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Original Article

Systematic analysis of rice (*Oryza sativa*) metabolic responses to herbivory

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ABSTRACT

Plants defend against attack from herbivores by direct and indirect defence mechanisms mediated by the accumulation of phytoalexins and release of volatile signals, respectively. While the defensive arsenals of some plants, such as tobacco and Arabidopsis are well known, most of rice's (*Oryza sativa*) defence metabolites and their effectiveness against herbivores remain uncharacterized. Here, we used a non-biassed metabolomics approach to identify many novel herbivory-regulated metabolic signatures in rice. Most were up-regulated by herbivore attack while only a few were suppressed. Two of the most prominent up-regulated signatures were characterized as phenolamides (PAs), *p*-coumaroylputrescine and feruloylputrescine. PAs accumulated in response to attack by both chewing insects, i.e. feeding of the lawn armyworm (*Spodoptera mauritia*) and the rice skipper (*Parnara guttata*) larvae, and the attack of the sucking insect, the brown planthopper (*Nilaparvata lugens*, BPH). In bioassays, BPH insects feeding on 15% sugar solution containing *p*-coumaroylputrescine or feruloylputrescine, at concentrations similar to those elicited by heavy BPH attack in rice, had a higher mortality compared to those feeding on sugar diet alone. Our results highlight PAs as a rapidly expanding new group of plant defence metabolites that are elicited by herbivore attack, and deter herbivores in rice and other plants.

Key-words: defence; diterpene phytoalexins (momilactones); feruloylputrescine (FP); herbivore damage; metabolomics; *p*-coumaroylputrescine (CoP); phenolamides (PAs); rice (*Oryza sativa*).

INTRODUCTION

Plants are regularly attacked by herbivorous insects from various feeding guilds. The most common feeding guilds are the leaf chewers, leaf miners, gall-makers, stem borers, root feeders and piercing–sucking herbivores; however even more categories exist in complex natural environments, such as tropical forests (Novotny *et al.* 2010). In addition, specialist insects feed on a few or even a single host species, usually belonging to the same or related plant family. Other herbivores, known as generalists, are able to exploit a wide(r) range of plants as alternative food sources (reviewed in Ali & Agrawal 2012). The existence of feeding guilds and different levels of specialization of insect herbivores are thought to be the consequences of natural selection that has guided the evolution of complex plant-herbivore food webs. The co-evolution of plants and insects caused vast diversity of natural products in terrestrial plants (McKey 1974; Awmack & Leather 2002; Fürstenberg-Hägg *et al.* 2013).

However, in another process, domestication by humans, potentially useful ancestors of modern crops were selected to increase their nutritional quality and yield (Bai & Lindhout 2007). Domestication was necessary for the worldwide expansion of human race, but it may have compromised the existing natural balance, chemical composition and innate defence of what has become a crop (Chaudhary 2013; Turcotte *et al.* 2014). The existence of trade-off between growth and defence against herbivores (Mayrose *et al.* 2011; Lind *et al.* 2013), based on limited energy and resource allocation in plants (Coley *et al.* 1985; reviewed in Strauss *et al.* 2002), could be a plausible mechanistic explanation for negative impact of domestication on innate plant defences. In addition, worldwide distribution of crops to entirely new ecosystems, and growing them in monocultures, exposed them to new insect pests and made them particularly vulnerable. This form of collateral damage from the breeding process may have contributed to the use of large amounts of agrochemicals, such as insecticides and herbicides for pest control. Alternative protection, for example through wider application of evolution-tested natural plant products against insects, applied to susceptible crops through modern technologies such as transgenesis or marker assisted breeding should be reconsidered as important measures for environmental protection and reduction of agrochemical pollution (Bai & Lindhout 2007). This goal calls for deeper analysis of the chemical potential existing in plants, and for better protection of natural diversity and preservation of valuable bioresources for future applications.

Rice is one of the most important staple crops in Asia and is highly susceptible to damage from insects, both directly from

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feeding exposure or indirectly from viruses transmitted by the insect vectors. For example, rice stripped stem borer, *Chilo suppressalis* (Lepidoptera: Pyralidae) is recognized as an important plant chewing insect in China (Jiang *et al.* 2011). Brown planthopper (BPH), *Nilaparvata lugens* (Homoptera: Delphacidae), is one of the most destructive sucking pests causing hopperburn and spreading rice ragged stunt virus (RRSV), as well as rice grassy stunt virus (RGSV) (Hibino 1996). At present, several quantitative trait loci (QTL) and genes responsible for resistance to BPH in rice have been identified (Kobayashi *et al.* 2014), showing that defence against sucking insects in rice depends on a specific gene cluster of three plasma membrane-localized lectin receptor kinases (OsLecRK1–OsLecRK3; Liu *et al.* 2015). However, the downstream molecular mechanisms of resistance such as direct involvement of chemical defence in rice remain unknown.

In addition to mechanical damage, herbivores can release/deposit specific elicitors contained in their oral secretions (OS; regurgitate) or saliva into the wounds (Maffei *et al.* 2012). Recognition of these elicitors allows plants to discriminate attack from various types of herbivores, as well as differentiate herbivory and simple mechanical damage (Halitschke *et al.* 2001). External herbivory-associated signals are mediated by jasmonate (JA) signaling that causes wide transcriptional reprogramming and changes in defence potential of plants (Woldemariam *et al.* 2011). For example, tobacco and Arabidopsis impaired in JA signalling accumulated less chemical defences and became more susceptible to insect pests (Heiling *et al.* 2010; Kaur *et al.* 2010; Guo *et al.* 2013). In rice, JA-deficient plants contained less proteinase inhibitors (PIs) that inhibit insect growth by interfering with insect digestion process (Zhou *et al.* 2009; Ye *et al.* 2012). Apart from PIs, however, the identity of rice defence metabolites targeted against insects remains elusive. Amongst small molecules, two classes of defence metabolites, flavonoids and diterpene phytoalexins are often linked to rice defence (Yamane 2013; Dong *et al.* 2014). In particular, momilactones A (MoA) and B (MoB) are well-known rice allelopathic chemicals against other plant competitors (Kato-Noguchi *et al.* 2002; Kato-Noguchi & Peters 2013). Momilactones also protect rice against some microbial pathogens, bacteria and fungi (Hasegawa *et al.* 2010; Toyomasu *et al.* 2014). Accordingly, enzymes in the momilactone pathway, namely OsCyc1 and OsKS4 genes, are stress inducible, for example by chitin oligosaccharide elicitor or ultraviolet (UV) irradiation (Shimura *et al.* 2007; Kato-Noguchi 2011). Other compounds, such as the flavonoids (e.g. sakuranetin) and phenolamides (PAs) N-*trans*-cinnamoyltryptamine and N-*p*-coumaroylserotonin were also induced by UV in rice, showing antibacterial activity to pathogenic microorganisms *Xanthomonas oryzae* pv. *Oryzae*, *X. oryzae* pv. *Oryzicola* and *Burkholderia glumae* (Park *et al.* 2014). JA-dependent accumulation of sakuranetin, together with MoA correlated with resistance to the rice blast fungus, *Magnaporthe oryzae* (Riemann *et al.* 2013; Hasegawa *et al.* 2014). Interestingly, sakuranetin accumulation was also associated with a rice variety resistant to the stem nematode *Ditylenchus angustus* (Plowright *et al.* 1996). Although herbivory leads to rapid accumulation of JA in rice (Fukumoto *et al.* 2013) and exogenous JA addition induced momilactone biosynthesis in rice (Nojiri *et al.* 1996), a direct role of momilactones in defence against chewing herbivores has never been clearly established.

