Herbivore-induced and constitutive volatiles are controlled by different oxylipin-dependent mechanisms in rice

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Running title: Control of volatile emission in rice

First submitted on: 1 March 2021
Word count: (body w/o Abstract, References, Legends): 5976
Number of Figures: 6
Number of Tables: 0
Supporting information: 4 Figures, 4 Tables

Summary statement: Herbivore-induced plant volatiles (HIPVs), such as linalool, are triggered by wounding and jasmonate (oxylipin) burst in rice. Other volatiles, such as β-caryophyllene, are released constitutively but still require an intact oxylipin pathway. These data suggest that various

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/pce.14126

volatile organic compounds (VOCs) are controlled by two or more independent oxylipin-dependent mechanisms in rice.

Funding information

This work was supported by Ohara Foundation and Grants-in-Aid for Scientific Research (No. 24570026 and 16K08143 to I.G., No. 18K05558 to T.S.). T.T. was supported by Pacific-LEADS program from JICA; I.S.S. by the JSPS postdoctoral fellowship P13379; and K.M. by IsDB 4 in 1 Project by Ministry of Research, Technology, and Higher Education, Republic of Indonesia.

Author contributions

KM - designed and conducted experiments, analyzed data; wrote paper; TT - conducted experiments, analyzed data; ISS - designed experiments, wrote paper; YH - conducted experiments, analyzed data; TS - designed experiments, wrote paper; IG - designed and conducted experiments, wrote paper.

Conflict of interest

The authors declare no conflict of interest.

Abstract

Despite the importance of volatile organic compounds (VOCs) for plants, control mechanisms for their basal and stress-induced biosynthesis and release remain unclear. We sampled and characterized headspace and internal leaf volatile pools in rice (*Oryza sativa*), after a simulated herbivory treatment which triggers an endogenous jasmonate burst. Certain volatiles, such as linalool, were strongly upregulated by simulated herbivory stress. In contrast, other volatiles, such as β-caryophyllene, were constitutively emitted and fluctuated according to time of day. Transcripts of the *LINALOOL SYNTHASE* gene transiently increased 1-3 hours after exposure of rice to simulated herbivory, while transcripts of *CARYOPHYLLENE SYNTHASE* peaked independently at dawn. Unexpectedly, although emission and accumulation patterns of rice inducible and constitutive VOCs were substantially different, both groups of volatiles were compromised in jasmonate-deficient *hebiba* mutants which lack the *ALLENE OXIDE CYCLASE* (*AOC*) gene. This suggests that rice employs at least two distinct oxylipin-dependent mechanisms downstream of AOC to control production of constitutive and herbivore-induced volatiles. Levels of the JA precursor, 12-oxo-phytodienoic acid (OPDA), were correlated with constitutive volatile levels suggesting that OPDA or its derivatives could be involved in control of volatile emission in rice.

Word count: 187

Introduction

Insect herbivory is associated with production of numerous volatile organic compounds (VOCs) in plants (Hare, 2011; Turlings & Erb, 2018), and majority of these herbivore-induced plant volatiles (HIPVs) then serve as call-for-help signals to attract natural enemies of herbivores (Dicke & Baldwin, 2010; Takabayashi & Shiojiri, 2019). Such "indirect" defense traits are ubiquitous in plants where they complement direct defenses, for example, the production of phytoalexins and building of mechanical barriers against herbivores (Mithofer & Boland, 2012; Schuman & Baldwin, 2016). A growing body of evidence highlights the ecological roles of volatiles (Brilli et al., 2009; Fontana et al., 2011; Lehrman et al., 2013; Wang et al., 2015; Simpraga et al., 2016; Bouwmeester et al., 2019), which arise from biochemical pathways for terpenoids, phenylpropanoids/benzenoids, fatty acid derivatives [also known as green leaf volatiles (GLVs)], and amino acid derivatives (Maffei, 2010; Dudareva et al., 2013; Degenhardt, 2009; Scala et al., 2013).

