 8 h), insects were removed, homogenized in liquid nitrogen, and 10-20 mg tissue was transferred to 1.5

mL tubes, and lyzed in 300 μL ice-cold 0.1 M perchloric acid on ice for 10 min. The homogenate were centrifuged at 14,000×*g* for 10 min at 4 °C. The supernatants were filtered (0.45μm filter), and stored at -20 °C until required for HPLC-MS analysis. Six replicates (30 insects each replicate) were performed for each time point.

Quantification of melatonin. Melatonin content was assayed according to Cai *et al.*[28] with modifications. Fresh samples (0.3 g) were ground and homogenized in 2 mL of methanol containing 50 ng/mL [$_2H^6$]- melatonin as an internal standard.

Screening for insect resistance in the field. For planthopper resistance, Jiazhe B and Jiazhe LM plants were grown in randomized plots (144 plants per plot) with three replicates at two different locations in China, Jiaxing (N30 °19'; E120 °17') and Sanya (N18 °10'; E108 °56'). No pesticides were used throughout the growing period and the number of planthoppers scored at the heading stage. For SSB resistance, Jiazhe B and Jiazhe LM plants were grown in randomized plots (30 plants per plot) with three replicates in Hangzhou, China. Each plant was infested with 5 newly hatched larvae (1^{st} or 2^{nd} instar) at the booting stage, and the number of dead tillers was recorded 10 and 20 days post infestation.

Artificial diet feeding studies with serotonin supplementation. BPH feeding studies were carried out as previously described (Ji *et al.* [29]), with addition of serotonin (0, 0.1, 1μg · mL^{-1}). Twenty second-instar BPH nymphs were released into individual feeding chambers and the number of surviving nymphs recorded every day. The experiment was replicated six times for each concentration. SSB feeding studies were carried out (Han *et al.* [30]) with addition of serotonin (0, 0.5, 1, 2, 4, 8 μg/g). Thirty second-instar SSB larvae were released into individual feeding chambers. The diet was replaced every ten days and body weight was recorded after 30 days. The experiment was replicated twice for each concentration of serotonin.

More information on the generation and growth of plant materials, quantification of aromatic amino acids, serotonin and melatonin, RNA isolation and qPCR analysis, is provided in Supplementary Methods.

Data analysis

Statistical analyses were performed using the one-way analysis of variance (ANOVA) programme StatView. Data are present in mean values with standard errors as error bars. The differences were considered to be significant when the probability (*p*) was less than 0.05 in Tukey test. Precise *p* values for all statistical comparisons in the figures and supplemental figures are given in Supplemental Information.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Author contributions

Q.Y.S., Y.G.L., A.M.R.G., G.Y.Y. and J.Z.H. contributed to study design and data analysis, HL contributed to overall study and data analysis, T.L. contributed to BPH and WBPH resistance studies, H.W.F. contributed to Jiazhe LM mutant development and field studies, L.W. contributed SSB resistance studies, Q.W. and Y.Y.T. contributed to the development and characterization of knockout mutants, A.M.R.G. and Q.Y.S. wrote the manuscript. All authors

read and approve the paper.

Competing financial interests

Authors declare no competing financial interests.

Materials & Correspondence

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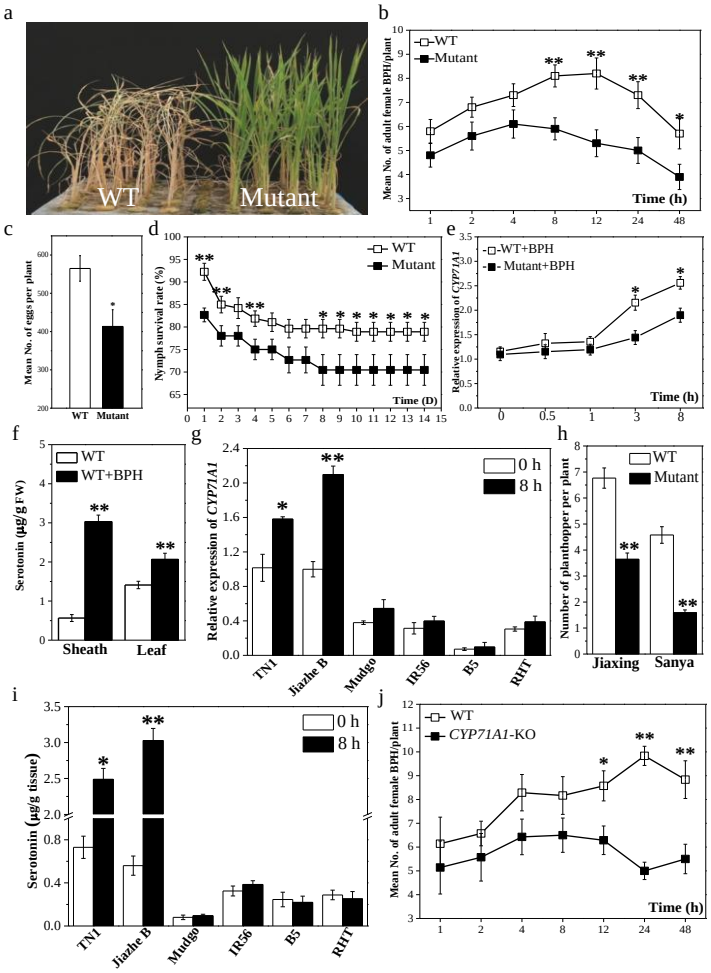


Figure 1. Mutation in *CYP71A1* resulting in the suppression of serotonin synthesis confers resistance to BPH. (a) Performance of WT (Jiazhe B) and *cyp71a1* mutant (Jiazhe LM) rice plants in response to BPH infestation; (b) host preference and (c) oviposition preference of BPH in free-choice studies 48 h post infestation; (d) BPH nymph survival is significantly greater on WT than mutant plants in "no-choice" studies ; BPH infestation induces: (e, g) expression of *CYP71A1* and (f, i) serotonin accumulation in susceptible (WT; TN1) but not in BPH-resistant (Mudgo, IR56, B5, RHT) rice lines (i); (h) planthoppers were less abundant on mutant (Jiazhe LM) than WT plants at two sites, Jianxing and Sanya; (j) *CYP71A1* knockout mutant (*CYP71A1*-KO), exhibits significantly enhanced resistance to BPH compared to the WT Xidao No.1. Data are mean and s.e. of 10 (b), 10 (c), 10 (d), 5 (e), 6 (f), 5 (g), 3 (h), 6 (i), 10 (j) biologically independent experiments, respectively. * p<0.05, ** p<0.01(Tukey test).