To strengthen our knowledge of chemical defence of rice against herbivores, and to provide novel tools for the improvement of rice resistance to insect pests, we conducted extensive metabolic analyses of *Oryza sativa* L. cv. Nipponbare (Japonica type), a model monocot plant. In the first experiment, a laboratory strain of the rice-adapted generalist pest, the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Groot *et al.* 2008) was used for untargeted metabolomics that revealed a broad spectrum of metabolic responses to chewing insects in rice. Amongst differentially regulated metabolic signatures, two PAs, *p*-coumaroylputrescine (CoP) and feruloylputrescine (FP) were identified and confirmed as novel herbivory-elicited chemicals in rice. Next, functional analysis using native rice herbivores collected in the rice paddy was performed to establish biological roles of PAs and other metabolites in these plants. Insects from different feeding guilds and specialization levels were used for generalization of our results. Namely, we used two chewing herbivores, a generalist lawn armyworm, *Spodoptera mauritia* (Lepidoptera: Noctuidae) and a rice specialist *Parnara guttata* (Lepidoptera; Hesperiidae), known as the common straight swift or rice plant skipper, together with a specialist phloem sucking pest BPH (*N. lugens*). Finally, we report that simulated herbivory and elicitors contained in the OS of feeding *S. mauritia* can amplify PA accumulation in rice plants.

MATERIALS AND METHODS

Plant cultivation

Rice plants (*O. sativa* L. var. Nipponbare) were germinated in nutrient-rich soil Kumiai Ube Baido No.2 (MC Ferticom, Tokyo, Japan). After 10 d, plants were transferred to larger pots with paddy field soil mixed in 5:1 (*v/v*) ratio with the same nutrient-rich substrate. The seedlings were maintained at 24–26 °C day/20–22 °C night temperatures and ambient humidity at 16 h photoperiod in the growth room supplemented with fluorescent light. In case of the metabolomics experiment conducted at Max Planck Institute (MPI) for Chemical Ecology in Jena, Germany, the plants were maintained in a glasshouse supplemented with light from 400 and 600 W sodium lamps (Philips Sun-T Agro; http://www.nam.lighting.philips.com).

Insects

Freshly hatched *S. frugiperda* larvae for metabolomics experiments were obtained from the Department of Entomology, MPI for Chemical Ecology (Jena, Germany). Larvae were kept on pinto bean-based artificial diet until second instar and transferred to rice leaf one day prior to metabolomics experiment. Colonies of two rice chewing herbivores, a generalist insect lawn armyworm, *S. mauritia* and a specialist rice herbivore, common straight swift (rice skipper), *P. guttata*, originally collected in the paddy field (Kurashiki, Okayama Prefecture, Japan),

were seasonally maintained in the laboratory. Although *P. guttata* is considered as rice specialist in this area, and larvae feed mostly on rice, the adults regularly migrate to southern regions of Japan for overwintering (Seko *et al.* 2006). Rice leaves were used as diet for *P. guttata*, while the generalist *S. mauritia* colony was supplied with a combined diet consisting of rice leaves and a pinto bean-based artificial diet. A colony of field-collected sucking insects, BPH (*N. lugens*) has been maintained in the laboratory since 2013 on a constant supply of young rice seedlings.

Wounding and herbivory treatments

In wounding and simulated-herbivory treatments, the youngest fully developed leaf on each seedling (5–6 weeks old in the growth room) was wounded with a fabric pattern wheel along the midvein, and the wounds were immediately treated with 20 μL of water (W) or 20 μL of water-diluted (1:5 *v/v*) OS collected from 4 to 5th instar *S. mauritia* or *P. guttata* larvae. Insects were always reared on rice prior to OS collection. In direct herbivore exposure, chewing herbivores were kept on the youngest developed leaves in small 6 × 4.5 cm clip cages attached to the leaves as shown in Fig. 6A. Two second-instar larvae starved for at least 3 h prior to the start of experiment were applied to each leaf. Five adult BPH insects (*N. lugens*) were applied in a larger 9 × 9 cm clip cage attached to a single leaf on each plant. The whole leaves including herbivore-exposed local (damaged) and surrounding systemic (undamaged) parts were collected and used for analysis. However, in local infestation experiment by high number of BPH shown in Fig. 6, BPH adults (10–15) were enclosed in a smaller clip cage 6 × 4.5 cm on the leaf (Fig. 6A), and only the insect-exposed areas (ca. 5 cm leaf length) were collected for analysis at designated time points.

Metabolomics treatment and sampling

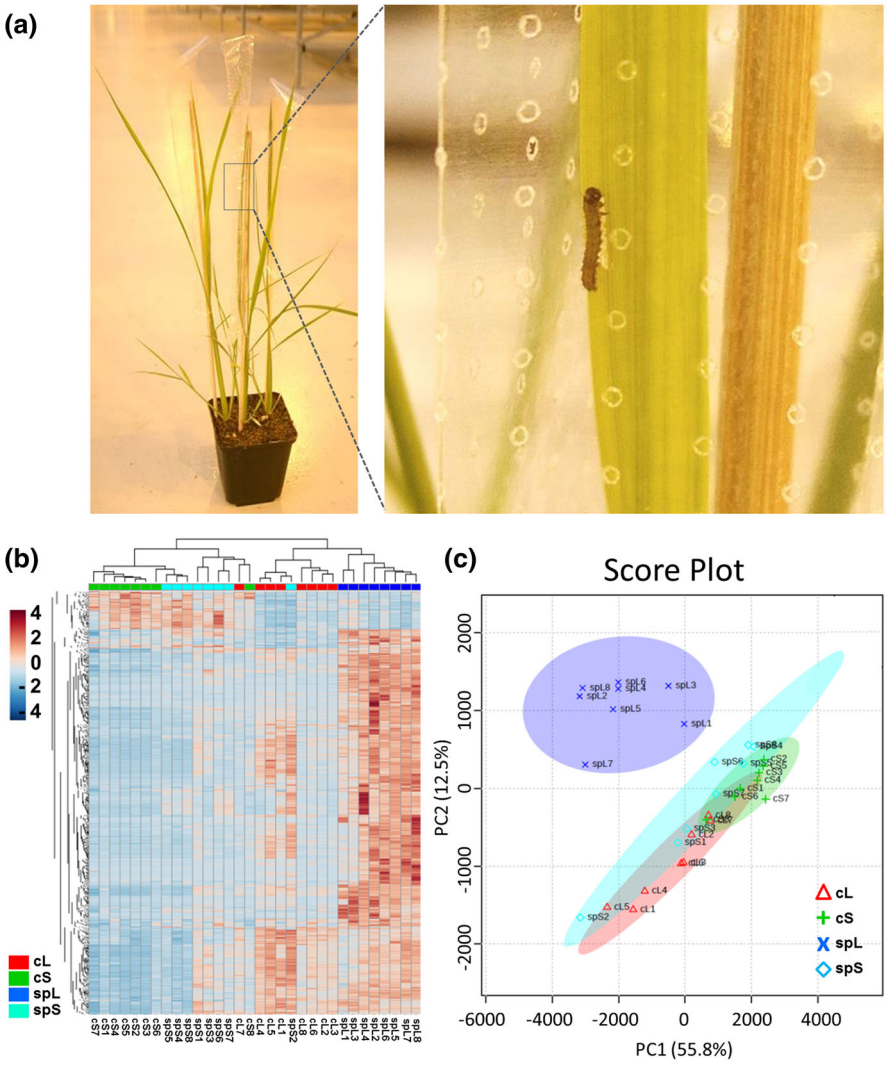
One starved second-instar *S. frugiperda* larva was placed on the youngest developed leaf in a 6-week-old rice seedling ($n = 24$) in the glasshouse, and its movement was limited by a perforated plastic bag covering the entire herbivory-exposed leaf (Fig. 1A). Identical sets of plants without herbivores but covered with perforated plastic bags were used as controls. After 3 d of continuous larval feeding, herbivory-damaged (local, designated as spL) and the second youngest (unattacked) leaves (systemic, spS) were collected from herbivore-attacked plants. Complementary leaves from the control set of untreated plants were harvested at the same time (designated as control local, cL; control systemic, cS). To reduce variability caused by differences in feeding rate of individual larvae, each local (L; $n = 8$) and systemic (S; $n = 8$) sample was prepared by pooling the leaves from three individually treated plants growing in the same pot. The same procedure was applied to the control set of samples. All samples were snap frozen in liquid nitrogen and maintained at −80 °C until metabolomics analysis.

