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## ORIGINAL ARTICLE

# Quantitative ^{1}H NMR metabolome profiling of Thai Jasmine rice (*Oryza sativa*) reveals primary metabolic response during brown planthopper infestation

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**Abstract** Brown planthopper (BPH; *Nilaparvata lugens*) is a phloem feeding insect which is one of the most serious threats to rice crops in many countries throughout Asia. ^{1}H NMR spectroscopy, combined with chemometrics, was used to analyze the polar metabolome from leaf extracts of Thai Jasmine rice (brown planthopper (BPH)-susceptible KD) and its BPH resistant isogenic lines (BPH-resistant IL7 and BPH-resistant[+] IL308 varieties) with and without BPH infestation at various time points (days 1, 2, 3, 4 and 8). Physiological changes of the rice isogenic lines were different based on the quantitative trait loci of BPH resistance. Multivariate models were capable of distinguishing between the susceptible and the resistant rice varieties throughout the infestation. The concentration of 10 metabolites were significantly altered ($p < 0.05$) between the infested and the control groups of each examined rice variety. Metabolic pathway analysis suggested that BPH infestation could perturb transamination during the early stages of infestation (days 1–3) for all rice varieties. In addition, the IL7 and IL308 varieties responded earlier (day 3) than the KD variety (day 8) by perturbing amino acid metabolism, shikimate and gluconeogenesis pathways. By day 8 of the infestation, the KD cultivar responded by activating the amino acid-mediated-de novo pathway whereas the IL308 variety activated the purine and pyrimidine compound-mediated-salvage pathway for nucleotide biosynthesis. This study has identified, for the first time, several potential metabolic pathways for acclimatization and defense mechanisms against BPH infestation. These findings provide a valuable, first insight into BPH resistance mechanisms in Thai Jasmine rice.

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## 1 Introduction

Brown planthopper (BPH; *Nilaparvata lugens*) is a phloem feeding insect which is one of the most serious threats to rice crops in many countries throughout Asia (Heong and Hardy 2009). It directly draws the nutrients from the leaf phloem causing the leaves to dry out and turn yellow. This process causes rice senescence and is called hopper burn. Additionally, BPH indirectly transmits viruses to the rice plant which cause grassy and ragged stunt diseases (Kerchev et al. 2012). In Thailand, BPH infestation is one of the major annual disease outbreaks in rice crops, causing huge cultural and economic burdens to Thai Jasmine rice producers. Khao Dawk Mali 105 (KDML 105; KD), a well-

known Thai Jasmine rice, is regarded as a premium rice due to its high quality and international consumer demand. However, due to its high susceptibility to BPH, production yields of KD rice have dramatically decreased, critically damaging Thailand's export trade over the past few decades (Toojinda et al. 2005). Therefore the use of pesticides are common place; however such chemical strategies are expensive, unfriendly to the environment and local ecology (Heinrichs 1979). Another economically and environmentally friendly strategy is to develop a rice breeding program to genetically increase BPH resistance. Currently, the breeding programs of resistant rice varieties has been done by backcross introgression of the major brown planthopper resistance gene (*Bph3*) from the original resistant donor Rathu Heenathi (RH) into KD. Consequently, the inherited BPH resistant isogenic lines (ILs) based on KD characteristics, namely UBN3078-101-342-4-7 (IL7), UBN3078-101-342-4-162 (IL162), UBN3078-101-342-4-283 (IL283), UBN3078-101-342-6-302 (IL302), and UBN3078-101-432-6-308 (IL308), have been available to improve the breeding program (Jairin et al. 2009). Interestingly, single-feature polymorphism (SFP) analysis demonstrated that Os04g2 7430 transcript was differentially expressed between the BPH-resistant rice varieties (IL162, IL283, IL302 and IL308) and the susceptible KD cultivar (Kamolsukyunyong et al. 2013). Os04g27430 encodes a putative sesquiterpene synthase (STPS) which may play an important role in the BPH resistance mechanism. Although these approaches have been developed against BPH attack, BPH has been reported to adapt to overcome both pesticides and resistant varieties (Peñalver Cruz et al. 2011; Lu et al. 2005). Moreover, an improper fertilizer management system can also stimulate BPH to feed on both susceptible and resistant varieties of rice (Heong et al. 1994). Thus, a comprehensive study of the BPH infestation mechanism is urgently required to decipher the responses in rice to BPH in order to find an effective way of preventing and controlling insect infestation.

Previously, the effect of BPH infestation using physiological (Watanabe and Kitagawa 2000) and biochemical analysis of rice leaves (Cagampang et al. 1974) has been reported. To obtain a comprehensive picture of the interaction between BPH and rice with and without BPH-resistant traits, several systematic studies using transcriptomic (Yuan et al. 2005), proteomic (Wei et al. 2009) and metabolomic approaches (Liu et al. 2010) were performed. These studies reveal alterations in gene expression, protein levels and metabolite levels related to abiotic stress, pathogen invasion and phytohormone signaling pathways, proteinase inhibitors and amino acids-derived secondary metabolites following the BPH infestation. However, these investigations were performed only in Chinese rice varieties with either the *Bph14* or *Bph15* resistant loci. The

response to BPH infestation in Thai Jasmine rice have not been previously reported, thus it is crucial to characterize responses to BPH infestation in Thai rice varieties to better understand the biological mechanism of infestation. Thai rice is genetically different from the Chinese rice varieties and contains different BPH resistant loci. To date, very little is known about the metabolomic response which is an important indicator of rice phenotypes and its relationship to the BPH.

Metabolomics analysis has been applied to study the interaction between plants and herbivores previously (Jansen et al. 2009). Among these reported studies various analytical platforms have been utilized to study the metabolome of plants. Proton nuclear magnetic resonance (${}^1$H NMR) spectroscopy is one of the major platforms used to biochemically profile biological samples. It is preferred to other techniques due to its nondestructive and highly reproducible qualities which require minimal sample preparation (Álvarez-Sánchez et al. 2010). Although it is limited in sensitivity as compared to high resolution mass spectrometry coupled with liquid chromatography, ${}^1$H NMR is very effective at measuring, characterizing and quantifying metabolites present at high concentrations (μM) (Bharti and Roy 2012). Numerous ${}^1$H NMR metabolomics studies have been reported in the literature to include: studies of abiotic stress due to increased salt concentrations (Ghosh et al. 2011), drought-salinity stress (Fumagalli et al. 2009), chromium (Cr) stress (Dubey et al. 2010), submergence stress (Barding et al. 2011) and studies of biotic stress due to brown planthopper infestation in rice leaves (Liu et al. 2010), tobacco mosaic virus infection in tobacco (*Nicotiana tabacum*) leaves (Choi et al. 2006) and phytoplasma infection in rosy periwinkle (*Catharanthus roseus*) leaves (Choi et al. 2004). This study is the first reported investigation using ${}^1$H NMR to analyze biochemical profiles of Thai Jasmine rice leaves and highlight the changes in the biochemical pathways between BPH resistance and BPH susceptible rice varieties.

## 2 Materials and methods

### 2.1 Rice and brown planthopper samples

For rice varieties, one BPH-susceptible cultivar, Thai Jasmine rice (KD) and two BPH-resistant isogenic lines (ILs); strain UBN03078-101-342-4-7 (IL7) and UBN3078-101-432-6-308 (IL308), were utilized to identify differential metabolomic profiles during BPH infestation at various time points. The IL7 and IL308 were the BPH-resistance-ILs of KD contain *Bph3* only and *Bph3* plus the *terpene synthase* (*TPS*) gene, respectively (Kamolsukyunyong et al. 2013).

For BPH, the Ubon Rachathani population (UBN-BPH) was cultured and manipulated in a standard BPH-suscepti-ble Taichung Native 1 (TN1) rice cultivar enclosure (Jairin et al. 2009). For rice samples, all examined rice varieties were grown in a total of 90 pots (30 pots/each; pot dimensions: 20 cm in diameter and 18 cm in height) with 3 plants per pot in a greenhouse with 13 h (06:00–19:00) light and 11 h (19:00–06:00) dark cycles for 45 days (tilling stage) before plants were equally divided into two groups (control and BPH treatment, 15 pots/group). For each group, samples were collected at five time points (1, 2, 3, 4, and 8 days; 3 pots/day/group). Effects of BPH infestation on the 3 varieties were investigated by dividing each variety into 2 groups: a control group (without BPH feeding) and a treatment group (with BPH feeding) (Fig. 1a).