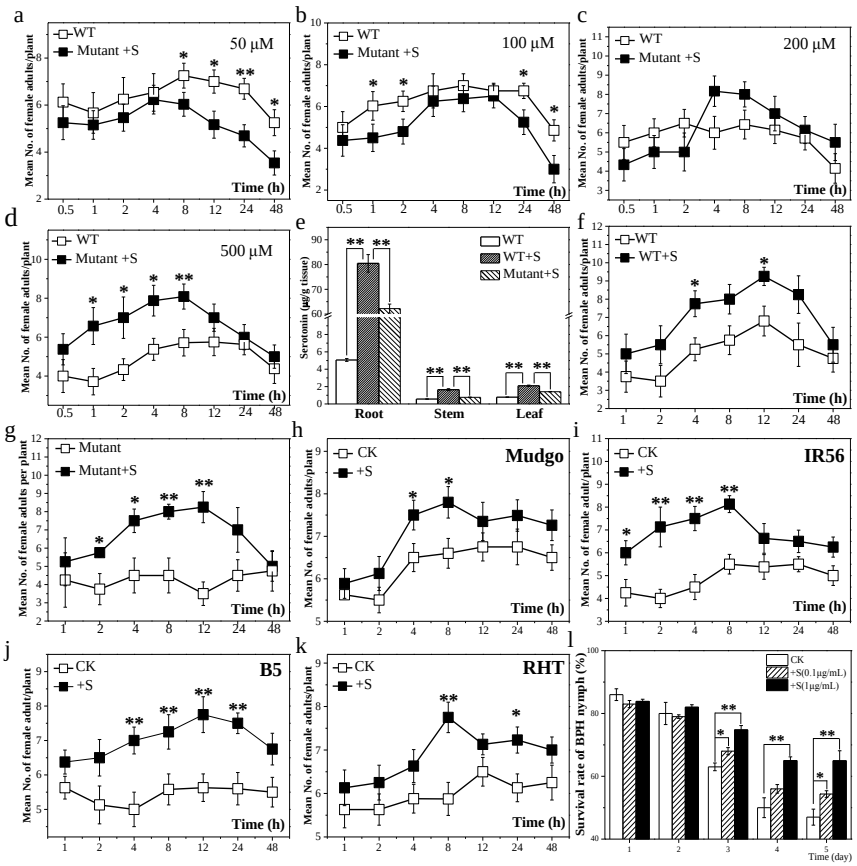


Figure 2 Addition of exogenous serotonin abolishes BPH resistance in rice.

(a-d) Supplementation of the BPH-resistant mutant (Jiazhe LM) with serotonin (+S) causes an increase in BPH survival in a dose-dependent manner; (e) serotonin concentration increases in different tissues in both WT and mutant in response to serotonin supplementation (200μM); (f-k) serotonin supplementation (300μM) results in enhanced BPH performance in free-choice studies in all rice lines: (f) WT Jiazhe B, classified as susceptible, (g) *cyp71a1* mutant, (h-k) BPH-resistant lines Mudgo, IR56, B5, RHT; (l) addition of serotonin to artificial diet increases BPH survival rates. Data are mean and s.e. of 10 (a-k) and 6 (l) biologically independent experiments, respectively. * $p<0.05$, ** $p<0.01$(Tukey test).

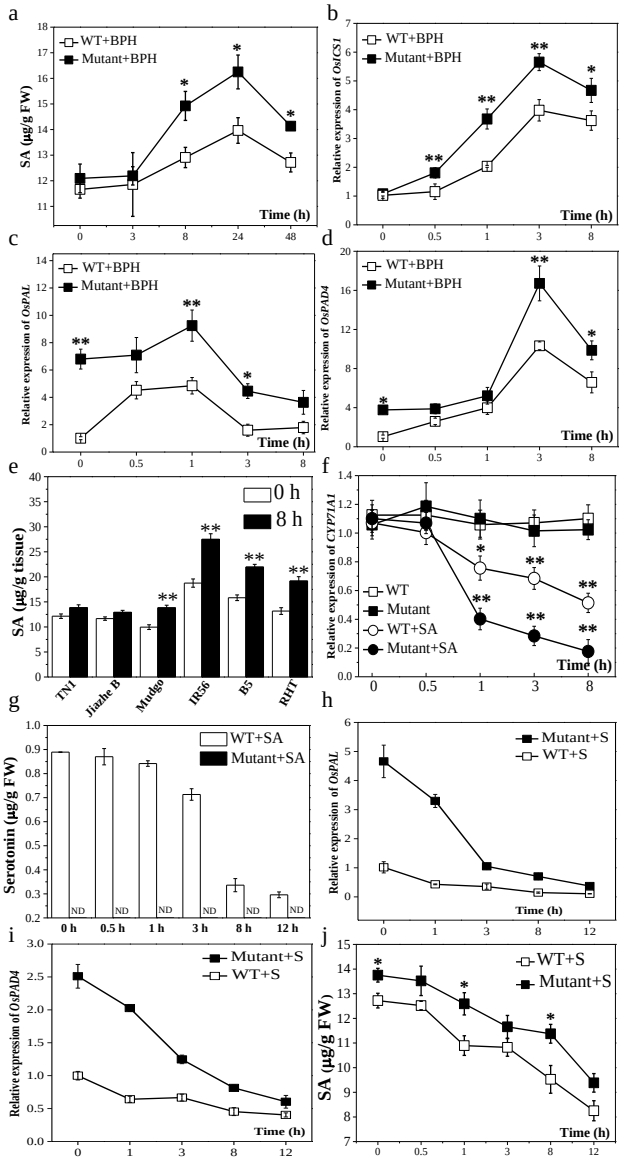


Figure 3 BPH infestation induces biosynthesis of salicylic acid (SA), whilst SA and serotonin suppress each other, suggesting mutual negative feedback. (a) BPH infestation and *cyp71a1* mutation increase SA accumulation and (b-d) expression of SA biosynthesis genes; (e) accumulation of SA in BPH-susceptible (Jiazhe B, TN1) and -resistant (Mudgo, IR56, B5, RHT) rice lines in response to BPH infestation; (f) addition of SA suppresses expression of *CYP71A1* and (g) serotonin content whilst supplementation of serotonin downregulates expression of (h) *OsPAL* and (i) *OsPAD4*, two genes involved in SA biosynthesis, and (j) lowers SA level. Data are mean and s.e. of 6 (a-f), 10 (g-k) and 6 (l) biologically independent experiments, respectively. * $p<0.05$, ** $p<0.01$(Tukey test).