Metabolomics analysis

The ultra performance liquid chromatography–electrospray ionization–time of flight–mass spectrometry (UPLC–ESI–TOF–MS) analysis was essentially carried out as described in Onkokesung *et al.* (2012) with minor modifications applied during sample preparation. About 100 mg of leaf powder prepared in liquid nitrogen was mixed with exactly 7.5 μL of extraction buffer [40% (*v/v*) MeOH in 84 mM ammonium acetate buffer, pH 4.8] per mg tissue in 2 mL screw-cap plastic tubes containing small metal balls. Samples were homogenized in a ball mill (Geno/Grinder 2000; SPEX CertiPrep) for 45 s at 250 strokes per min and centrifuged at 16 000 *g*, 4 °C for 30 min. Supernatants were transferred into clean set of 2 mL microcentrifuge tubes, and pellets were re-extracted with the same volume 80% (*v/v*) MeOH in buffer as before. Homogenate was mixed at 4 °C in extraction buffer for 10 min and centrifuged as before. Supernatants from both extractions were pooled, briefly centrifuged to remove solid particles and used for UPLC–ESI–TOF–MS analysis. Two microlitres of extract was separated by a Dionex Rapid Separation Liquid Chromatography (LC) system using a Dionex Acclaim 2.2 μm 120 A, 2.1 × 150 mm column. Binary separation gradient was applied at flow rate of 300 μL per min: 0 to 5 min, isocratic 95% A, 5% B; 5 to 20 min, linear gradient to 32% B; 20 to 22 min, linear gradient to 80% B; isocratic run for 6 min. Eluted metabolites were detected by a MicroToF mass spectrometer (Bruker Daltonics) with an ESI source operating in either positive or negative ionization modes. Typical instrument settings were capillary voltage, 4500 V; capillary exit, 130 V; dry gas temperature, 200 °C; dry gas flow 8 L min^{-1}. Detected ion range was set between *m/z* 75 and 1400 at a repetition rate of 1 Hz. Mass calibration was performed using sodium formate clusters occurring in 10 mM solution of NaOH in 50% *v/v* isopropanol/water with 0.2% formic acid. Atropine (200 ng mL^{-1} extraction solvent, LABEL p1147 in Supplemental Table 1), reserpine (600 ng mL^{-1}; LABEL p3062) and glycyrrhizinic acid (1000 ng mL^{-1}; LABEL n3045) were added prior to extractions as positive controls to each sample.

UPLC–ESI–TOF–MS data analysis

The UPLC–ESI–TOF–MS data were processed as described previously (Onkokesung *et al.* 2012; Gaquerel *et al.* 2013b). Raw data files in netCDF format were processed with the XCMS package (http://metlin.scripps.edu/download/) in R software. Peak detection was performed using the 'centWave' method [ppm = 20, snthresh = 10, peakwidth = c(5,18)]. For alignments, XCMS 'group' method was repeated twice with a retention time correction in between (minfrac = 0.5, bw1 = 10, bw2 = 3, mzwid = 0.01, span = 1, extra = 0, missing = 0). Areas of missing features were estimated using the 'fillPeaks' function in XCMS. Ion traces were deconvoluted, and putative in-source pseudospectra were reconstructed with the R package CAMERA (http://www.bioconductor.org/packages/release/bioc/html/CAMERA.html) and default parameters (Kuhl *et al.* 2012; Supplemental Table 1). Data matrixes including both positive and negative MS data were imported into



(d)

	spL/cL (local)		spS/cS (systemic)	
	Positive	Negative	Positive	Negative
Up 2-fold	526	545	479	340
Down 2-fold	23	20	15	21
others	3094	2601	3149	2805

Figure 1. Metabolomics analysis of herbivory-elicited rice seedlings. (A) Second instar larvae of a rice-adapted strain of *Spodoptera frugiperda* were allowed to feed for 3 d on an isolated youngest rice leaf. Samples from herbivory treated and control leaf samples were subjected to ultra performance liquid chromatography–electrospray ionization–time of flight–mass spectrometry (UPLC–ESI–TOF–MS) analysis. (B) Heatmap of top 2105 analysis of variance (ANOVA) ($P < 0.001$; Fisher's least significant difference (LSD)) metabolic features obtained in positive and negative MS modes. (C) Principal component analysis of filtered 4085 metabolic features obtained in positive and negative MS modes. (D) Numbers of significantly down- and up-regulated features analysed by Student's *t*-test ($P < 0.05$; >twofold). Sample labels: cL, control local leaf; spL, *S. frugiperda*-fed local leaf; cS, control systemic leaf; spS, leaf systemic to *S. frugiperda*-fed leaf.

Microsoft Excel for further analysis in the statistical module of MetaboAnalyst 3.0 suite online (http://www.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtml). Features with >75% missing values were automatically removed in the uploaded peak intensity table and replaced by a small value (half of the minimum positive value in the original data). Sample set was filtered based on relative standard deviation (SD/mean) to remove the non-informative near constant variables throughout the experiment (40% of features; Supplemental Table 1). Data were normalized by Pareto scaling (mean centred and divided by the square root of standard deviation of each variable; Supplemental Table 1) and subjected to one-way analysis of variance (ANOVA, $P < 0.05$, Fisher's least significant difference (LSD) post-hoc test; Supplemental Table 1). Principal

component analysis (PCA) was conducted with 4085 filtered features and Heatmap visualization was performed with a subset of 2105 ANOVA significant features (Pearson distance measure, complete clustering algorithm; $P < 0.001$). Fold changes were calculated in Microsoft Excel spreadsheet and significant +/− twofold regulated features were analysed by Student's *t*-test ($P < 0.05$) (Fig. 1D and Supplemental Table 1).

Triple quadrupole LC-MS

Using identical extraction procedure as above, selected differentially accumulated metabolites from metabolomics analysis were analysed with triple quadrupole LC-MS/MS 6410 (Agilent Technologies, Santa Clara, CA, USA) equipped with a Zorbax SB-C18 column [2.1 mm id × 50 mm, (1.8 μm), Agilent Technologies]. Metabolite separation in 10 μL extract was performed with solvent A [0.1% (*v/v*) formic acid in water] and solvent B [0.1% (*v/v*) formic acid in acetonitrile] in time (min)/B (%) gradient: 0/5, 0.5/5, 2/40, 6/40, 10/95, 15/95, 16/5, 20/5 at a constant flow rate of 0.4 mL min^{-1}. Two detection modes of low resolution MS were used: (1) selected ion monitoring (SIM) mode was used to detect specific *m/z* of metabolites found during UPLC–ESI–TOF–MS. Signal intensities of peaks (*m/z*) at estimated retention times were integrated, corrected to fresh masses of extracted samples and compared between control and *S. mauritia*-infested leaf samples (Supplemental Figure S2). (2) multiple reaction monitoring (MRM) mode was used for routine quantification of PAs, and MoA and MoB. Prior to MRM analysis of PAs and momilactones, crude metabolite extracts were diluted in 84 mM ammonium acetate buffer (pH 4.8) to obtain 20% MeOH extract, which was then loaded to 3 mL Bond Elut C18 SPE cartridges (Agilent Technologies). Samples were eluted with 1.5 mL of MeOH, briefly centrifuged to remove any particles and used for analysis. MRM conditions and compound quantification were optimized using synthetic standards of PAs, CoP, caffeoylputrescine (CP) and FP, and purified MoA and MoB, kindly provided by K. Okada, University of Tokyo (Shimizu *et al.* 2008). Mass transitions: metabolite/Q1 precursor ion (*m/z*)/Q3 product ion (*m/z*) were monitored for each compound: CoP/235.3/147.2; CP/251.3/163.2; FP/265.3/177.1; MoA/315.2/271.2; MoB/331.0/269.0. The fragmentor (V)/collision energy (V) were set to 100/15 for CoP; 100/16 for CP; 110/12 for FP; 110/10 for MoA and MoB.