## 2.2 Brown planthopper (BPH) treatment and rice sample collection

For the BPH treatment group, each rice variety in the group was separately transferred into an aluminum cage before being treated with BPH. The cage was covered with plastic mesh (1 mm diameter) to prevent the BPH to migrate be-tween the different rice isogenic lines. In each cage, 25–30 second- to third-instar BPH nymphs were introduced to each plant (15 pots/variety/cage). In parallel, control groups were maintained in separate aluminum cages covered with plastic mesh without the introduction of BPH nymphs (Fig. 1a). For sample collection, a homogeneous pool of 3 plants/pot of leaf samples from three different varieties in both control and treatment groups were collected on days 1, 2, 3, 4 and 8, respectively. The samples were collected at the same time daily beginning at 09:00 for control groups fol-lowed by BPH treatment groups at 13:00. Rice varieties were collected in the same order for both control and BPH treatment groups. The samples were cut using scissors on the lowest point of the leaf sheath at ground level. Leaf samples (approximately 60–100 cm tall) including leaf sheathes and leaf blades were collected and flash frozen in liquid nitrogen. All frozen samples were lyophilized (Mo-dulyo®, Thermo Scientific, USA), ground to a fine powder (Phillip HR2020, USA) and sieved to remove any large particulates. All dried powder samples were stored at −80 °C prior to metabolomic analysis (Fig. 1b).

## 2.3 Polar metabolite extraction

The polar extraction method was modified from Graham et al. 2009. Briefly, 0.5 grams of leaf samples were extracted with 10 and 15 mL of ice-cold 80:20 MeOH:$H_2O$, respec-tively, by rigorously mixing samples (MINIMIX, merris, UK) for 10 min before sonicating for 15 min. Samples were subsequently centrifuged at 4000×$g$ followed by a

16,000×$g$ cycle for 20 min at 4 °C each. The supernatants were collected and lyophilized (Christ, UK) overnight until completely dried.

The dried samples were reconstituted in 1 mL of deu-terium oxide (Sigma, cat no. 151882) containing 0.081 μM of the internal standard 3-(trimethylsilyl) propionic-2,2,3,3-$d_4$ acid sodium salt (TSP; Sigma, cat no. 269913) in 0.1 M phosphate buffer (PB, pH 7.0). The reconstituted samples were centrifuged at 16,000 $g$ for 20 min at 4 °C and 600 μL of the supernatant was transferred into 5 mm NMR tubes for analysis.

## 2.4 NMR data acquisition and data processing

All spectral data were acquired on a 400 MHz ^{1}H Avance III NMR spectrometer (Bruker, Germany) at 300 K. Using a randomized running order one-dimensional ^{1}H NMR spectra were acquired across a spectral width of 8.2 kHz into 32 K data points: Relaxation delay = 5 s, t1 = 6 ms. A total of 128 transients were acquired. As with standard one-dimensional spectra, the summed transients were multiplied by a 0.3 Hz apodisation factor prior to Fourier Transformation; chemical shifts ($\delta$) are reported in parts per million (ppm) of the operating frequency and were refer-enced to the TSP resonance ($\delta = 0.00$). Replication of re-sults was insured by running a single sample throughout the experiment and direct comparison of the acquired spectra was used as a measure of reproducibility and quality control (Graham et al. 2012). All the sample spectra were phased and baselines were manually corrected using ACDlabs version 12.0 (Toronto, Canada). Data reduction was carried out by manually binning the spectra and measuring the in-tegral for each bin/bucket between $\delta$0.00 and $\delta$8.50 ppm—a total of 197 buckets were recorded. The region from $\delta$4.70 to $\delta$4.90 ppm, which contained the residual water signal, was excluded. The integrals were normalized to the total spectral intensity prior to chemometric analysis.

## 2.5 Metabolite identification and chemometric analysis

The spectral integrals were identified by comparing their chemical shift and multiplicity to public and commercial databases such as Chenomx NMR Profiler version 7.6 (Chenomx NMR suite 7.6, Chenomx Inc., Canada), the Human Metabolome database (Wishart et al. 2007, 2009, 2013) and the Madison metabolome consortium database (Cui et al. 2008). The identified metabolites were con-firmed using 2D [1]H-^{1}H correlation spectroscopy (COSY), [1]H-^{13}C heteronuclear single quantum correlation (HSQC) and spiking with pure compounds. The metabolite were identified according to minimum reporting standards for chemical analysis (Sumner et al. 2007) (Table S1).

**Fig. 1** Experimental design for brown planthopper (BPH) treatment and rice sample collection. **a** BPH was cultured on a standard susceptible Taichung Native 1 (TN1) cultivar for 2 months (enlarged image showed the BPH in the experiment). In the meantime, three different rice varieties (BPH-susceptible KD cultivar (S), BPH-resistant IL7 containing *Bph3* (R) and BPH-resistant$^+$ IL308 containing *Bph3* and *terpene synthase* (*TPS*) genes (RR) were cultivated in pots until tilling stage for 2 months. Each rice variety was divided into 2 groups, i.e., control group (without BPH feeding) and treatment group (BPH feeding). Each rice variety in treatment group was transferred into an aluminum cage before BPH feeding. Leaf samples of the rice in control and treatment groups were collected at 5 different time points (days 1, 2, 3, 4 and 8), **b** leaf samples were collected by cutting on the lowest point of leaf sheath at the ground level. The collected samples were quickly frozen in liquid nitrogen, ground into fine powders and stored at −80 °C prior to metabolite extraction, and **c** the rice varieties in control and treatment groups were observed for any physiological changes along the time point of the experiment
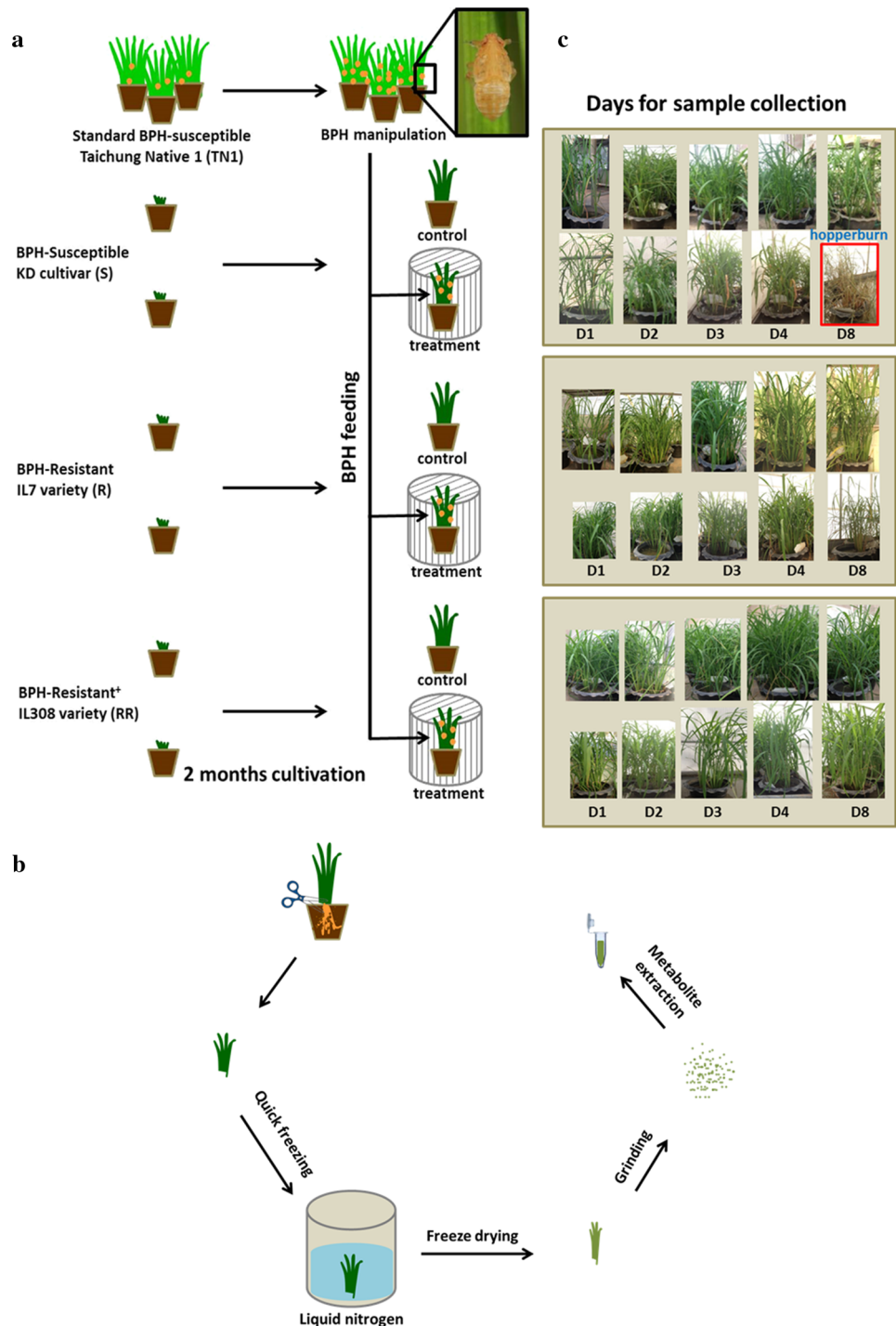