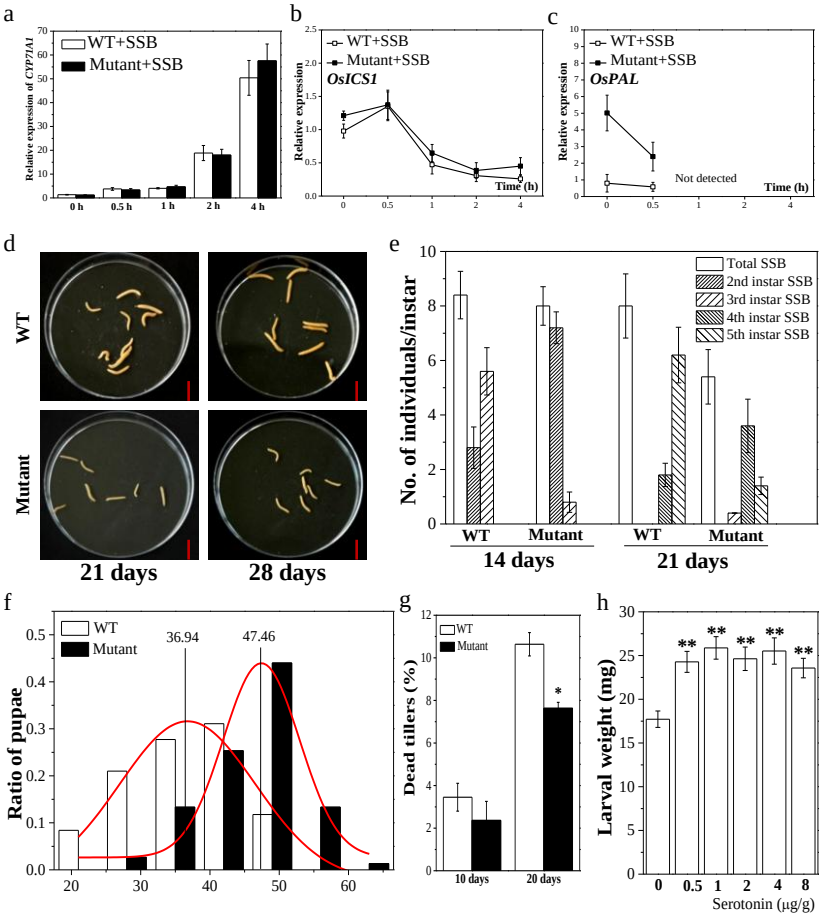


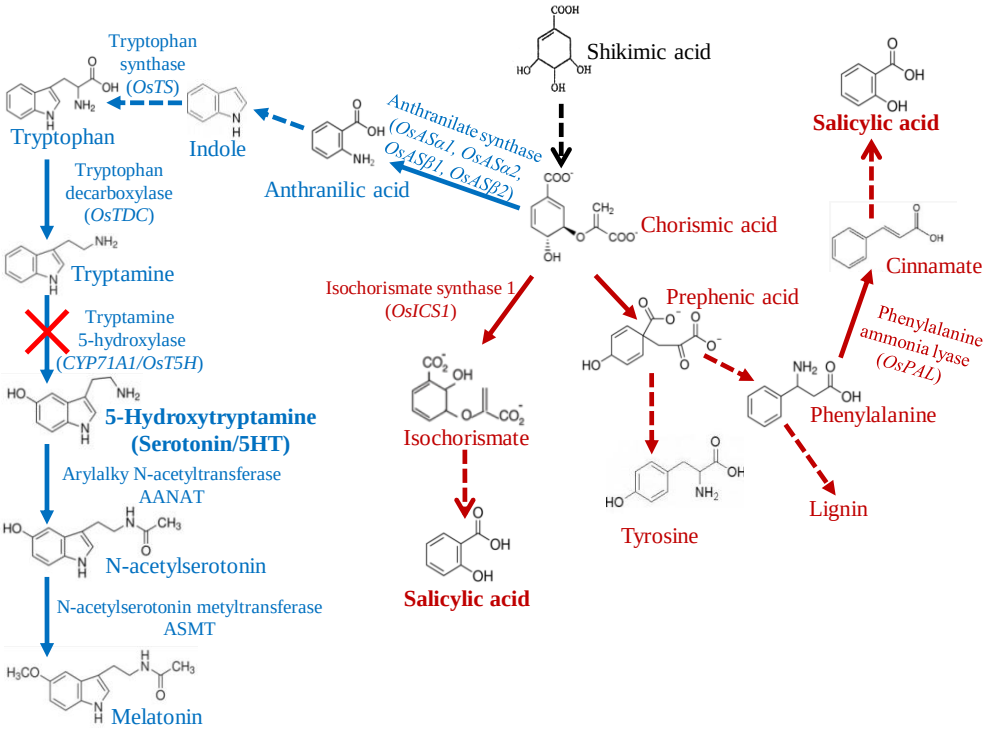
Figure 4 Striped stem borer (SSB) infestation induces expression of *CYP71A1* whilst mutation in this gene enhances resistance to SSB. (a) SSB challenge induces transcription of *CYP71A1*, but not of the SA biosynthesis genes (b) *OsICS1* and (c) *OsPAL*; SSBs feeding on the mutant plants (Jiazhe LM) show (d) reduced body size (bar: 1 cm), (e) retarded development, and (f) extended time to pupation; (g) the mutant line (Jiazhe LM) shows increased field resistance to SSB compared to WT plants (Jiazhe B) in terms of number of dead tillers; (h) addition of serotonin in artificial diet enhances SSB performance. Data are mean and s.e. of 10 (a), 6 (b-c), , 20 (d-f), 3 (g) and 2 (h) biologically independent experiments, respectively. * p<0.05, ** p<0.01(Tukey test).