High-performance liquid chromatography (HPLC) analysis

Previously described chromatographic conditions (Onkokesung *et al.* 2012) and Agilent HPLC 1100 series system (http://www.chem.agilent.com) were used for the detection of UV-absorbing materials in rice extracts. Briefly, 1 μL aliquots of extracts from metabolomics experiment were applied by an autoinjector in a Chromolith FastGradient RP 18-e column (Merck) guarded by a precolumn (Gemini NX RP18). The mobile phases A [0.1% (*v/v*) formic acid + 0.1% (*v/v*) ammonium water, pH 3.5] and B (methanol) were used in a gradient mode time/concentration (min/%) for B: 0.0/0, 0.5/0, 6.5/80, 9.5/80, followed by 5 min at 0% B for reconditioning. The solvent flow rate was 0.8 mL min^{-1}, and column oven temperature was set at 40 °C. Chromatograms were analysed at 254, 320 and 360 nm wavelengths to find corresponding UV-absorbing peaks. A set of 13 distinct peaks was integrated and provided in the Supplemental Figure S1, after adjusting the signal intensities to the same amount of initial material used for extractions (i.e. 1 mg tissue was extracted in 15 μL of buffer).

PA synthesis

Either hydroxycinnamic acid (3 meq) and dicyclohexylcarbodiimide (DCC) (3.3 meq) was dissolved in 40 mL tetrahydrofuran (THF) and stirred at room temperature for 2 h. The acid–DCC mixture was then added dropwise to 3.3 meq putrescine dissolved in 40 mL THF under continuous stirring. After an additional 24 h of stirring, the reaction mixture was filtered and precipitate washed with THF until yellow colour was maximally reduced. THF was evaporated, and the solid residue was dissolved in 25 mL 1 M HCl, centrifuged to remove insoluble materials and extracted with 3 × 25 mL dichloromethane (DCM). The DCM phase was discarded, and the aqueous acid fraction containing PAs was evaporated by rotary evaporator at 55 °C. Dry material was dissolved in 1–2 mL water and briefly centrifuged to remove remaining insoluble materials. PA solution was applied to silica 60 column (30–60 g) equilibrated in water. After collecting the fractions, PA-containing fraction(s) were identified by UV-spectral analysis with Nanodrop (Thermo Scientific, Wilmington, USA) instrument (peaks/shoulders around 295 and 315 nm). Water was evaporated under vacuum in the SpeedVac system (Sakuma, Tokyo, Japan), and dry material was stored with silica gel beads at 4 °C until use.

Herbivore bioassays

Chewing herbivores, *S. mauritia* and *P. guttata*, were reared on rice leaf diet until they became second instars. For bioassays, a single larva was inserted into a 15 mL glass tube with a rice leaf fixed by small piece of soft sponge at the bottom. The tubes (upside down) were set in racks with sponge partially submerged in water to prevent wilting of rice leaves. Leaves were painted with PA solution in water (or water as control) to distribute evenly 200 μg of the compound on the surface. The fresh mass of larva was determined before, and after 4 and 6 d of feeding. Fresh leaves were introduced at each larval mass determination. Survival rate of BPH on 15% sugar solution containing 0, 25 and 100 μg mL^{-1} PAs was determined using 50 mL plastic tubes with perforated lids. A smaller 2 mL tube with diet covered with thin layer of parafilm was inserted in lid opening, and 10 BPH adults were allowed to feed inside for 3 d. Surviving versus dead animals were counted after visual observation every day.

Statistical analysis

Statistical analyses were carried out with open source OpenStat software (ANOVA) or Microsoft Excel (Student's *t*-test).

Statistics implemented in the MetaboAnalyst 3.0 suite online (http://www.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtml) were used where applicable as described above (Fig. 1B–D; Supplemental Table1).

RESULTS

Metabolomics analysis of rice responses to herbivory

To examine the full potential of rice defence against herbivores, we used an untargeted metabolomics approach with a laboratory-reared rice-adapted strain of fall armyworms, *S. frugiperda* (Lepidoptera; Noctuidae) and seedlings of rice (*O. sativa*) cultivar Nipponbare (Fig. 1A). The UPLC–ESI–TOF–MS chromatograms were processed with the XCMS package of R software to extract individual metabolic features characterized by (1) precise molecular or ion-source-generated fragment masses and (2) corresponding retention times (RT). A metabolic heatmap of positive and negative *m/z*–RT pairs (Fig. 1B) was generated by MetaboAnalyst suite from the set of 2105 most significant metabolomics features identified by ANOVA ($P < 0.001$; Fisher's LSD; Supplemental Table 1). Here, we found a surprisingly large number of potential metabolites up-regulated locally by *S. frugiperda* feeding. PCA with 4085 MetaboAnalyst-filtered features (see Materials and Methods for details) showed a clear separation of *S. frugiperda* locally fed leaves (spL) from the remaining three groups (control-Local, cL; control-Systemic, cS; *S. frugiperda*-systemic, spS). In contrast, cL and spS leaves, and the leaves in the same position on control plants (cS), were not clearly separated from each other in the PCA plots (Fig. 1C). When the samples were analysed separately in local and systemic pairs (Student's *t*-test, $P < 0.05$, fold change > 2; Fig. 1D), similar numbers of positively regulated ions by *S. frugiperda* herbivory were observed in the local and systemic leaves. Interestingly, we found only a minor fraction of down-regulated metabolic features (fold change > 2; Fig. 1D), both in local and systemic leaves.

In addition to UPLC–ESI–TOF–MS analysis, the contribution of UV-absorbing compounds to herbivory-induced metabolic differentiation has also been analysed in the same set of metabolomics extracts by a HPLC coupled to a photodiode array detector (PDA). While in tobacco several UV-absorbing compounds such as phenylpropanoids and flavonoids strongly responded to herbivory (Kaur *et al.* 2010), in rice leaves, significant but only relatively small increases of UV-absorbing materials were detected. Relative peak areas of 13 distinguishable HPLC chromatographic peaks are shown in the Supplemental Figure S1A–M. Interestingly, some peaks with a typical UV-absorbance spectrum of flavonoid compounds significantly increased only in the systemic leaves, while they remained unchanged in local leaves (Supplemental Figure S1I, J and K). We currently identify these compounds as potential herbivory systemic defence metabolite markers in rice.

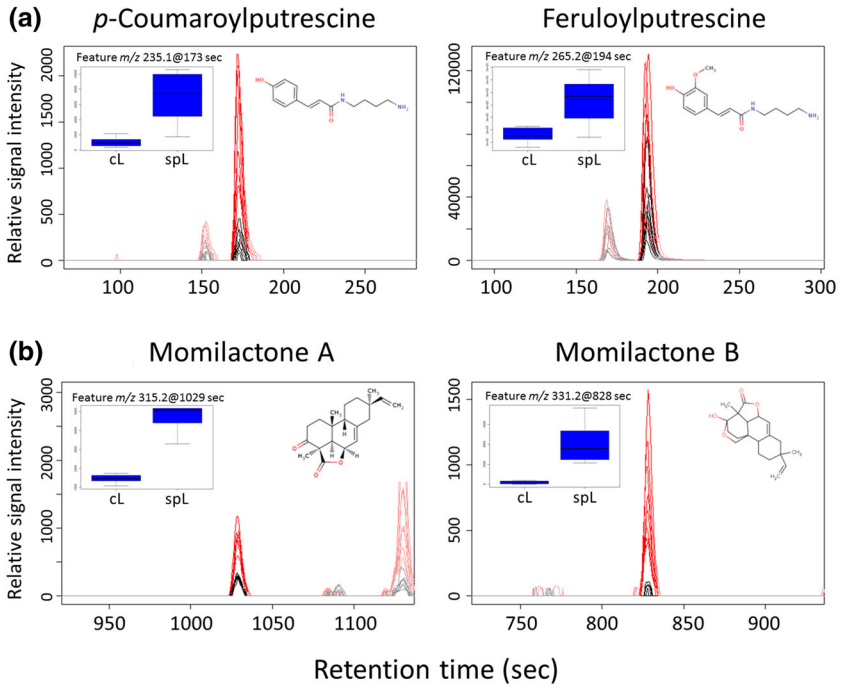


Figure 2. Extracted-ion chromatograms (EICs) of herbivory-elicited and control rice seedlings. Second instar larvae of a rice-adapted strain of *S. frugiperda* were fed on the youngest rice leaves and subjected to UPLC–ESI–TOF–MS analysis. Selected positive EICs corresponding to putative (A) phenolamides (PAs), *p*-coumaroylputrescine (*m/z* 235.14–235.15) and feruloylputrescine (*m/z* 265.15), and (B) diterpene phytoalexins, momilactone A (*m/z* 315.19–315.20) and momilactone B (*m/z* 331.19) were extracted by XCMS package in R software (black, control local leaves; red, *S. frugiperda*-fed local leaves). Compound structures and accurate masses were obtained from the Metlin metabolite database (http://metlin.scripps.edu/index.php).