Plant volatile emission depends on biosynthetic and/or release levels but the underpinning mechanisms controlling volatile emission remain poorly understood. As the majority of plant metabolic responses to herbivory are orchestrated by jasmonates (JA) (Larrieu & Vernoux, 2016; Wasternack & Song, 2017; Ruan et al., 2019), it is expected that HIPVs should be also controlled by oxylipins. Indeed, a key study on native tobacco (*Nicotiana attenuata*) demonstrated that jasmonate-dependent HIPVs confer higher fitness and defense of plants against herbivores in nature (Kessler & Baldwin, 2001; Schuman et al., 2015). However, the role of JA signaling in control of HIPV emission is less well known in rice. For instance, independent RNA interference (RNAi)-based approaches for silencing of JA pathway in rice (*Oryza sativa*) resulted in somewhat variable effects on volatiles (Zhou et al., 2009; Qi et al., 2011). In addition, as HIPVs exist on background of many other non-inducible (constitutive) volatiles (Simpraga et al., 2016), additional approaches are necessary to fully understand the role(s) of JA in overall regulation of VOCs in rice.

In contrast to RNAi lines used in previous studies in rice (Zhou et al., 2009; Qi et al., 2011), it appears that a rice mutant line known as "*hebiba"* shows much more complete shut-down of its JA pathway (Riemann et al. 2003). This is due to a disruption of an essential JA biosynthetic gene, *ALLENE OXIDE CYCLASE* (*AOC*) in these plants (Riemann et al. 2013). The phenotype was first

discovered in screens for photomorphogenesis defective rice by Riemann et al. (2003); *hebiba* was then used to show that degradation of photoactivated phytochrome A in rice coleoptiles in dark requires jasmonate pathway (Riemann et al., 2009). Furthermore, *hebiba* was used to show the importance of JA in rice defense against pathogens, where it was more susceptible than the wild-type to an incompatible strain of the rice blast fungus, *Magnaporthe oryzae*. Interestingly, such disease susceptibility was associated with *hebiba*'s highly reduced levels of blast-induced phytoalexins, flavonoid sakuranetin and diterpene momilactones (Riemann et al. 2013). While this indicates that rice secondary metabolites induced by pathogens are controlled by JA, so far, neither HIPVs nor other VOCs have been thoroughly examined in *hebiba* plants.

After biosynthesis, systems for the release and retention of volatiles are likely to affect emission levels in plant headspace (Tissier et al., 2017). Previously, it was proposed that volatiles diffuse passively from plant cells (reviewed in Widhalm et al., 2015). A more detailed examination of passive release model in this review, however, suggested that alternative VOC release mechanisms may exist in plants (Widhalm et al., 2015). For example, as VOCs are mainly produced in the cytosol, and favorably partitioned into various membrane structures, they could be trafficked *via* endoplasmic reticulum (ER) or vesicles in cells (Pichersky et al., 2006). Furthermore, as transfer of VOCs into the apoplast involves crossing lipophilic layers into aqueous environments, membrane-localized transporters could be engaged in the process. Notably, it was shown that plasma membrane adenosine triphosphate–binding cassette (ABC) transporters are capable of such roles. For example, the AtABCG29 protein is known to mediate transport of monolignol *p*-coumaryl alcohols in Arabidopsis (Alejandro et al., 2012), and emission of volatiles from *Petunia hybrida* flowers is facilitated by the PhABCG1 transporter (Adebesin et al., 2017). In analogy, specific transporters for HIPVs, and possibly all VOCs, could affect volatile emission from plants. However, other plant volatile transporters remain elusive and await discovery. Here, we hypothesize that plant volatile emission depends partly on dynamics that occur between internal plant space and surrounding headspace. In particular, this is likely to be the case for volatiles which are not emitted following their expected concentration gradients, and therefore are likely to employ some specific transport and/or retention mechanisms for their genetically programmable release and/or storage.

Studies of metabolite dynamics mentioned above typically involve detailed observations of multiple plant metabolites, resolved in time and space, ideally supported by enzyme activities,

and gene transcription levels. This approach could also be applicable to leaf VOCs for comparison of emission in headspace with internal pools of stored volatiles. However, such studies are rare, possibly because of technical challenges associated with synchronized extraction of volatiles from leaves and headspace, and subsequent measurement of numerous samples by individual Solid Phase Microextraction (SPME) fibers on GC-MS (Vas & Vekey, 2004; Hou et al., 2017). As we already possessed an efficient system for trapping headspace volatiles in rice (Sobhy et al., 2017), and recently developed easily applicable methods for extraction of internal leaf volatiles from rice (Mujiono et al., 2020), a high throughput approach was applied in this study to capture, for the first time, both basal and stress-induced volatile dynamics in rice. Using various molecular approaches, we first aimed to firmly establish the role of oxylipins in volatile emission in rice. Next, we focused on discrepancies that exist between leaf-contained and headspace-released volatiles, thus proposing an existence of yet unknown transport mechanisms and/or structural transformations for rice volatiles.