Supplemental Information

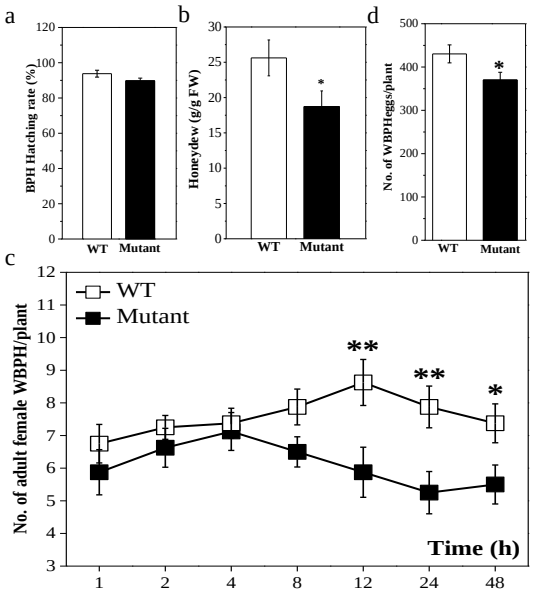
Resistance of Rice to Insect Pests Mediated by Suppression of Serotonin Biosynthesis

Supplemental Inventory

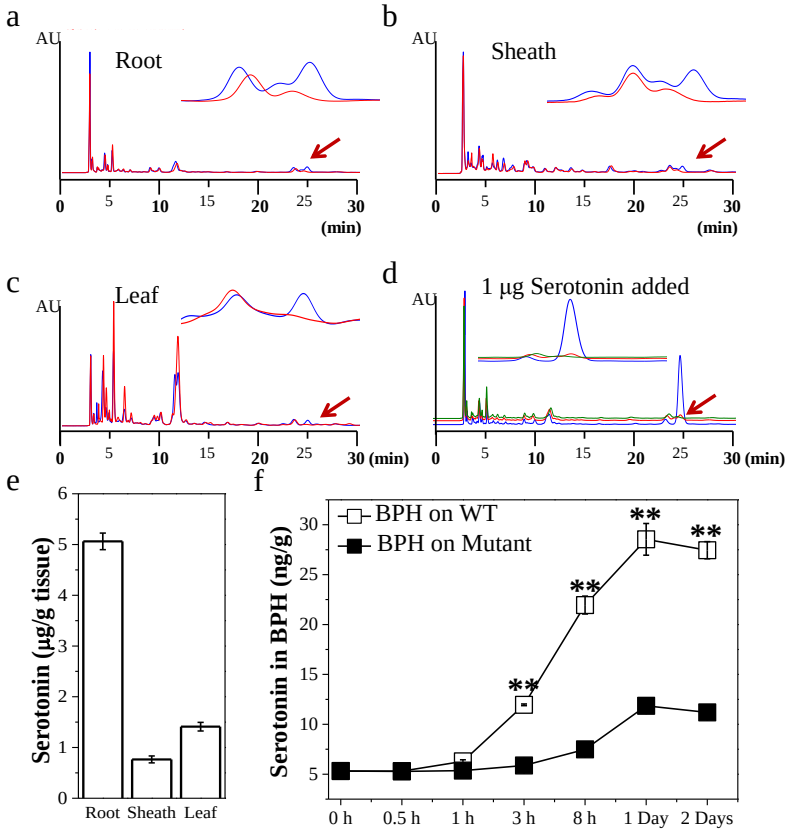
1, Supplemental Figures and Tables



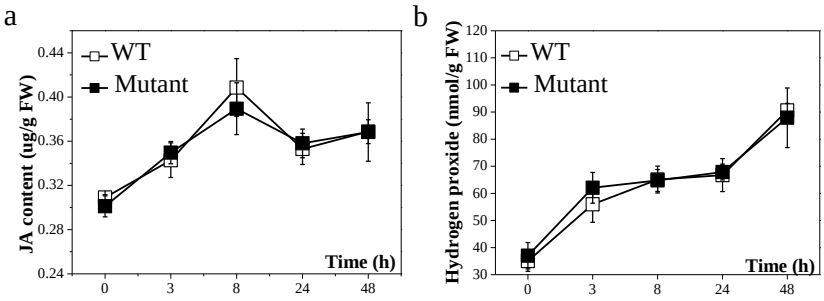
Supplementary Figure 1 The biosynthesis of salicylic acid (SA), serotonin (5-hydroxytryptamine ,5-HT), melatonin, and aromatic amino acids (AAA). These aromatic compounds are produced from core primary metabolites via the shikimate pathway, leading to the synthesis of chorismate. Chorismate is the initial branch point metabolite in the synthesis of all three AAAs (Trp, Phe, Tyr), SA, serotonin and a wide range of other aromatic secondary metabolites derived from it. Genes encoding respective enzymes are presented in parenthesis and in italics; broken arrows indicate two or more steps between substrate and product.