For the chemometric analysis, all spectral integrals were mean centered and Pareto scaled using SIMCA P version 13.0.1 (Umetric, Sweden). Principle Component Analysis (PCA) was employed to generate models to examine the effects of different factors, namely: the rice varieties, the BPH treatment and the time points of the experiment. The metadata has been made available at the MetaboLights metabolomics data repository with accession number MTBLS151 (http://www.ebi.ac.uk/metabolights/MTBLS151).

## 2.6 Metabolite quantification and univariate statistical analysis

The metabolites were characterized by their chemical shift and multiplicity to select the peak region where no overlapping

peaks were observed. The metabolites were quantified using the following equation (Chenomx Inc., Canada):

$$[\text{metabolite}]$$
$$= \frac{\text{No. proton of TSP} \times [\text{TSP}] \times \text{integral of metabolite}}{\text{No. proton of metabolite} \times \text{integral of TSP}}$$

The quantified metabolites of all sample groups in each time point were statistically tested using two-way ANOVA analysis to identify the metabolites significantly affected ($p < 0.05$) by genotype and treatment. The identified metabolites were further analyzed for multiple comparisons across the examined rice varieties by Tukey's post hoc test ($p < 0.05$). In addition, quantified metabolites measured in both control and treatment groups for each time point were also examined using an Unpaired $t$ test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). The univariate statistical analyses were performed using SPSS version 11.5 (SPSS Inc., USA).

## 3 Results and discussions

### 3.1 $^1$H NMR spectra analysis from leaves

Untargeted metabolic profiles of polar extracts were investigated in rice leaves before and after brown planthopper (BPH) infestation at different time points using $^1$H NMR spectrometry. The reproducible and robust nature of $^1$H NMR for accurately quantifying metabolites in a variety of biological samples has been extensively reported in the literature (Holzgrabe et al. 1998; Ward et al. 2010; Bharti and Roy 2012). As previously reported by Graham et al. (2012), as a measurement of quality control, one randomly selected sample was analyzed repeatedly throughout the

entire acquisition (Graham et al. 2012). Direct comparison of the spectra showed us that the data acquisition using $^1$H NMR was stable and highly reproducible throughout.

Firstly, physiological changes among three rice varieties, namely the BPH-susceptible KD cultivar (KD), BPH-resistant IL7 containing *Bph3* (IL7) and BPH-resistant[+] IL308 containing *Bph3* and the *terpene synthase (TPS)* gene (IL308), were observed (Fig. 1c). Different physiological responses were observed in leaves which were dependent on the level of BPH resistance. Specifically, the effect of BPH infestation was obvious on day 8 in the KD cultivars in which the leaves dried out and turned brown exhibiting the classic symptoms of hopper burn while the effect of the BPH infestation was only marginally observed on day 8 in the IL7 cultivar. Contrastingly, there was no effect of BPH treatment on the growth of the IL308.

To examine the differential metabolomic responses to BPH infestation, $^1$H NMR spectroscopy was used to analyze the polar extracts from leaves of all the rice varieties at various time points throughout the experiment. For leaves from all rice varieties, distinct metabolomic changes were observed across the different time points during the infestation and the metabolic differences between the control and the BPH-treated groups could also be identified.

Thirty metabolites associated with BPH infestation were identified to include: 13 amino acids (valine, leucine, isoleucine, alanine, glycine, serine, glutamine, glutamate, asparagine, aspartate, phenylalanine, tryptophan, tyrosine), 6 organic acids (lactate, acetate, γ-aminobutyrate (GABA), formate, fumarate, succinate), 2 sugars (sucrose, glucose), 1 sugar alcohol (mannitol), 4 purine and pyrimidine compounds (uracil, guanosine, uridine and hypoxanthine), 1 phenolic acid (gallic acid) and 3 other chemical compounds (glycine-betaine, choline and allantoin) (Fig. 2). The



1  valine/ leucine /isoleucine
2  fatty acids
3  lactate
4  alanine
5  γ-aminobutyrate
6  acetate
7  glutamine
8  glutamate
9  succinate
10 aspartate
11 asparagine
12 choline
13 betaine
14 glycine
15 mannitol
16 serine
17 sucrose
18 β-glucose
19 α-glucose
20 allantoin
21 uracil
22 uridine
23 fumarate
24 tyrosine
25 gallic acid
26 tryptophan
27 phenylalanine
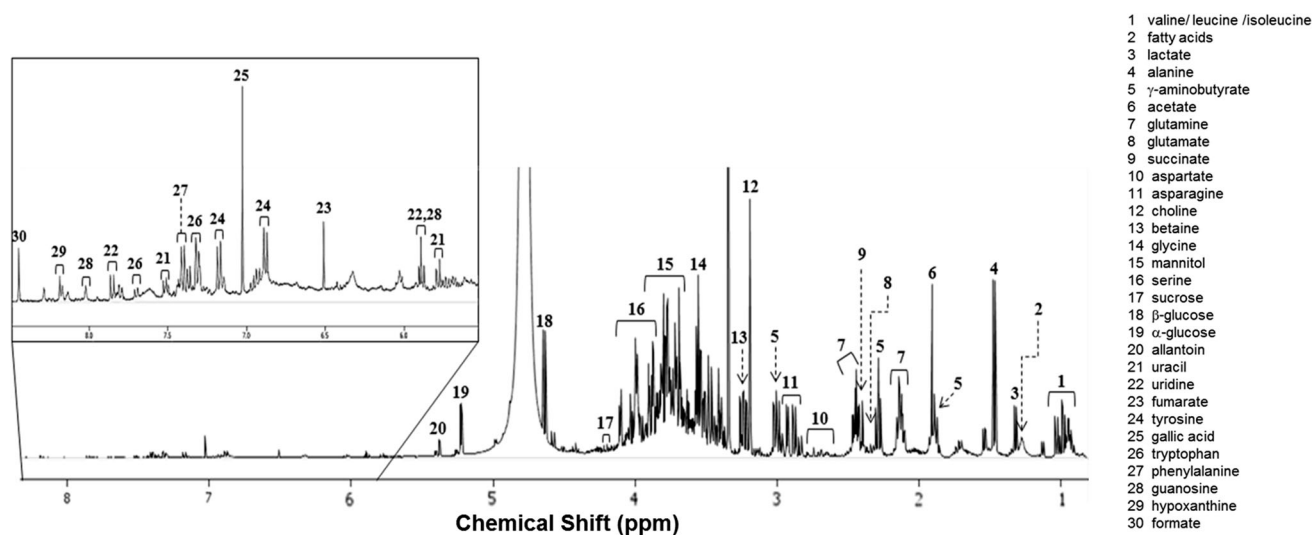28 guanosine
29 hypoxanthine
30 formate

**Fig. 2** Representative of $^1$H NMR spectra of leaf sample in BPH-susceptible KD cultivar after treatment by day 4

identified metabolites were confirmed by 2D NMR spectroscopy (Fig. S1). Most of the identified metabolites were similar to previous studies in rice (*Oryza sativa*) (Barding et al. 2011; Ratcliffe 1997; Liu et al. 2010); however, we report for the first time that there were minor differences in concentrations of guanosine, uracil, mannitol, gallic acid and hypoxanthine. This might be a result of different solvent extractions, different traits and different rice varieties. In this study, the optimal extraction method was chosen based on the signal to noise ratio and coverage of metabolites prior to metabolite analysis. All of the obtained signals were then analyzed for any distinct pattern using chemometric analysis in order to determine significant alteration of patterns between the groups.