Materials and Methods

Plant materials and growth conditions

Japonica rice *Oryza sativa* L. cv. Nipponbare (wild-type) was used in all experiments, except for *hebiba* trials where wild-type cv. Nihonmasari (genetic background of *hebiba*) was used as proper control. Heterozygous seeds of *hebiba* mutant (Riemann et al., 2003) were obtained from Dr. K. Okada, University of Tokyo (with permissions of Drs. M. Riemann and P. Nick). Seeds were germinated in trays soaked in water on nutrient-rich soil pellets Kumiai Ube Baido No.2 (MC Ferticom, Tokyo, Japan). After two weeks, germinated seedlings were transferred to individual 100 mL pots with sterilized field soil nutritionally amended with Ube Baido in 4:1 (v/v) ratio. Unless specified, plants were maintained in the cultivation room at 14 h photoperiod, temperature 28 ± 3°C, and natural light complemented with fluorescent lamps (80-120 µmol m^{-2} s^{-1}). Germinated *hebiba* seedlings were screened at 4-weeks by genomic PCR and DNA extracted from the first or second leaf removed from plants. Gene specific primers (Table S4) were used to discriminate among homozygotes (*h*/*h*), heterozygotes (*h*/H) and wild-types (H/H). Subsequently, *h*/*h* and H/H were used in all experiments with oxylipin-deficient (*hebiba*) and control plants. Insect oral secretions (OS) were collected from the 4th and 5th instar larvae of generalist chewing

herbivore, *Mythimna loreyi* (Lepidoptera: Noctuidae). Larvae were maintained on rice leaf diet preceding OS collection as described in Shinya et al. (2016).

Plant treatments and sampling intervals

Simulated herbivory was performed using a standardized wounding and oral secretion (WOS) method with 6-7-week-old plants (Fukumoto et al., 2013), except for *hebiba* experiments that were postponed to 8-weeks to allow full recovery after leaf removal for genotyping. Plants were typically elicited at 15:00 o'clock to avoid midday heat in outdoor experiments. In brief, small mechanical wounds were created by rolling a fabric pattern wheel along each side of midvein on the youngest fully developed leaf of each rice plant, and subsequently, 20 µL water-diluted 3:1 (v/v) oral secretion (OS) was applied on the freshly wounded areas. Slow pipetting and gentle rubbing was used to evenly distribute OS. Treated and control plants were then separately maintained in the cultivation room, or enclosed in custom-made headspace VOC trapping chambers as described later. For leaf VOCs, the first sampling was done at the time of treatment, and then continued every three hours for 24 h (n = 3). For headspace volatiles, samples were collected over 3 h (n = 3), except for a shorter ½ initial period (15:00-16:30). Each headspace collection was separated by attaching a clean volatile filter to outlet of each trapping container.

In outdoor experiments, four-week-old rice plants were moved from the laboratory to a wire-mesh screen house and acclimated to outdoor conditions for additional three weeks. On days of experiment, conditions varied in range of day $temp^{MAX}$ 35°C; night $temp^{MIN}$ 19.5°C; $RH^{MIN-MAX}$ 18-58%, and maximal light intensity 200 µmol m^{-2} s^{-1} (see Tables S1, S2). After treating plants at 15:00 as before, samples for internal leaf volatiles, hormones, and gene analyses (n = 3) were collected every hour for 24 h. For gene expression and hormones in leaves of outside plants, an additional 0.5 h time point after treatment was introduced to monitor early occurring transcriptional and hormonal changes. Due to technical challenges associates with headspace trapping under local Japan conditions, such as the overheating of closed containers exposed to direct sunshine in summer, only internal leaf but not headspace VOCs were examined in all outside experiments.