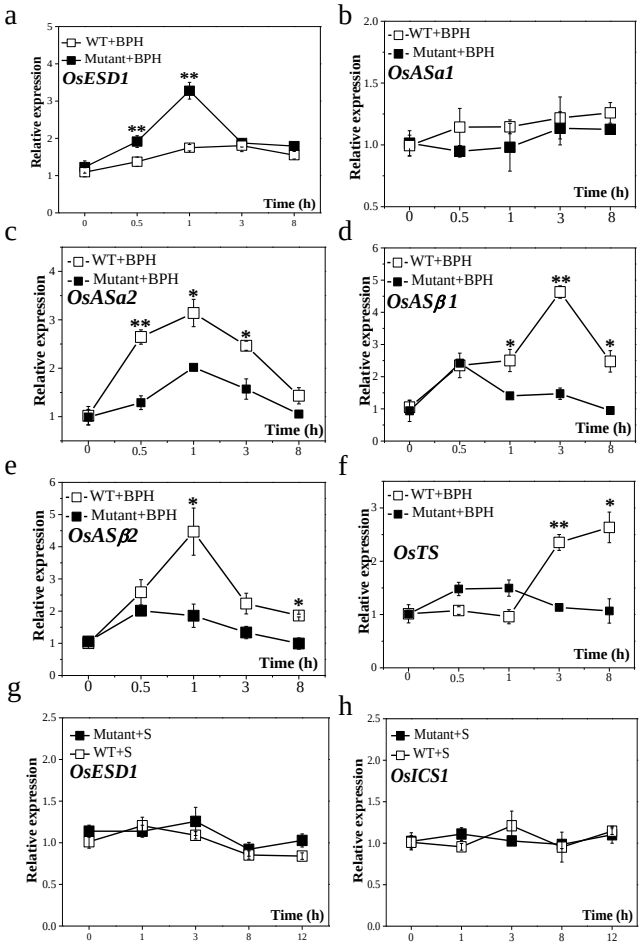
Supplementary Figure 2 Comparison between brown planthopper (BPH) and white-backed planthopper (WBPH) performance on WT (Jiazhe B) and mutant (Jiazhe LM) rice lines. (a) BPH egg hatch and (b) BPH honeydew production 48 h post infestation; (c) WBPH host preference and (d) WBPH oviposition preference 48 h post infestation. Data are mean and s.e. of 10 (a), 30 (b), 10 (c), 10 (d), biologically independent samples, respectively. *p<0.05, ** p<0.01 (Tukey test).



Supplementary Figure 3 Serotonin content in different rice tissues prior to BPH infestation and in BPH female adults feeding on either WT or mutant rice lines. HPLC chromatogram of serotonin levels between the WT (Jiazhe B, blue lines) and the mutant (Jiazhe LM, red lines) in: (a) root, (b) sheath, and (c) leaf; (d) addition of serotonin to the WT sample confirms that the peak is serotonin; (e) serotonin content in different tissues of WT Jiazhe B; (f) serotonin content in BPH females after feeding on WT (Jiazhe B) or mutant (Jiazhe LM) rice plants. Prior to the assay, female adults were starved for 12 h. Data are mean and s.e. of 3 (e), 6 (f), biologically independent samples, respectively. ** p<0.01 (Tukey test).



Supplementary Figure 4 Changes in levels of signalling molecules in WT (Jiazhe B) and mutant (Jiazhe LM) rice plants in response to BPH infestation. (a) Jasmonic acid (JA) content and (b) H_2O_2 (b) content. Data are mean and s.e. of 6 (a), 6 (b), biologically independent samples, respectively.



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655 **Supplementary Figure 5** Expression of genes involved in the biosynthesis of
656 salicylic acid (SA) and serotonin in WT (Jiazhe B) and mutant (Jiazhe LM) rice lines
657 in response to BPH infestation (+BPH) and serotonin supplementation (+S). Gene
658 expression in response to BPH of: (a) *OsESD1* involved in SA biosynthesis; (b)
659 *OsASa1*, (c) *OsASa2*, (d) *OsAS1*, (e) *OsAS2*, and (f) *OsTS* involved in serotonin
660 biosyntheis*;* gene expression in response to addition of 300 μM serotonin of: (g)
661 *OsESD1* and (h) *OsICS1*, involved in SA biosynthesis. Data are mean and s.e. of 6
662 biologically independent samples, respectively. **p*<0.05, ** *p*<0.01 (Tukey test).

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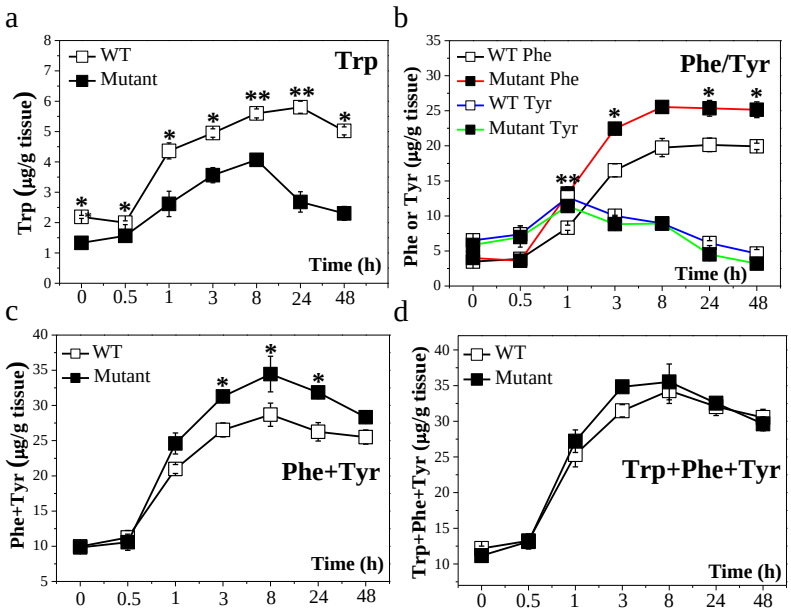
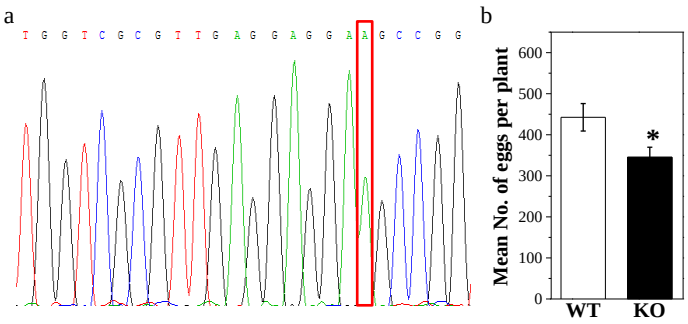
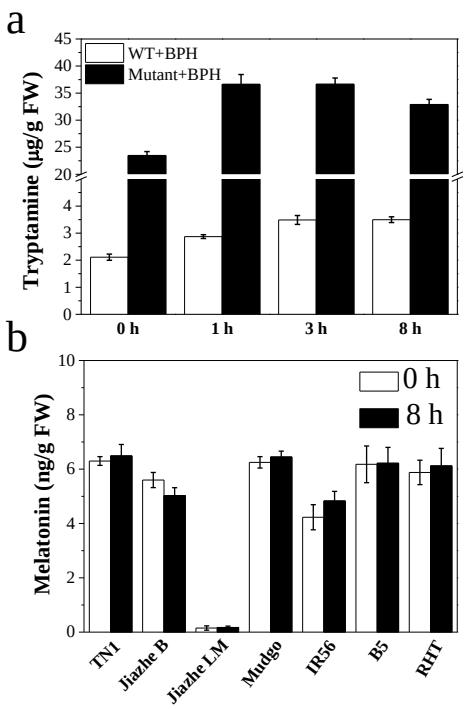