Validation of metabolomics data

To validate and expand the data from the metabolomics experiment conducted with *S. frugiperda*, another generalist herbivore *S. mauritia* (see introduction of new herbivores below) fed *O. sativa* cv. Nipponbare was used. Ten differentially accumulated ions from the metabolomics dataset with good MS response were analysed. We could confirm seven out of 10 features to be significantly up-regulated by low resolution LC-MS operating in SIM mode in 2 d *S. mauritia*-fed leaf samples compared to control leaves (Supplemental Figure S2). Although another two features were also up-regulated by herbivory, these differences were not statistically significant.

PAs and momilactones in rice leaves

Next, we examined if some of the already known plant defence compounds could be found in the rice metabolomics dataset. At first, metabolic features corresponding by accurate masses to MoA and MoB (Nojiri *et al.* 1996) were putatively assigned (Fig. 2). Because of the lack of authentic momilactone standards at the time of metabolomics experiment, the peaks could not be directly verified. However, up-regulation in spL leaves indirectly supported the annotation of *m/z* 315.1 [M + H] at 1029 s as MoA and *m/z* 331.1 [M + H] at 828 s as MoB. Furthermore, we searched for a typical *m/z* 251.2 representing a well-known tobacco defence PA against insects, CP (Kaur *et al.* 2010), in rice extracts but no corresponding peak was found in rice. Instead, two other herbivory-induced peaks, *m/z* 235. 1 [M + H] at 173 s and *m/z* 265. 1 [M + H] at 194 s, putatively belonging to CoP and FP from the same metabolic pathway, were identified (Fig. 2). In support, two UV-absorbing peaks with the typical absorbance spectrum of phenylpropanoid compounds (or their conjugates, such as PAs) were also found in the preceding HPLC-PDA analyses (Supplemental Figure S1E, F).

PAs strongly accumulate in herbivory exposed rice leaves

To examine further the defensive role of PAs and momilactones, we exposed young rice seedlings to feeding of the three species of herbivores: *S. mauritia*, *P. guttata* and BPH (Fig. 3). The switch to another insect system was partially forced by the laboratory relocation from Germany to Japan but it, eventually, broadened the ecological relevance of our research as we now use natural herbivores of rice already existing in Japan. We also upgraded analytical method for PA and momilactones, using triple quadrupole LC-MS operating in MRM mode, to increase detection limit and reliability of our measurements.

CoP accumulation was low and/or delayed in *S. mauritia* and *P. guttata*-fed leaves examined over the 3 day feeding period (Fig. 3). The induction of CoP in BPH-fed leaves seemed more pronounced but remained below statistically significant levels. In contrast, FP was significantly elevated in all insect-attacked leaves/systems, suggesting an important role of this metabolite in rice defence. Compared to PAs, MoA and MoB induction was restricted to BPH attack (Supplemental Figure S3), and it was only significant in case of MoB after 6 d of BPH treatment. This is in contrast with metabolomics data obtained with *S. frugiperda*-challenged leaves (3 day exposure). Working with various rice–insect systems, we find the levels of momilactones highly variable and, more importantly, rarely correlated with the feeding of chewing insects on rice plants. Because a defence role of diterpene phytoalexins against phytopathogens has also been proposed (Hasegawa *et al.* 2010; Toyomasu *et al.* 2014), these irregular patterns might be explained by secondary infections in chewing herbivore-attacked leaves that are difficult to control for under the non-aseptic conditions of our experiments.

PA accumulation is promoted by herbivore-derived elicitors

We next conducted simulated herbivory experiments (Fukumoto *et al.* 2013) with rice seedlings to emphasize the role of PAs in rice defence against herbivores, and to dissect the contribution of insect elicitors in PA response. Young leaves were mechanically damaged with a fabric pattern wheel to create one row of wounds on each side of the youngest expanded leaf lamina, and the resulting wounds were immediately treated with either 20 μL of water, or with 20 μL of diluted OS isolated from *S. mauritia* larvae (this treatment efficiently mimics herbivore feeding but with a standardized amount of wounding). As shown in Fig. 4, mechanical wounding induced both CoP and FP levels in rice leaves; however, the accumulation of both metabolites was enhanced when *S. mauritia* regurgitate was added to the mechanical wounds (also see Supplemental Figure S4). *P. guttata* OS only promoted FP accumulation in wounded rice leaves. These results further support defence function of PAs (and FP in particular) against rice herbivores, one that is enhanced by the plant's recognition of herbivore feeding.

Consistent with results of direct feeding experiments, treatments with chewing insect elicitors or wounding alone did not lead to any consistent increases in MoA and MoB (data not shown).

PAs directly function against BPH

Metabolite accumulation patterns in response to herbivory suggest but do not prove biological role of these metabolites in defence. Hence, a defence role of CoP and FP against herbivores was examined by directly feeding the synthetic compounds to the insects. Despite several efforts and application methods (artificial diet, leaf spray), chewing herbivore larvae did not respond to PAs in terms of delayed growth (Fig. 5) or increased mortality (nearly all larvae survived experiment until day six).

In contrast to chewing herbivores, both CoP and FP directly enhanced mortality in BPHs when suspended at a concentration 100 μg mL^{-1} in the feeding solution consisting of 15% sucrose in water (Fig. 5A). However, the level of PAs required to suppress BPH on artificial diet was high, compared to