## 3.2 Chemometric analysis from leaves

Chemometric analysis was performed to determine why control and treatment groups among the examined rice varieties differ at various time points post infestation. Normalized integrals were Pareto scaled before principal component analysis (PCA) was performed. Firstly, PCA of all the examined samples (N = 3/variety/time point; 90 samples in total) produced a model with $R^2 = 0.950$ and $Q^2 = 0.770$ (Fig. 3a). The PCA scores plot defined two different groups: early response (days 1–3 in the control group and days 1–4 in the treatment group) and late response (days 4–8 in the control group and day 8 in the treatment group). Secondly, the PCA model of each rice variety was obtained (N = 3/time point; 30 samples in total; Fig. 3b–d).

The PCA scores plots of the BPH-susceptible KD variety ($R^2 = 0.890$ and $Q^2 = 0.746$; Fig. 3b) revealed differences in the metabolic profiles among early response (days 1–3 in the control group and days 1–4 in the treatment group) and late response (days 4–8 in the control group and day 8 in the treatment group). The profiles obviously differed during days 4–8 between the control and the treatment groups whereas the profiles during days 1–3 did not show any clear differences. Principle component (PC) 1 accounts for 50.7 % of the variation and PC2 is equal to 19.3 %. Sucrose, glutamine, glutamate and asparagine were found to be at higher concentrations in the early response group whereas choline, glucose, mannitol, glycine, and γ-aminobutyrate (GABA) were found to be at higher concentrations in the late response in the control group. Furthermore, succinate, lactate, acetate and valine were found to be at higher concentrations in the late response in the treatment group.

On the other hand, the PCA scores plots of the BPH-resistant IL7 ($R^2 = 0.891$ and $Q^2 = 0.697$; Fig. 3c) and resistant$^+$ IL308 varieties ($R^2 = 0.874$ and $Q^2 = 0.735$; Fig. 3d) indicated similar profiles which differed from

**Fig. 3** Multivariate analyses of metabolomic responses in rice leaves. **a** Principle component analysis (PCA) scores plot of all the examined rice varieties, **b** PCA scores plot and loading plot of BPH-susceptible KD samples, **c** PCA scores plot and loading plot of BPH-resistant IL7 samples and **d** PCA scores plot and loading plot of BPH-resistant$^+$ IL308 samples. *Red color* represents BPH-susceptible KD variety, *green color* represents BPH-resistant IL7 variety and *blue color* represents BPH-resistant$^+$ IL308 variety. *Circle symbol* represents the samples in control group and *triangle symbol* represents the samples in treatment group. The numbers (*1, 2, 3, 4* and *8*) represent days after BPH infestation (Color figure online)

those of the susceptible KD cultivar. The metabolic profiles between the early and the late response groups could be clearly distinguished. Comparing the control and the treatment groups, the profiles evidently differed on day 4 but showed slight differences on days 1, 2, 3 and 8. The concentrations of sucrose, glutamine, glutamate and asparagine were found to be increased in the early response group whereas the concentrations of acetate, succinate, glycine, glycine-betaine, choline, glucose, mannitol, valine, alanine, lactate and GABA were found to be increased in the late response group.

Chemometric results demonstrated that the metabolic profiles in leaves were responsive to the BPH infestation, and this correlated to the visual physiological changes. Although the metabolite levels of the susceptible and the resistant rice varieties were different, the identified metabolites in this study were the same in all rice varieties. There might be two possible explanations: (i) the genetic background of resistant varieties (IL7 and IL308) are 83–91 % similar to the susceptible variety (KD) because both resistant varieties harboring the resistant genes were backcrossed to KD (Jairin et al. 2009); (ii) the obtained metabolites only represented in the polar extracts, consisting of mainly primary metabolites which might not directly play a role in the resistance mechanism. Previous studies have reported that secondary metabolites such as terpenoids are the major compounds to defend against herbivores attack. For instance, s-linalool, a product of rice monoterpene synthases (Os02g02930), is the abundant volatile compound emitted from armyworm (*Spodoptera frugiperda*)-damaged-rice (Yuan et al. 2008). Furthermore, (E)-β-caryophyllene, a product of OsTPS3, has been identified to be a major inducible volatile sesquiterpene which is indirectly involved in the defense of rice cultivars by attracting parasitoid wasps (*Anagrus nilaparvatae*) (Cheng et al. 2007). To complete a more holistic coverage of the metabolome, other analytical methods such as GC–MS and LC-QTof-MS may be necessary and will provide additional information on primary and secondary metabolites in addition to volatile compounds. This study demonstrates that primary metabolites are influenced by BPH infestation and may also be used for acclimatization
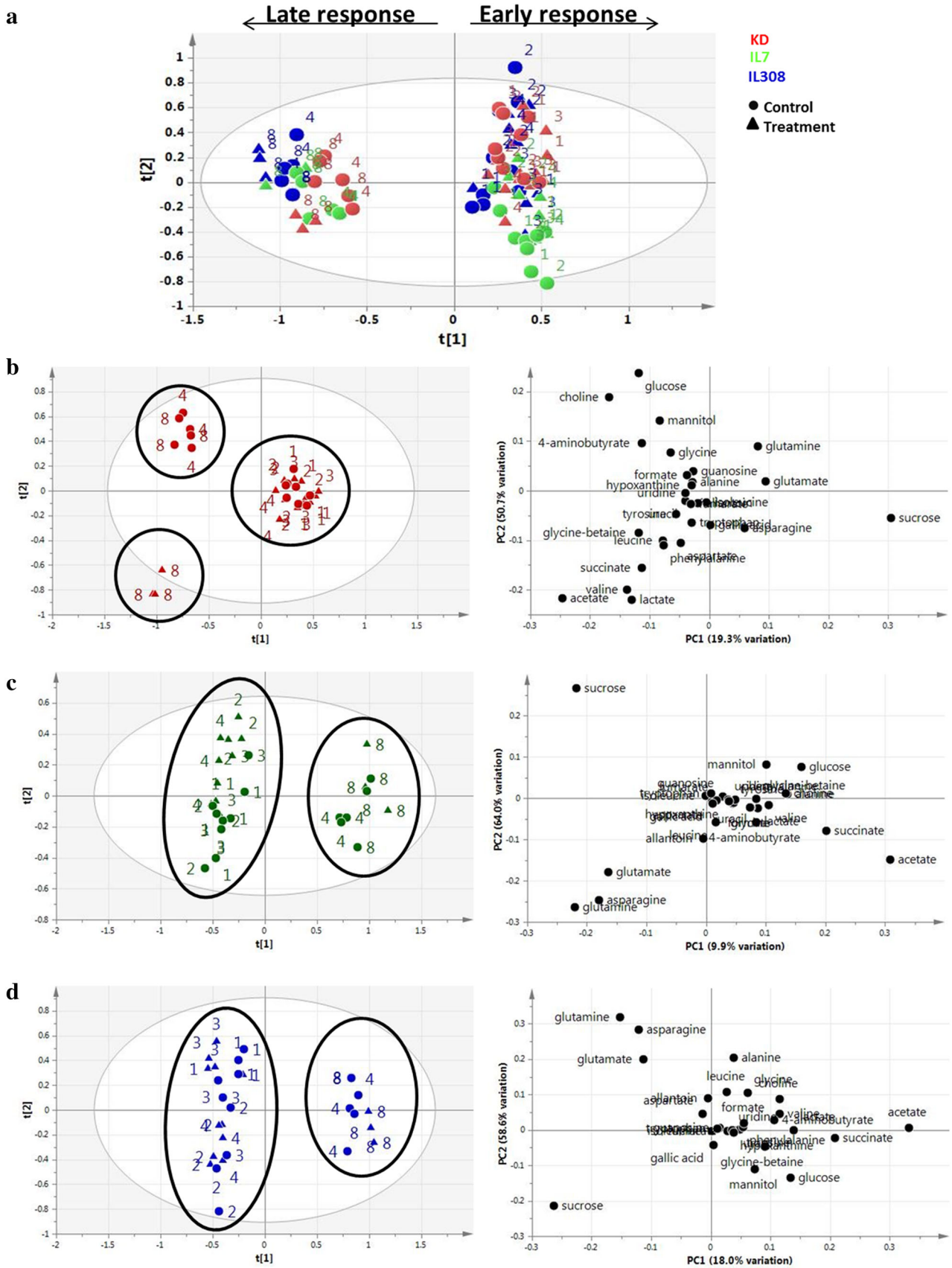
to biotic stress and/or important precursors of secondary metabolites to fight against insect infestation. Consequently, all identified metabolites in the different varieties were quantified to determine the differential metabolite levels before and after BPH infestation at different time points.