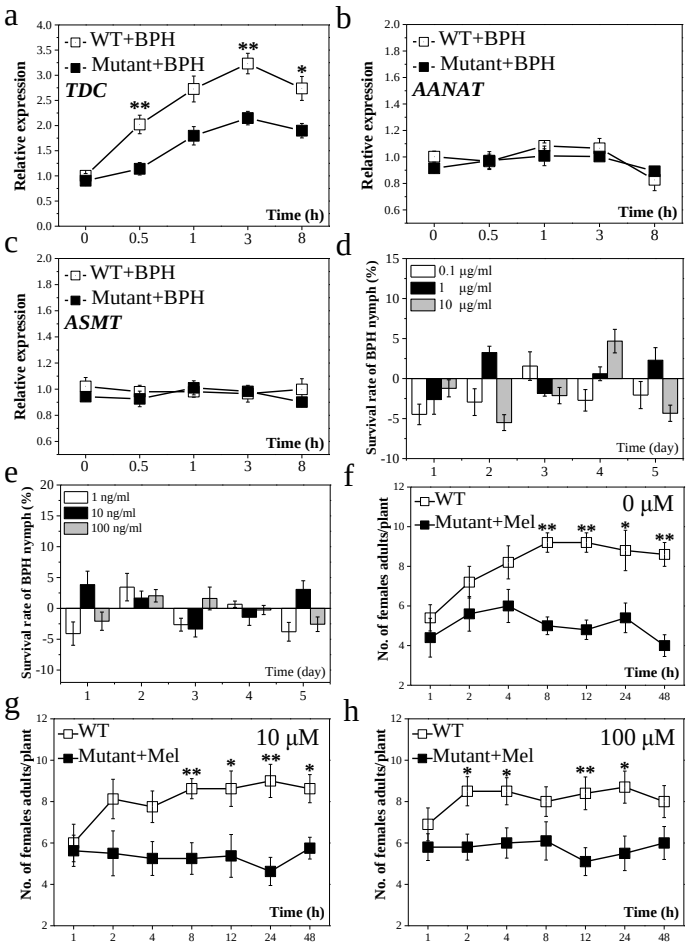
Supplementary Figure 6 Aromatic amino acid (AAA) content in WT (Jiazhe B) and mutant (Jiazhe LM) rice lines post BPH infestation. (a) tryptophan (Trp); (b) phenylalanine (Phe) and tyrosine (Tyr) in Jiazhe B and Jiazhe LM, respectively. All plants were infested with 15 BPH female adults. (c) Sum of the two AAAs, Phe and Tyr. (d) Sum of all three AAAs. Data are mean and s.e. of 6, biologically independent samples, respectively. *p<0.05, ** p<0.01 (Tukey test).



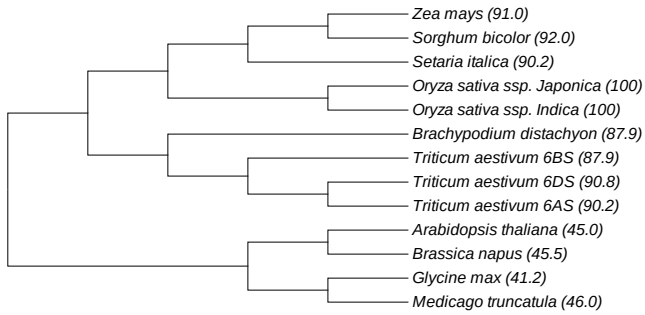
Supplementary Figure 7 *CYP71A1* knockout out (*CYP71A1*-KO) rice mutant generated by CRISPR/Cas9 and its resistance to BPH. (a) sequence chromatograph of the target *CYP71A1* fragment of the KO mutant showing a nucleotide insertion (boxed in the red frame); (b) mean number of eggs on pairs of plants (the parental line Xidao No.1 vs *CYP71A1*-KO) 48 h post BPH infestation. Data are mean and s.e. of 10 (b), biologically independent samples, respectively. *p<0.05 (Tukey test).



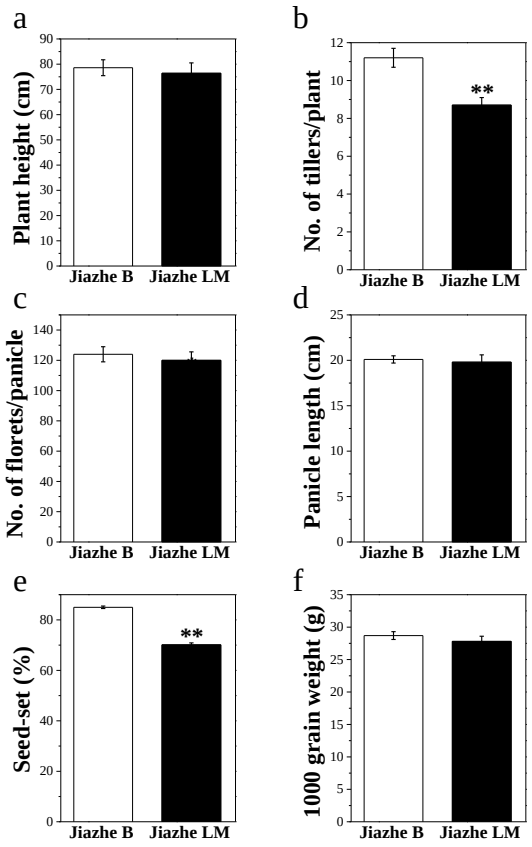
Supplementary Figure 8 Changes in levels of tryptamine and melatonin in response to BPH infestation. (a) changes in tryptamine content in WT (Jiazhe B) and mutant (Jiazhe LM) plants 0, 1, 3, 8 h h post BPH infestation. (b) Melatonin content in BPH susceptible (TN1, Jiazhe B) and resistant (Jiazhe LM, Mudgo, IR56, B5 and RHT) genotypes before and 8 h post BPH infestation. Data are mean and s.e. of 6 (a), 6 (b), biologically independent samples, respectively.