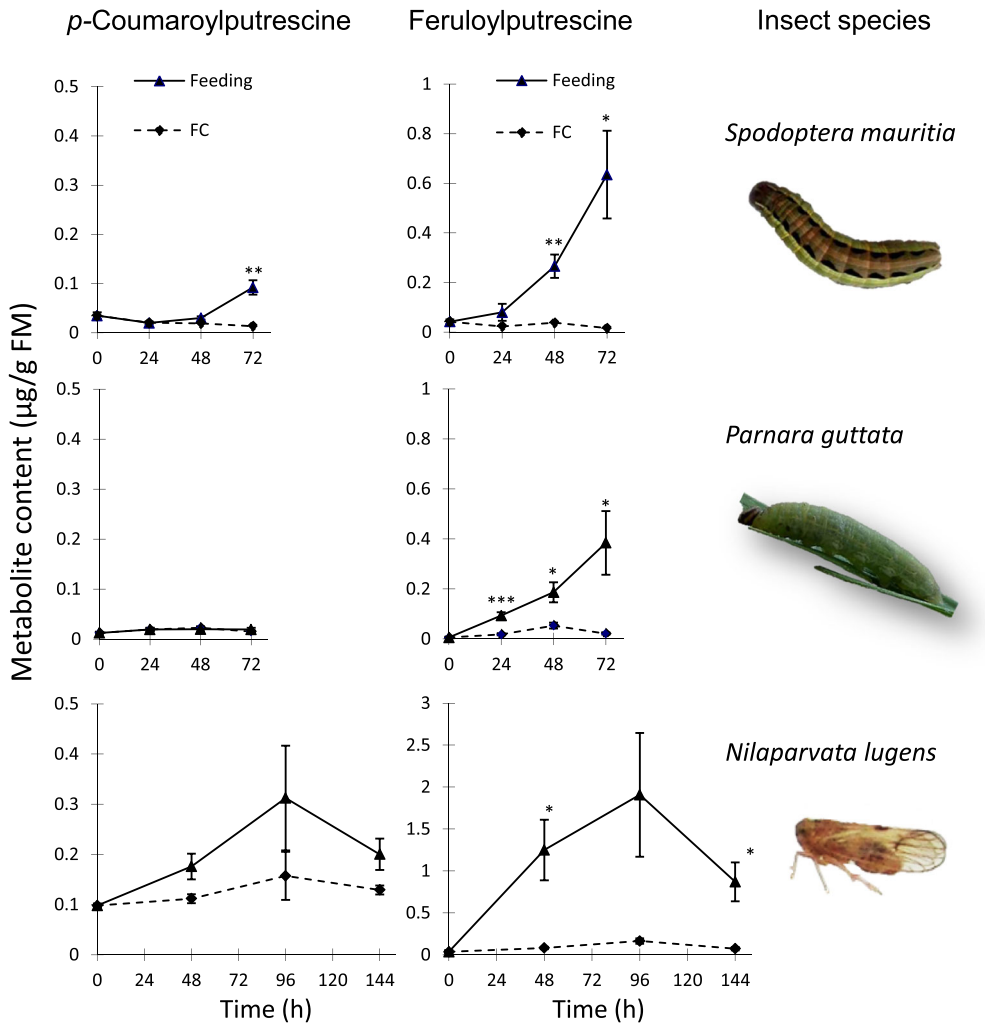


Figure 3. Herbivore feeding elicits accumulation of PAs in rice seedlings. Rice leaves exposed to continuous feeding of larvae of lawn armyworms, *Spodoptera mauritia* (upper), rice skippers, *Parnara guttata* (middle) and brown planthoppers, *Nilaparvata lugens* (bottom) were extracted and subjected to triple quadrupole liquid chromatography (LC)-MS analysis for quantification of PAs, *p*-coumaroylputrescine and feruloylputrescine. Schematic representations of rice herbivores used in experiments are shown on the right. Values are means of four to six biological replicate measurements with Standard Error (SE) indicated. Asterisks show statistically significant differences between treatments determined at each time point by Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). FM, fresh mass; FC, feeding control.

BPH-elicited levels shown in Fig. 3C. In this latter experiment, however, only five insects per 9 cm enclosed leaf length were used, and both local and systemic parts of the leaves were collected and analysed for PA levels. Thus, another experiment was established with higher herbivory loads (10–15 adults per 5 cm exposed leaf length, see Fig. 6A, B), and only locally attacked tissues were collected and analysed for PA and momilactone contents as before. FP levels increased more rapidly and attained concentrations of 60 µg FP per gram FM after 6 d of BPH attack (Fig. 6C). At this point, damage to the leaves was already visible (Fig. 6B), and the majority of BPH in the clip cages died. However, it was not possible to distinguish if BPH death was because of entrapment on a sticky honeydew that accumulated over time in the smaller clip cage, or because of direct leaf toxicity, or both (i.e. intoxicated animals were more easily trapped on a sticky surface). MoA and MoB accumulations (Fig. 6D) were also more clearly elicited compared to previous experiment reported in Supplemental Figure S3. Unfortunately, direct contribution of momilactones on BPH survival, and their possible interactions with PAs could not be examined in the present study because of a very limited supply of purified MoA and MoB standards.

DISCUSSION

Despite practical importance, most defence metabolites in plants and molecular mechanisms of their biosynthesis remain unknown. This is because of extreme diversity of secondary products currently existing in plants. We examined metabolic responses of cultivated rice to herbivory using both untargeted and targeted metabolic approaches. A large number of potentially novel metabolites elicited by chewing herbivores have been identified. Interestingly, two of them (CoP and FP)

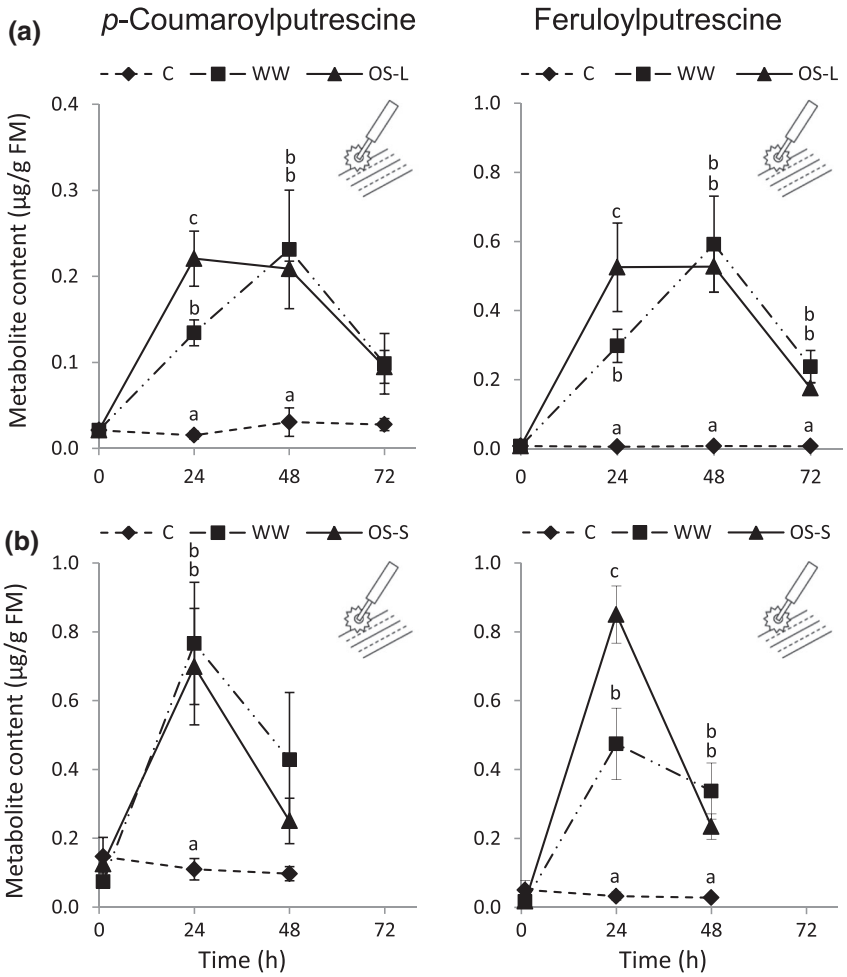


Figure 4. Wounding- and simulated herbivory-induced PA accumulation in rice seedlings. Rice leaves were wounded with a fabric pattern wheel on each side of the midvein and immediately treated with 20 μL water (WW) or fivefold diluted oral secretions (OS) from lawn armyworm *S. mauritia* (A, OS-L), or rice skippers *P. guttata* (B, OS-S) larvae. Plants were incubated for the indicated time periods, and PA levels were determined by triple quadrupole LC-MS. Values are means of four biological replicate measurements with SE indicated. Different letters show statistically significant differences between treatments determined at each time point by ANOVA ($P < 0.05$; Fisher's LSD test). FM, fresh mass.

accumulated in response to wounding as well as feeding by insects from different feeding guilds and levels of specialization.

Untargeted metabolomics uncovers potentially novel defence metabolites in rice

Although major secondary metabolic pathways in plants are similar, such as the phenylpropanoid or terpenoid pathways, the final products and active metabolites can be highly diverse. Untargeted metabolomics approach provides unique potential for unraveling of chemical defence in plants. This approach can be particularly efficient when combined with molecular genetic tools, plant bioresources and sophisticated bioinformatics methods (Gaquerel *et al.* 2014). Recently, software for data processing and routine identification of differentially regulated metabolomics features became available for a broad spectrum of non-specialist users (Kuhl *et al.* 2012; Patti *et al.* 2012).