### 3.3 Metabolic changes in response to brown planthopper (BPH) infestation

From the identified metabolites, the observed peaks of each metabolite were selected to be quantified as shown in Table S1. Of these, 29 of 30 identified metabolites were quantified to determine the significantly altered metabolites in response to BPH infestation. Quantified metabolites were analyzed by two-way ANOVA to determine whether the metabolite concentrations were affected by two main factors (genotype × treatment) at each time point (Fig. 4). The results demonstrate that the number of significantly different metabolites at the early time points (days 1–3) were less than those at the late time points (days 4–8). With regards to metabolites affected by both main factors and the interaction between the main factors, the concentrations of 21 metabolites were significantly different to include: asparagine, glutamate, glutamine at day 1, lactate, phenylalanine and tryptophan at day 3, GABA, acetate, formate, fumarate, glucose, guanosine, lactate, leucine, phenylalanine, succinate, sucrose, tyrosine, uracil, uridine and valine at day 4 and GABA, aspartate, betaine, formate, glutamine, phenylalanine and tryptophan at day 8. However, there were no metabolites with significantly different concentrations found at day 2.

The metabolites found to be statistically different by the two-way ANOVA were subsequently analyzed by Tukey's post hoc multiple comparisons test to determine whether their concentrations were significantly altered ($p < 0.05$) across the time points and among the three different rice varieties (Table S2). Additionally, three different rice varieties were compared between no BPH feeding (control) and BPH feeding (treatment) groups in parallel at five different time points in order to avoid the metabolic changes caused by any environmental factors. The results of the multivariate analysis demonstrate that the overall spectra of all samples tended to discriminate between control and treatment groups at the different time points. To evaluate which metabolites were significantly altered after the infestation in each rice varieties, an Unpaired Student's *t* test was used to compare between the groups at 5 different time points (Table S2). Consequently, metabolites with significantly altered concentrations were determined based on two-way ANOVA, Tukey's multiple comparison test and an Unpaired Student's *t* test analyses. Of these, 10 metabolites (asparagine, aspartate, glutamate,

glutamine, lactate, leucine, phenylalanine, tryptophan, uracil and valine) had significantly different concentrations between control and treatment groups along the experimental period (early and late response) for each rice variety (Fig. 5a–c).

Three major groups based on the metabolite alteration patterns among the examined rice varieties were identified. The first group with a similar metabolite alteration pattern in all the examined rice varieties contains three metabolites: asparagine, glutamine and glutamate. The concentrations of asparagine, glutamine and glutamate were significantly elevated in the treatment group ($p < 0.05$) at the early time point (day 1 in the susceptible (KD) and the resistant (IL7) varieties, day 3 in the resistant⁺ (IL308) variety; Fig. 5a). The second group with a different pattern between the KD and the resistant (IL7 and IL308) rice varieties had five metabolites which were significantly different: lactate, leucine, phenylalanine, tryptophan and valine. The concentrations of these five metabolites were significantly increased by day 3 in the IL7 and IL308 rice varieties whereas those concentrations were significantly increased by day 8 in the KD variety (Fig. 5b). In the final group, two metabolites (uracil and aspartate) were found to have different patterns in the KD and in the IL308 rice varieties at day 8 of the infestation. The concentration of uracil was significantly elevated by day 8 in the IL308 variety but decreased in the KD variety. Contrastingly, the concentration of aspartate was significantly elevated by day 8 in the KD variety but decreased in the IL7 and IL308 varieties (Fig. 5c).

The results correlated to the multivariate analysis which clearly distinguishes two major groups based on the response patterns (i.e., early/late responses and susceptible/resistant varieties). The effect of both factors (genotype & treatment) and their interactions by day 4 from two-way ANOVA could be suggested that the metabolites with significantly different concentrations might be caused by plant circadian rhythm. As the circadian rhythm may affect metabolite concentrations during growth and development, the time of harvest in all experiments was controlled to avoid such effect in the rice samples; however, we report significant changes ($p < 0.05$) in the concentration of all identified metabolites in the control group at day 4 with an exception to asparagine, glutamine and glutamate. These changes in metabolite concentrations were due to photosynthesis and photorespiration which were essential for plants growth and development (Lorimer and Andrews 1973; Osmond 1981; Sharkey 1985). These findings were consistent with previous studies on the effects of environmental and biological stresses on photosynthetic rate in leaves. For example: Fujita et al. (2003) reported the effect of P-deficiency in tomato fruit and stem (Fujita et al. 2003), Hill and Littlefield 1969 reported the effect of elevated

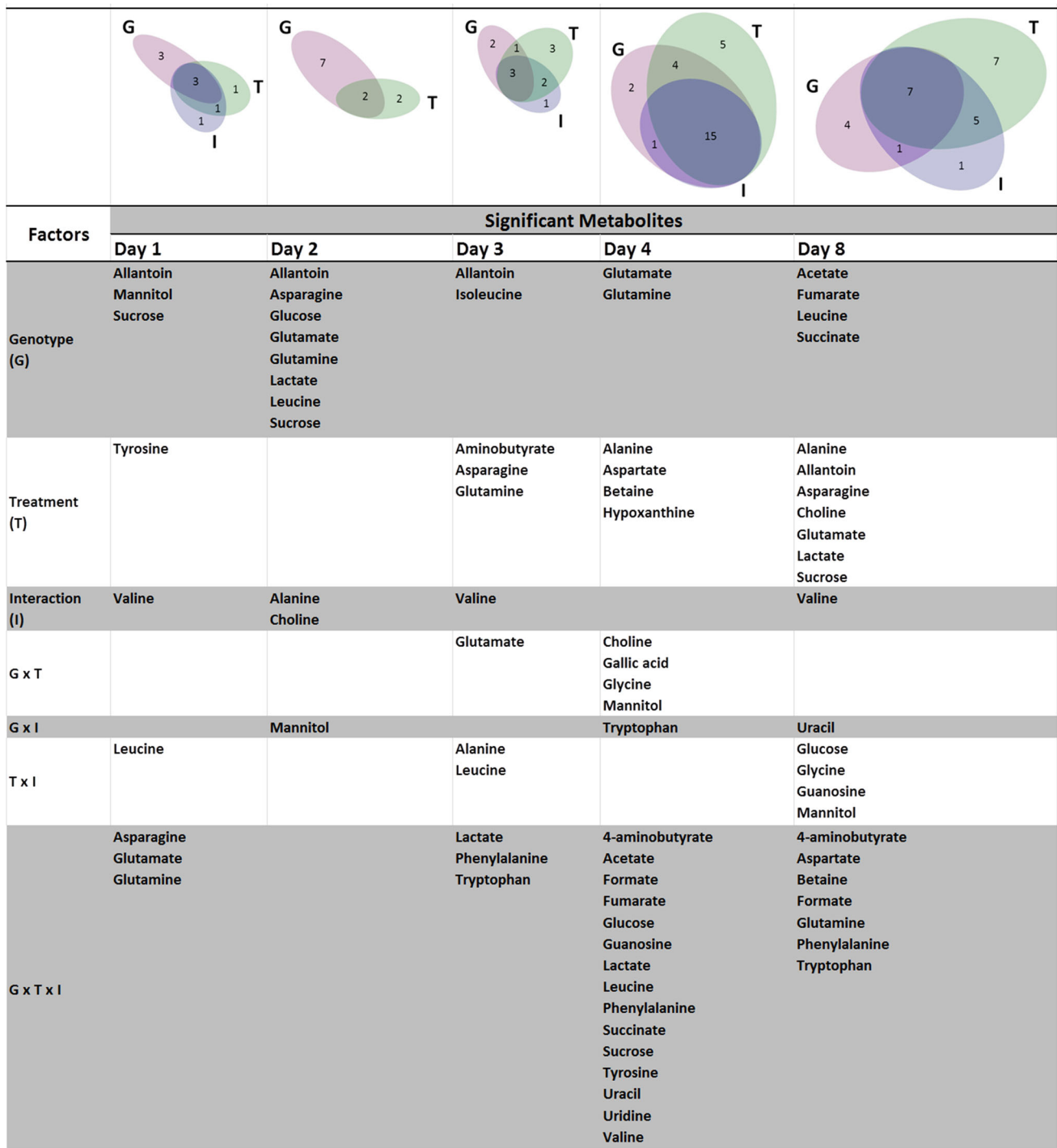| Factors | Significant Metabolites | | | | |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 8 |
| Genotype (G) | Allantoin<br>Mannitol<br>Sucrose | Allantoin<br>Asparagine<br>Glucose<br>Glutamate<br>Glutamine<br>Lactate<br>Leucine<br>Sucrose | Allantoin<br>Isoleucine | Glutamate<br>Glutamine | Acetate<br>Fumarate<br>Leucine<br>Succinate |
| Treatment (T) | Tyrosine | | Aminobutyrate<br>Asparagine<br>Glutamine | Alanine<br>Aspartate<br>Betaine<br>Hypoxanthine | Alanine<br>Allantoin<br>Asparagine<br>Choline<br>Glutamate<br>Lactate<br>Sucrose |
| Interaction (I) | Valine | Alanine<br>Choline | Valine | | Valine |
| G x T | | | Glutamate | Choline<br>Gallic acid<br>Glycine<br>Mannitol | |
| G x I | | Mannitol | | Tryptophan | Uracil |
| T x I | Leucine | | Alanine<br>Leucine | | Glucose<br>Glycine<br>Guanosine<br>Mannitol |
| G x T x I | Asparagine<br>Glutamate<br>Glutamine | | Lactate<br>Phenylalanine<br>Tryptophan | 4-aminobutyrate<br>Acetate<br>Formate<br>Fumarate<br>Glucose<br>Guanosine<br>Lactate<br>Leucine<br>Phenylalanine<br>Succinate<br>Sucrose<br>Tyrosine<br>Uracil<br>Uridine<br>Valine | 4-aminobutyrate<br>Aspartate<br>Betaine<br>Formate<br>Glutamine<br>Phenylalanine<br>Tryptophan |