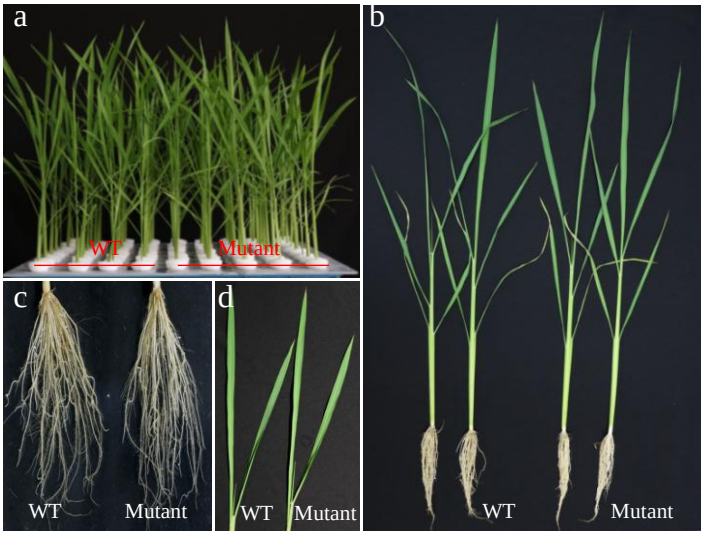
Supplementary Figure 9. Expression of genes involved in tryptamine and melatonin biosynthesis in response to BPH infestation and effects of tryptamine and melatonin on BPH survival. (a-c) The relative transcript abundance of *TDC*, *AANAT* and *ASMT* in mutant (Jiazhe LM) and WT (Jiazhe B) plants pre and post BPH infestation (0.5 -8 h); changes in survival rate of BPH nymphs fed artificial diets supplemented with tryptamine (d) and melatonin (e); (f-h) host preference of BPH on mutant plants grown in culture media supplemented with melatonin at different concentrations (0, 10, 100 μM) as compared with WT plants. Data are mean and s.e. of 6 (a), 6 (b), 6 (c), 6 (d), 6 (e), 10 (f), 10 (g), 10 (h), biologically independent samples, respectively. *p<0.05, ** p<0.01 (Tukey test).



Supplementary Figure 10 Sequence similarity of CYP71A1 homologues in a range of plant species. CYP71A1 homologues were obtained using a blastP (protein to protein) search of the Gramene database and similarity values (shown in parenthesis) are calculated in relation to CYP71A1 of *Oryza sativa* ssp. *japonica*.



Supplementary Figure 11. Performance of agronomic and yield traits of Jiazhe B and Jiazhe LM grown side by side in a field experiment in Jiaxing, Zhejiang Province, 2015.Data are mean and s.e. of 30, biologically independent samples, respectively. ** p<0.01 (Tukey test).



Supplementary Figure 12 The phenotype of Jiazhe B (WT) and mutant (Jiazhe LM) plants. (a) Plants grown in a liquid culture medium at the age used for BPH resistance screening (Figure 1a). Enlarged view of: (b) plants; (c) root system; (d) leaves, demonstrating that prior to infestation the WT and mutant plants were morphologically similar to one another.

Supplementary Table 1 Performance of the striped stem borer (*Chilo suppressalis*) on WT (Jiazhe B) and mutant (Jiazhe LM) rice lines

Items	Jiazhe B	Jiazhe LM
No. of larvae infested	200	200
No. of pupae	119	75
Pupal weight (mg)	37.8	35.6
Eclosion ratio (%)	82.4	69.3
No. of eggs	2478	691
Hatching ratio (%)	83.2	61.6
Multiplication index (MI) *	10.31	2.13

*MI = Number of new larvae produced per larva through one generation

Supplementary Table 2 Forward (F) and reverse (R) primers used for real-time quantitative PCR

Gene	Primersequence (5′→3′)
CYP71A1	F: CACCATCGGCGACTTCTTCCC
	R: AGCTCCGTCATCACCCACTCC
OsEDS1	F: CATTCCAAGAACGAGGACACTG
	R: CAAGACTCAAGGCTAGAACCGA
OsPAD4	F: CCAACATGTACCGCATCAAG
	R: GGTTGTTTCGGTGGTAGTGG
OsPAL	F: GCACATCTTGGAGGGAAGCT
	R: GCGCGGATAACCTCAATTTG
OsICS1	F: TATGGTGCTATCCGCTTCGAT
	R: CGAGAACCGAGCTCTCTTCAA
OsNPR1	F: TTTCCGATGGAGGCAAGAG
	R: GCTGTCATCCGAGCTAAGTGTT
OsPR1	F: GGCAACTTCGTCGGACAGA
	R: CCGTGGACCTGTTTACATTTTCA
OsASα1	F: AATTTGGGTCAGCACTACAG
	R: AACTTTGTCTTCTGCTTTCGA
OsASα2	F: CAGTTTGGTACACCTTTGAAG
	R: ACAAACATCTTCCTTCTCTGT
OsASβ1	F: ATGAACTTACCATAGAGGATG
	R: ATGATCCTCTTGCCTTCTGG
OsASβ2	F: GATATCACCGTGGAAGAAATT
	R: CATGAGCCTCCCTTCGTGG
TDC	F: ATGACCTGCCTCGACTGCACC
	R: CTTGTTCAGCCGCTCCATCAG
AANAT	F: GGGCTGCGGCAACTTGGTCC
	R: GCTGGCACTAAAATCTGGGGTACC
ASMT	F: TACCGTCCATGACGGCG
	R: CGGCCGCCTTCTCGACA
OsActin	F: CAGCACATTCCAGCAGAT
	R: GGCTTAGCATTCTTGGGT