A surprisingly large number of up-regulated metabolomics features by *S. frugiperda* herbivory were identified in the young rice seedlings. In our validation experiment, using a different herbivore system (*S. mauritia*), we confirmed the accumulation of seven of ten metabolomics outputs (Supplemental Figure S2). In the following experiments, two additional ions belonging to PAs were also confirmed while significant MoA and MoB accumulations were confined to samples subjected to feeding of a sucking insect pest. We reason that some of the observed differences could be related to differences in herbivore systems and the increased accumulation of momilactones in *S. frugiperda* attacked leaves could be, therefore, a specific response to this insect.

PA accumulation patterns in rice

We strongly anticipated finding inducible CP levels in rice plants; instead, we identified two other closely related metabolites, CoP and FP, with no detectable levels of CP in the rice samples. Previously, by comparative analysis of plants silenced in the expression of the key pathway regulator, *MYB8* gene (Galis *et al.* 2006; Kaur *et al.* 2010; Onkokesung *et al.* 2012;

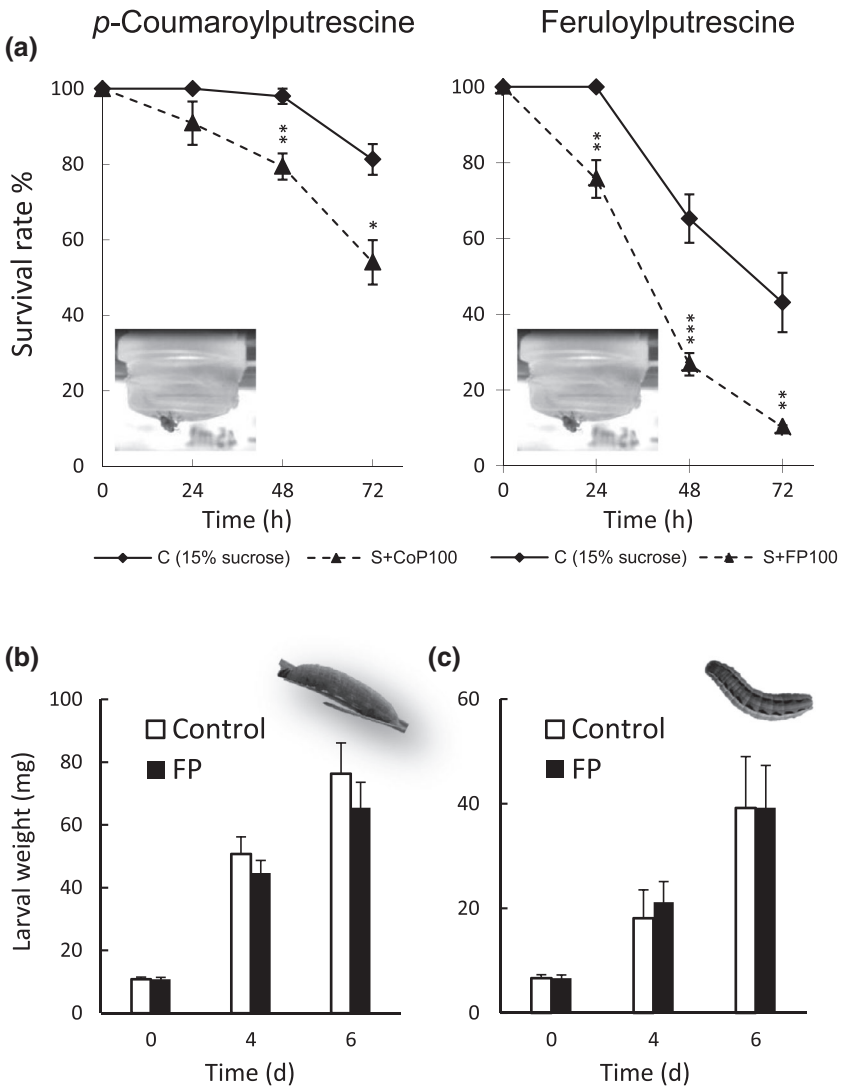


Figure 5. Bioassays and biological activity of PAs against rice herbivores. (A) Brown planthopper (BPH), *N. lugens* (*n = 10*) adults were fed with 15% sucrose or sucrose supplemented with 100 μg mL^{-1} PAs, *p*-coumaroylputrescine or feruloylputrescine (*n = 5*). Survival of insects was determined by visual observations. Asterisks show statistically significant differences between treatments determined at each time-point by Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$); C, control treatment; S, 15% sucrose solution. Second- to third-instar larvae of *P. guttata* (B) and *S. mauritia* (C) were individually fed on control (*n = 15*) or 200 μg water-solubilized feruloylputrescine-treated rice leaves (*n = 15*) kept in a glass tube. No statistically significant differences amongst treatments were found in B and C.

Gaquerel *et al.* 2013a), a large number of PAs was identified in wild tobacco species *Nicotiana attenuata*. A very diverse *N. attenuata* network of PA metabolites acting in defence was further refined by Gaquerel *et al.* (2014), suggesting that rice plants are likely to contain even more PAs that still need to be identified.

PA metabolites represent a large and ubiquitously occurring group of defence metabolites with yet underestimated role(s) in plant defence (Edreva *et al.* 2007). In particular, various mono-and polyamines exist in plants together with numerous phenylpropanoids that can be combined in a large number of conjugates by specific plant acyltransferases (Onkokesung *et al.* 2012). Future characterization of these enzymes will allow the targeted manipulation of PAs, and the subsequent improvement of plant defence. Recent genome-wide association analysis of 529 diverse accessions provided first tentative annotation of several PA biosynthetic genes in rice (Chen *et al.* 2014). However, experiments with single knockout plants are necessary for the conclusive functional annotation of these genes.

Although CoP and FP accumulated to similar levels in wounded or simulated herbivory-treated rice leaves, herbivory feeding *per se* preferentially elicited the accumulation of FP. Possibly CoP is a precursor for FP biosynthesis. It purports that FP is the more active metabolite against insects in rice. Alternatively, CoP and FP could be produced by separate acyltransferases specific for each metabolite. Previously, tobacco AT1 enzyme mediated biosynthesis of CP, but it almost completely failed to produce CoP. Furthermore, the activity with feruloyl-CoA substrate was only 10% when