Fig. 4 Univariate analyses of metabolomic responses in rice leaves. Two-way ANOVA analysis with two main factors (genotype; G × treatment; T) and the interaction (I) was performed and the metabolites with significant differences at each time point were visualized by Venn's diagram

ozone in barley, wheat, tobacco and sugar beet leaves, Delucia et al. (1985) published their findings on the effect of elevated $CO_2$ levels in cotton leaves (Delucia et al. 1985) and Balachandran et al. (1997) published on the effect of viral infection in tobacco leaves. These previous studies found that their photosynthetic rates were slightly increased in normal state whereas those rates were reduced after exposure to stress. Therefore, one could hypothesize that the metabolite alterations in the control group indicate a normal metabolism under non-stress conditions whereas
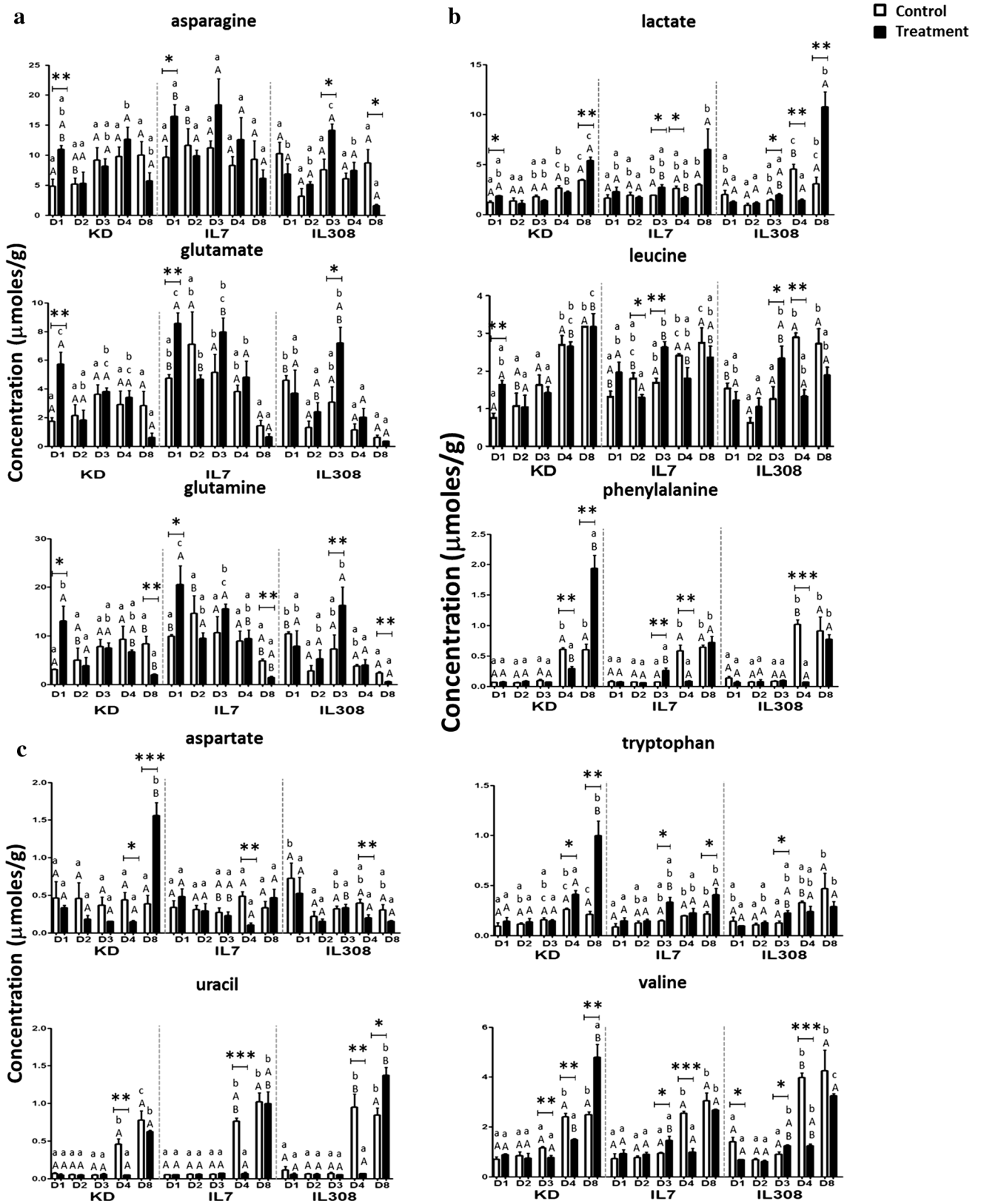
◄**Fig. 5** Significantly altered metabolites (μmoles/g) influenced by BPH infestation with Unpaired *t* test (\**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001). **a** similar pattern of metabolite alteration found in both the susceptible (KD) and the resistant (IL7 and IL308) rice varieties after the BPH treatment by days 1–3, **b** different pattern of metabolite alteration between the susceptible (KD) by day 8 and the resistant (IL7 and IL308) rice varieties after BPH treatment by day 3 and **c** different pattern of metabolite alteration between the susceptible (KD) and the resistant (IL7 and IL308) rice varieties after BPH treatment by day 8. Open bar represents metabolite concentration in control group and solid bar represents metabolite concentration in treatment group. [a,b,c]The *same alphabet* represents no significant differences, *different alphabets* represent significant differences among the time points of the experiment. [A,B,C]The *same alphabet* represents no significant differences, *different alphabets* represent significant differences among the three different rice varieties

the BPH profile represent the metabolism of rice under stress. To further understand the response of Thai Jasmine rice post-infestation, the significantly altered metabolites ($p < 0.05$) were mapped to the metabolic pathways in order to reveal how all examined rice varieties responded to the BPH infestation.