2, Supplemental Experimental Procedures

Generation, growth and treatment of plant materials. In the production of *CYP71A1* knockout (*CYP71A1*-KO) mutants, a 20 bp fragment (5'-TGGTCGCGTTGAGGAGGAGC-3') in the *CYP71A1* gene was designed as the target and inserted into the vector pHun4c12 for CRISPR/Cas9 knockout. The vector was transformed into rice var. Xidao No.1 via *Agrobacterium* –mediated (strain EHA 105) transformation. A total of 18 plants were regenerated from hygromycin-resistant calli (T_0 plants). The genotypes of each transgenic plant was verified by PCR amplification and sequencing. Six CYP71A1-KO plants were obtained, and the T-DNA free plants were selected out in the T_1 generation to confirm the role of CYP71A1 in the resistance of Jiazhe LM to BPH.

Pre-germinated seeds of the different varieties were cultured in plastic bottles under controlled environmental conditions (28℃, 14 h light, 10 h dark). Ten-day-old seedlings were transferred to 20-L hydroponic boxes containing a rice nutrient solution[1]. After 40 days, seedlings were transferred to individual 300 mL hydroponic plastic pots for 4-5 days prior to experimentation.

For SA treatment 20 μL SA (3.5 μg/mL) in lanolin paste was individually applied to plant stems. For serotonin treatment, plants were grown hydroponically in nutrient solution supplemented with serotonin to final concentrations of 0, 50, 100, 200 and 500 μM for 12 h prior to subsequent studies.

Analysis of aromatic amino acids. Tryptamine was analyzed on HPLC according to Li *et al.* (2016)[2]. In brief, frozen samples (0.3g) were ground and homogenized in 2 mL of methanol. The homogenates were centrifuged at 11,500 *g* for 10 min. The supernatants were analyzed by reverse-phase HPLC. Compounds were separated on an Atlantis C18 column with an isocratic elution profile of 5% (v/v) methanol in water containing 0.3% trifluoroacetic acid at a flow rate of 0.8mL/min. The detection of compounds was monitored at 280 nm.

For analysis of other aromatic acids, 0.5 g of nitrogen treated leaf sheath powder was suspended in 10 mL 3% trichloroacetic acid solution. Samples were agitated on an orbital shaker at 200 rpm for 1 h at RT, centrifuged at 12,000 × *g* for 15 min and the supernatant filtered using Millipore 0.25 μm syringe filters. The free amino acid (FAA) content in the filtrate was determined through the ninhydrin method using an L-8800

high-speed amino acid analyser (Hitachi, Japan)[3]. Six replicates (2 seedling each replicate) were performed for each time point.

Quantification of serotonin levels in plants and BPH. One gm fresh tissue were ground in liquid nitrogen and immediately extracted in 8 mL methanol for 10 min on an orbital shaker at 4 °C followed by centrifugation at 13,500 × *g* for 5 min at 4 °C. The supernatant was filtered through Millex-LG (Waters, USA) and 2 mL (1/4 volume) dist. water was added to the filtered solution. The serotonin was partially purified on a Sep-pak C18 cartridge (previously washed with methanol and water), and the cartridge was washed with 10mL (same volume as sample) of 80% methanol. The flow-through and wash (containing the serotonin) were combined, evaporated to dryness and dissolved in 500 µL 50% methanol. This was then analyzed by reverse-phase HPLC using a C18 column with isocratic solution of 10% methanol in water containing 0.3% trifluoroacetic acid at a flow rate of 0.8 mL/min. The eluates were detected at 280 nm.

Quantification of melatonin levels. Fresh samples (0.3 g) were ground and homogenized in 2 mL of methanol containing 50 ng/mL [$_2H^6$]- melatonin as an internal standard. The homogenate was shaken overnight at 4 ℃ in the dark and then centrifuged at 15 000 *g* for 10 min. The supernatant was transferred to a new tube, and the pellet was re-extracted with 2 mL of methanol. The supernatant from two extractions was combined and eluted through a Sep- Pak C18 cartridge. The flow-through was concentrated to dryness under nitrogen. The residue was dissolved in 0.5 mL 70% methanol and subjected to HPLC electrospray ionization/MS-MS analysis on an Agilent 6460 triple quad LC/MS with an Agilent- XDB C18 column.

RNA isolation and qPCR analysis. Total RNA was isolated using the SV Total RNA Isolation System (Promega), following the manufacturer's instructions. 500 ng of each total RNA sample was reversed transcribed using the PrimeScript®RT-PCR Kit (TaKaRa). qRT-PCR was performed on an Eppendorf MasterCycler® ep RealPlex4 (Wesseling Berzdorf, Germany) in 10 µL aliquots, containing 1 µL cDNA, 0.2 µLof each primer (10 µmol/L), 5 µl 2 x mix buffer (Master mix，TOYOBO), supplemented with sterile dist. water, with the following program: 94 ℃ 2 min; 40

cycles of 94 ℃ 30 s and 68 ℃ 1 min. The relative quantification of gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method, where the Ct value is the cycle number at which the fluorescent signal rises statistically above the background. A rice *Actin* gene was used as an internal standard to normalize cDNA concentration. Primers for qRT-PCR for all tested genes are provided in Table S2. For qRT-PCR analysis, two independent plants were combined to form one biological sample, with five biological replicates and two technical replicates for each sample.

3, Supplemental References

1. Yoshida, S., Forno, D.A., Cock, J.H., & Gomez,K.A. Laboratory Manual for Physiological Studies of Rice. Manila: International Rice Research Institute, (1976).

2. Li, M.Q. *et al.* Melatonin mediates selenium-induced tolerance to cadmium stress in tomato plants. *J. Pineal Res.* **61**, 291-302 (2016).

3. Kim, M.Y. *et al.* Comparison of free amino acid, carbohydrates concentrations in Korean edible and medicinal mushrooms. *Food Chemistry*, **113,** 386-393 (2009).