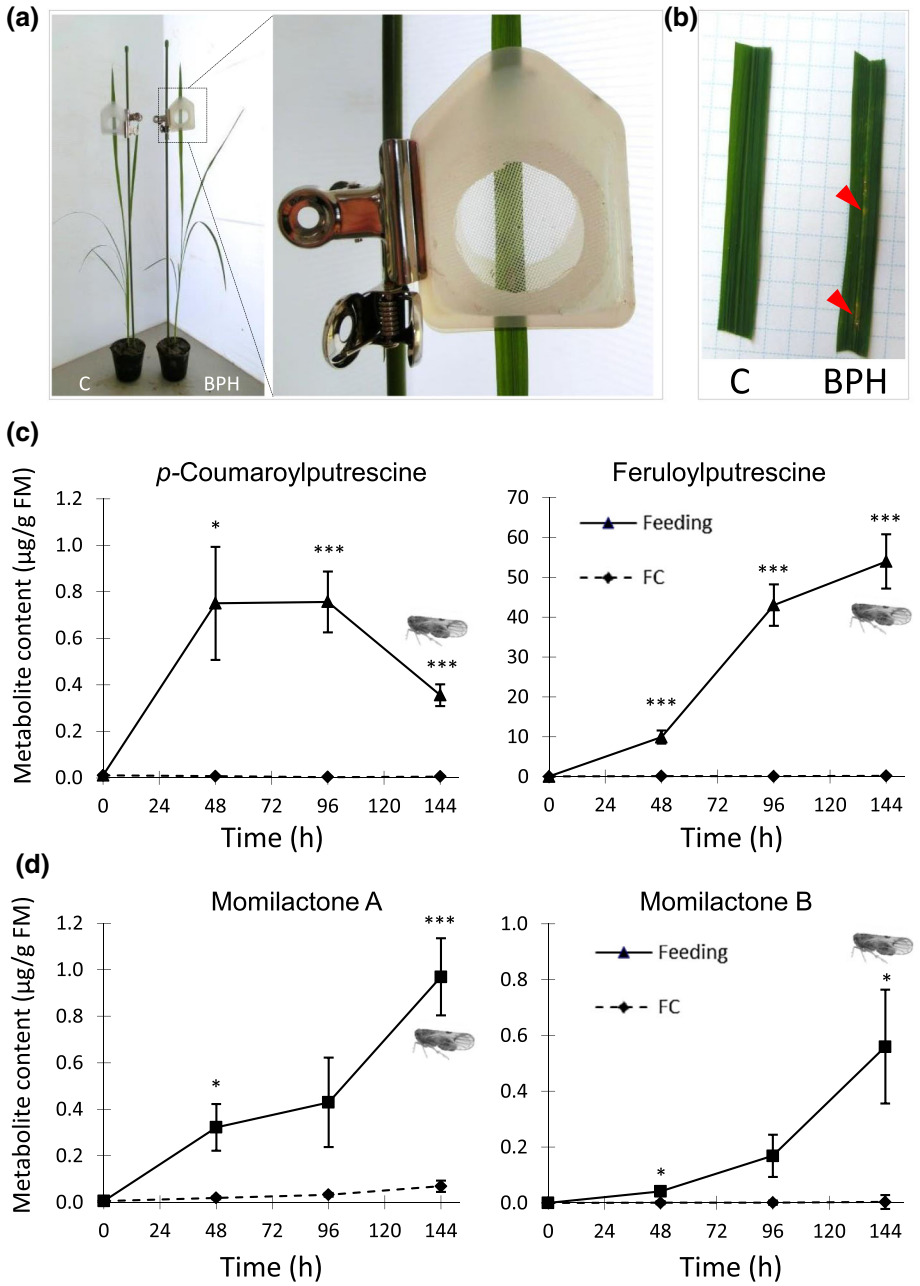


Figure 6. Local infestation of BPH induces strong accumulations of feruloylputrescine in rice leaves. (A) BPH, *N. lugens* adults ($n = 10–15$) were enclosed in a clip cage covering 5 cm of length of the youngest leaf on a 5-week-old rice plant ($n = 5$). (B) BPH feeding caused visible chlorosis and necrosis on the BPH exposed leaf areas after 6 d (red arrows). (C) *p*-Coumaroylputrescine (CoP) and feruloylputrescine levels in the BPH locally attacked areas before, and 2, 4 and 6 d after insect attack. (D) Momilactone levels in local areas attacked by BPH. Asterisks (C, D) show statistically significant differences between treatments determined at each time point (Student's *t*-test; $*P < 0.05$; $***P < 0.001$). FM, fresh mass; FC, feeding control.

compared to caffeoyl-CoA *in vitro*. Silencing of *AT1* gene did not affect *in vivo* contents of FP (Onkokesung *et al.* 2012), but strongly suppressed CP, suggesting the existence of a specific acyltransferase for FP biosynthesis in tobacco. Similarly, CoP and FP in rice may be the products of separate enzymes, in contrast to genome-wide association analysis that assigned a single gene to CoP, CP and FP biosynthesis in rice (Chen *et al.* 2014). We could not even detect CP in our rice system.

Rice plants metabolically discriminate between mechanical damage and herbivory

In our experiments, PAs accumulated in response to feeding of both chewing and sucking insects. We then examined if rice plants could respond differentially to mechanical wounding and simulated herbivory treatments. In the latter, OS from two rice lepidopteran herbivores, *S. mauritia* and *P. guttata*

were used as insect elicitors. Supporting our previous data and enhanced production of phytohomones after *S. mauritia* OS application (Fukumoto *et al.* 2013), rice plants accumulated more FP in response to both regurgitates (Fig. 4) than mechanical wounding. Conjugates of fatty acids, commonly linolenic acid or 17-hydroxylinolenic acid, and amino acids, Glu or Gln, known as FACs are common elicitors found in Lepidoptera larval regurgitate (Halitschke *et al.* 2001). Interestingly, *S. mauritia* regurgitate contained large amounts of easily detectable FACs but OS from *P. guttata* were virtually FAC-free (unpublished data). Thus comparable metabolic responses to both OS suggest the presence of additional elicitors, other than FACs that are responsible for PA accumulation during herbivory. Both CoP and FP were clearly promoted by BPH feeding in rice. In this case, mechanical damage was limited to the penetration site of a stylet into the lamina of the rice leaf. It is tempting to speculate that CoP and FP accumulations are triggered by some, yet unknown type of elicitor(s) from BPH saliva, possibly perceived by the recently identified set of LecRK proteins (Liu *et al.* 2015).

Defence functions of PAs

Previously documented diversity of PAs in *N. attenuata* and their preferential accumulation in young vegetative and reproductive tissues suggested a critical defence function of these metabolites in plants (Kaur *et al.* 2010). PAs are likely to play multiple defence roles in plants (reviewed in Bassard *et al.* 2010). For example, PAs accumulated in response to virus infections in tobacco plants (Martintanguy *et al.* 1976; Torrigiani *et al.* 1997; Rabiti *et al.* 1998), and PAs showed antimicrobial properties in several other plant–pathogen interactions (Grandmaison *et al.* 1993; Ramos *et al.* 1997; Von Röpenack *et al.* 1998; Campos *et al.* 2014). With regard to virus transmission by sucking insects, BPH-induced CoP and FP accumulations (Fig. 3) could be controlling the viruses that infect rice. Indeed, the up-regulation of a committed phenylpropanoid enzyme, phenylalanine ammonia lyase (PAL) in small BPH (*Laodelphax striatellus*)-infected rice correlated with the resistant rice variety (Duan *et al.* 2014).

Our current results show that PA accumulation can be dose dependent and plants under heavy BPH attack elicit very high local levels of FP (50–60 $\mu g\, g^{-1}$ FM; Fig. 6). Although these concentrations were still slightly below the concentrations effective in the bioassays (Fig. 4A), it should be emphasized that rice defence system, apart from PAs, involves other known (e.g. PIs) and unknown factors. Some of the unknowns could be amongst the candidate metabolites identified in our broad metabolomics screen (Fig. 1).

In contrast to BPH (Fig. 5A), and our previous results with tobacco (Kaur *et al.* 2010), growth of chewing insects was not significantly affected by exogenously applied PAs (Fig. 5B, C). Perhaps, PAs accumulate very locally at wound sites in rice. Alternatively, specific combination of PAs may be required to exert strong biological activity against insects, or PA internalization and/or modifications are required for defence activation. Previously, reverse genetic tools were used to show positive association of PAs with tobacco anti-herbivory defences (Kaur *et al.* 2010). Plants silenced in the expression of the key transcriptional regulator NaMYB8 lacked PAs that enhanced the susceptibility of transgenic plants to Lepidopteran larvae, *Manduca sexta* and *Spodoptera littoralis*. Identification of rice NaMYB8 homologue(s) or other regulators for PAs will help us to elucidate the function of PAs in monocot plants. In parallel, alternative hypotheses such as the function of CoP and FP against secondary invaders, bacterial and fungal pathogens, associated with chewing damage should be considered.

Conclusions and perspectives

Metabolomics analyses typically provide an overwhelming amount of information. However, the main challenge remains to identify differentially regulated metabolites, such as many of those found in rice leaves induced by herbivory. Additional systematic work is required to discover new defence pathways in plants and suggest new functions for metabolites in plant defence against insects. Such tools will aid the more efficient development of sustainable insect resistant plants in the future.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Summary of UV-absorbing compounds in rice seedlings subjected to herbivory.
Figure S2. Herbivore feeding elicits metabolites in rice seedlings.
Figure S3. Herbivore feeding elicits accumulation of momilactones in rice seedlings.
Figure S4. Wounding and simulated herbivory induces PA accumulation in rice seedlings (repeated experiment in the Figure 4).
Table S1. Processing of metabolomics data.