For the first group (days 1–3), asparagine, glutamine and glutamate concentrations significantly ($p < 0.05$) increased during early response (day 1 in the KD and IL7 rice varieties and day 3 in the IL308 rice variety) and were found to be involved in nitrogen assimilation and transamination process, γ-aminobutyric acid (GABA) shunt and gluconeogenesis pathways (Fig. 6). This early response could potentially be induced by the BPH infestation. Generally, nitrogen assimilation employs an ammonium ion ($NH_4^+$) to produce glutamate via the glutamine-synthase/glutamate-synthase (GS/GOGAT) pathway and then glutamate is used to produce asparagine via the transamination process for amino acid biosynthesis (Walker et al. 1984; Rainbird et al. 1984), and for energy production via the TCA cycle (Lehninger et al. 2000). In addition, glutamate can produce GABA via the GABA shunt pathway which is a short metabolic pathway for production of succinic semialdehyde bypassing 2 steps in the TCA cycle. In this relationship, asparagine, glutamine and glutamate may have significant roles in BPH infestation which is one of the biotic stresses in rice. These metabolic processes have been reported to be induced after abiotic and biotic stresses due to reactive oxygen species (ROS) production resulting in hyper-ammonia in plant cells which causes cytotoxicity. To decrease the elevated levels of ammonium in the plant cells, glutamate and asparagine are used for both nitrogen assimilation and transamination of ammonia (Skopelitis et al. 2006; Zhang et al. 2011). For the GABA shunt, the concentration of GABA post-infestation was also found to be elevated though not significantly (Table S2). The results were also correlated to the study of the BPH-resistant B5 (carrying *Bph14* and *Bph15*) and the BPH-susceptible TN1

varieties (Liu et al. 2010). Therefore, it could suggest that BPH attack may affect transamination and GABA shunt which was commonly found in all the examined rice varieties but not for defensive mechanism.

For the second group (days 3–8), four amino acids (leucine, phenylalanine, tryptophan and valine) and an organic acid (lactate) concentrations were significantly increased following BPH infestation by day 3 in the resistant varieties but only reached significance at day 8 in the susceptible variety. These compounds are involved in amino acids metabolism, secondary metabolite biosynthesis and gluconeogenesis pathways (Fig. 6). Tryptophan and phenylalanine act as signaling molecules for growth and development (Luan 2002). These aromatic amino acids can be mediators for nucleotide synthesis via the de novo pathway by cooperating with aspartate (Ashihara et al. 2005). In addition, they were found to be precursors for secondary metabolites in plants such as pigments (Tanaka et al. 2008), fragrance (Gonda et al. 2010) as well as alkaloids (Leete and Marion 1953) and phenolic compounds (Loomis and Battaile 1966) for defense against herbivore attack. With respect to defensive molecules against herbivore attack, tryptophan is a precursor for indole alkaloid and glucosinolate compounds which act as insecticides. One such example is gramine in insect-resistant barley varieties after aphid (*Rhopalosiphum padi*) infestation (Zúñiga and Corcuera 1986). Phenylalanine is a precursor for salicylic acid which was found to be increased after BPH infestation in the B5 rice (Liu et al. 2010). The results suggest that the activation of tryptophan and phenylalanine in the early stage of the infestation may be caused by the activation of *Bph3* in the resistant rice varieties.

Valine and leucine are important amino acids for nitrogen balance in plants (Binder et al. 2007). These glucogenic amino acids also promote gluconeogenesis and are intermediates of TCA cycle; for example, they support respiration in tomato fruit (Kochevenko et al. 2012) and produce succinate which is a mediator for energy production (Satyanarayana and Radhakrishnan 1962). However, valine and leucine were found to be significantly affected by the interaction and treatment × interaction, respectively, from the two-way ANOVA analyses which suggests that these metabolites may not play a role in the defense mechanism in the resistant rice varieties but may be a results of a response to stress.

Additionally, lactate is known to be involved in gluconeogenesis. It is an intermediate for anaerobic glycolysis for the production of energy and it is also an alternative pathway for plants to adapt to different environments (Kennedy et al. 1992). When considered, indicators for photosynthesis and carbon utilization in plants, we found that the glucose concentration was lower in the BPH-treated group than the control group during days 1–8
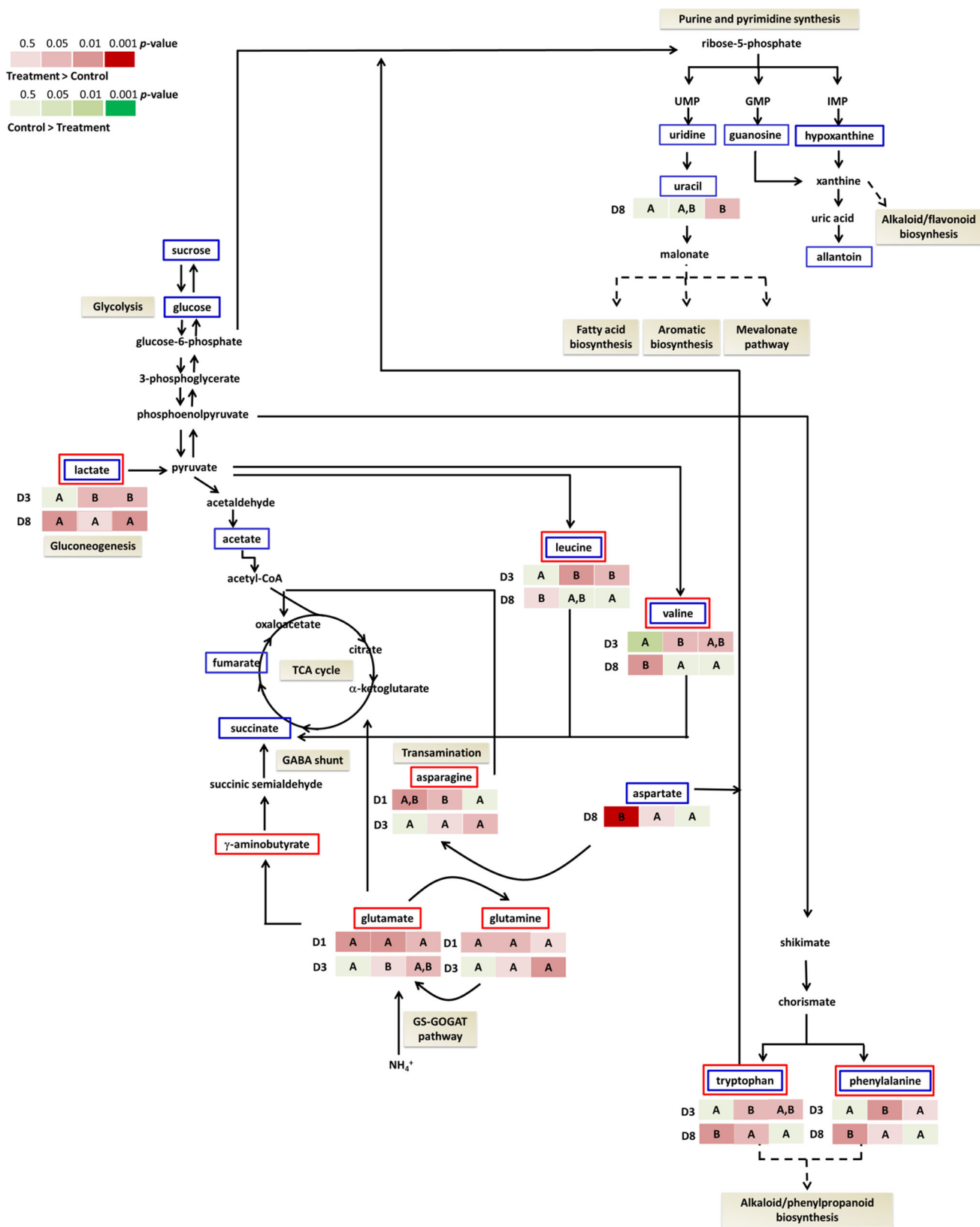
Purine and pyrimidine synthesis

ribose-5-phosphate

0.5  0.05  0.01  0.001 *p*-value
**Treatment > Control**

0.5  0.05  0.01  0.001 *p*-value
**Control > Treatment**

UMP          GMP          IMP

uridine    guanosine    hypoxanthine

uracil                  xanthine

| D8 | A | A,B | B |
|----|---|-----|---|

uric acid          Alkaloid/flavonoid biosynhesis

malonate           allantoin

Fatty acid biosynthesis    Aromatic biosynthesis    Mevalonate pathway

sucrose

**Glycolysis**    glucose

glucose-6-phosphate

3-phosphoglycerate

phosphoenolpyruvate

lactate → pyruvate

| D3 | A | B | B |
|----|---|---|---|
| D8 | A | A | A |

**Gluconeogenesis**

acetaldehyde

acetate          leucine

| D3 | A | B | B |
|----|---|---|---|
| D8 | B | A,B | A |

acetyl-CoA

valine

| D3 | A | B | A,B |
|----|---|---|-----|
| D8 | B | A | A |

oxaloacetate    citrate

fumarate    **TCA cycle**    α-ketoglutarate

succinate

**GABA shunt**

**Transamination**

succinic semialdehyde    asparagine

| D1 | A,B | B | A |
|----|-----|---|---|
| D3 | A | A | A |

aspartate

| D8 | B | A | A |
|----|---|---|---|

γ-aminobutyrate

glutamate       glutamine

| D1 | A | A | A |   | D1 | A | A | A |
|----|---|---|---|---|----|---|---|---|
| D3 | A | B | A,B |   | D3 | A | A | A |

**GS-GOGAT pathway**

NH$_4^+$

shikimate

chorismate

tryptophan          phenylalanine

| D3 | A | B | A,B |   | D3 | A | B | A |
|----|---|---|-----|---|----|---|---|---|
| D8 | B | A | A |   | D8 | B | A | A |

Alkaloid/phenylpropanoid biosynthesis

◄ **Fig. 6** Possible metabolic pathways influenced by BPH infestation with similar and different patterns of metabolite alteration between the susceptible (KD) and the resistant (IL7 and IL308) rice varieties at early and late time points of the BPH infestation. Metabolites in *red* represent the metabolites whose levels elevated at early time points after BPH treatment. Metabolites in *blue* represent the metabolites whose levels elevated at late time points after the BPH treatment. Metabolites in *red* and *blue* represent the metabolites whose levels elevated at early time points in the resistant varieties but at late time point in the susceptible variety after BPH treatment. Different shades of boxes under the names of metabolites represent different levels of significant changes between control and treatment groups indicated by different *p* value calculated from Unpaired *t* test. The *same alphabet* in the *boxes* represents no significant differences whereas the *different alphabets* in the *boxes* represent significant differences between the susceptible and the resistant rice varieties (Color figure online)

whereas the sucrose concentration was higher during days 1–4 and further decreased by day 8. For carbon utilization in plants, sucrose and glucose concentrations are inversely correlated, i.e., sucrose is used as carbon source by being degraded into glucose for production of energy necessary for growth (Koch 2004; Katahira and Ashihara 2002; Zrenner et al. 2006). From these results, it could be hypothesized that the BPH attack may affect the utilization of carbon via sucrose degradation in rice which might lead to an alternative pathway that involves lactate for adaptation post-infestation. Our results suggest that the faster response (day 3) of the five metabolites in the resistant varieties may be from the adaptability for carbon and nitrogen balancing as well as secondary metabolites for defensive mechanisms against the infestation. In contrast, the slower response (day 8) of those metabolites in the susceptible variety might be from stress-induced senescence (as shown in the physiological changes). Thus, the five metabolite concentrations (lactate, leucine, phenylalanine, tryptophan and valine) with different responses in term of time point between the susceptible and the resistant rice varieties might play significant roles on the acclimatization and the BPH resistance.

For the last group (day 8), two metabolites (aspartate and uracil) were found to have different concentrations among the three rice varieties according to the levels of the BPH resistance. The concentration of aspartate was significantly increased in the susceptible compared to the resistant rice varieties. Aspartate is the first amino acid to produce asparagine, glutamine and glutamate which are precursors for nitrogen containing compounds (Lea and Azevedo 2007). Furthermore, aspartate is also used to synthesize nucleotides via the de novo pathway (Zrenner et al. 2006) cooperating with the aromatic amino acids as aforementioned. In contrast, the concentration of uracil was higher in the IL308 variety which is more resistant to BPH than the IL7 and the KD varieties. The pyrimidine nucleotide, uracil serves as coenzymes, in redox reactions, as

energy sources and as important nitrogen compounds (Ashihara et al. 1997; Stasolla et al. 2003). They are also responsible for nucleotide synthesis via salvage pathways which permits recycling of nucleoside, phosphate, nitrogen and carbon (Zrenner et al. 2006). Besides, the purine nucleoside-hypoxanthine, which has the same role as uracil was also found to be increased in the resistant (IL7 and IL308) as compared to the KD varieties which correlated to the QTL of BPH resistance. Hypoxanthine concentration was also higher in treated groups than in the control in the IL308 variety although it was not found to be significantly different (Table S2). These findings are in agreement with gene expression analysis in the BPH-susceptible Minghui63 rice variety that an inosine 5′-monophosphate dehydrogenase (IMPDH) transcript, which is involved in IMP regulation for nucleotide synthesis, was found to be downregulated (Yuan et al. 2005).

Interestingly, hypoxanthine can also be used as a precursor of secondary metabolites such as alkaloid compounds (Ashihara et al. 2008) whereas uracil can be converted to malonate which is a precursor for fatty acid biosynthesis, aromatic biosynthesis and mevalonate biosynthesis (Kim 2002). These secondary metabolites derived from hypoxanthine and uracil have been reported to play a role as defensive metabolites to herbivores attack (Ashihara et al. 2008; Kim 2002). As plant terpenoids can be synthesized from mevalonate pathway and from the induction of fatty acid synthesis pathway (Singh and Sharma 2015), the IL308 containing *TPS* may cause the alteration of these metabolites. Therefore, it could be hypothesized that the resistant[+] IL308 rice variety might respond to the BPH infestation through an alternative pathway such as purine/pyrimidine pathways to produce nucleotides for growth because these pathways consume less energy than de novo pathways that the susceptible KD variety employed. Additionally, the resistant[+] IL308 rice variety could perhaps respond to infestation through the secondary metabolites for the BPH resistance.

In summary, the study of the global metabolic changes associated with the BPH infestation by NMR-based metabolomics reveal both metabolic response between treated and untreated with BPH and between the susceptible and the resistant rice varieties. The metabolites were identified and quantified to determine how these key metabolites are influenced by BPH infestation. A total of 10 key metabolites were significantly altered by the BPH infestation. BPH infestation has caused systematic metabolite alterations involved in GABA shunt and transamination processes in early stage for all the examined rice varieties. Interestingly, in the resistant (IL7 and IL308) varieties, the metabolite alterations involved in amino acid metabolism, shikimate and the gluconeogenesis pathways were altered in the early stage (day 3) of the infestation suggesting it may be caused

by *Bph3* in the resistant rice varieties. In addition, only the resistant[+] IL308 rice variety may utilize purine/pyrimidine-mediated-nucleotides via salvage pathway as well as secondary metabolites synthesis via mevalonate and fatty acids synthesis pathways in the late stage (day 8) of the infestation suggesting it might be caused by *TPS* in the resistant[+] variety. In contrast, in the susceptible (KD) variety, the metabolites involved in amino acid metabolism, gluconeogenesis and amino acid-mediated-nucleotides via de novo pathways were altered at the late stage (day 8) of the infestation suggesting that it may be from stress-induced senescence.

These findings provide important evidence of the acclimatization and defense mechanism of the rice plants via oxidative stress response, balancing C:N ratio and energy production with alternative pathways post-infestation between the susceptible and the resistant rice varieties. Although the primary metabolic responses in this study were similar to the response in the BPH-resistant B5 variety carrying *Bph 14* and *Bph 15* on C and N balancing and oxidative stress response, the metabolites involved in the alternative pathways for energy production and secondary metabolite synthesis were found to be different. The different metabolic responses between the Thai and Chinese rice carrying different *Bph* resistant genes may be due to genotypic difference and *Bph* resistant loci difference. Ultimately, the pathways with elevated levels in the resistant rice varieties (carrying *Bph3* and *TPS*) could be further investigated to uncover further information as to the resistance mechanism.

## 4 Concluding remarks

The application of combining tools such as NMR, multivariate and univariate statistical analysis can rapidly help identify the major changes and illustrate the effect of the BPH infestation in Thai Jasmine rice variety and its different levels of BPH resistant varieties. The significantly ($p < 0.05$) altered post-infestation have been reported to be involved in several relevant functions such as nitrogen assimilation, transamination, oxidative stress response, gluconeogenesis, nucleotide synthesis and secondary metabolite processes. Therefore, metabolic profiling obtained from these approaches could be preliminarily employed for the comprehensive study of plant–insect interaction to find effective ways to control and prevent the disease in the near future. Further studies will be required to focus on examination of secondary metabolites and non-polar compounds using LC-QTof-MS and GC–MS to help finding the potential metabolites important for resistance mechanism.

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