Headspace collection and elution method

The headspace VOC collection method followed the protocol described in Sobhy et al. (2017), with slight modifications. Briefly, treated and control plants were inserted into individual 50 ×15 cm (height × diameter) acrylic cylinders with two separate ports, designated as inlet and outlet. To seal the collection space, the basal part of each cylinder was placed in a tray with 5 cm water level. All chambers were connected by tubing to a single ULVAC DAP-12S pump (ULVAC KIKO Inc., Japan). Inlet air was filtered through an activated charcoal cartridge, while pulling ambient outside air into chambers at approximately 10 L/min. Four volatile traps were set without plants as background references. After passing through the chamber containing the plant, air was directed through individual volatile traps containing two serially arranged MonoTrap devices (cylindrical monolithic silica adsorbents; GL Sciences Inc., Japan; Morita et al., 2019). After trapping, both MonoTraps were eluted together by sonication in a GC vial (Tomsic Ltd., Japan) using 300 µL dichloromethane as solvent (DCM; FUJIFILM Wako Pure Chemical Corporation). Each sample was spiked with 400 ng tetralin (1,2,3,4-tetrahydronaphthalene; FUJIFILM Wako Pure Chemical Corporation) used as an internal standard (IS). MonoTraps placed in GC vials were sonicated for 5 min, three times, while allowing 2 min cooling breaks between sonication. After removing MonoTraps from vials, samples were analyzed by standard GC-MS method described below.

Internal leaf volatile extraction

Solid phase micro-extraction (SPME) was used according to Mujiono et al. (2020). Frozen leaves in 16 mL glass tubes were defrosted in ice to reduce enzyme activities, and spiked with 5 µL of tetralin (400 ng) in DCM as internal standard. The MonoTrap device was suspended in a tube on a stainless steel pin that was pushed into the nitrile packing of the polytetrafluoroethylene (PTFE) lid. Airtight closed tubes were then incubated in programmable aluminum heat block (hole diameter 1.8 cm; depth 6.5 cm) using a volatile heat-elution program: rapid increase from RT to 150ºC, hold for 30 min, natural cool-down to 60ºC, and another hold for 30 min. Before use, volatile extraction was optimized with standards to achieve recovery rates of 60-80% for GLVs, 80-100% for monoterpenes, and 80-90% for sesquiterpenes under static trapping conditions in airtight closed tubes. One exception was (*Z*)-3-hexenal, providing poor recovery suspected due to heat instability, although it was still recovered from leaf samples at unknown recovery rate. Therefore, (*Z*)-3-hexenal should be treated with caution as these levels are most likely underestimates of true leaf values. After heating cycle, MonoTraps were detached and water

removed by stream of nitrogen gas. Volatiles were eluted from MonoTraps kept on pins with 200 µL DCM by sonication as before. MonoTraps and pins were removed and samples analyzed on GC-MS.

Analysis of volatile organic compounds by GC-MS

Volatile samples (1 µL) were analyzed in split mode on an Agilent 7890A GC (HP-5MS capillary column, 30 m, 0.25 mm i.d., 0.25 µm film thickness, Agilent Technologies, USA) which was coupled to MS (Agilent 240 system). Instrument settings and parameters were basically as reported in Sobhy et al. (2017) and raw MS spectra were processed with Agilent Workstation ver. 7.02. Quantification of known compounds with standards was performed by comparison of peak areas to authentic standards at a concentration range of 0.1-5 ng/µL. Compounds with no available standards were calculated and presented as relative peak areas normalized by tetraline IS.

Phytohormone analyses

Phytohormone methods followed standard procedure described in Fukumoto et al. (2013). Phytohormone contents were measured on a 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].

Quantitative RT-PCR

Gene expression was determined by quantitative RT-PCR as before (Fukumoto et al. 2013). Total RNA was extracted from 100 mg tissue by Trizol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was prepared from aliquots of total RNA with PrimeScript reverse transcriptase (1 µg; Takara Bio Inc., Japan). DNase treatment and cleanup of RNA was used before each cDNA synthesis. Transcript levels were determined by THUNDERBIRD qPCR Mix (Toyobo, Osaka, Japan) on a CFX Connect TM Real-Time System (Bio-Rad Laboratories, Inc., USA). *OsEF1α* (Os03g0177900) or *OsActin* (Os03g0718100) housekeeping genes were used for normalization of relative transcript levels. Gene-specific oligonucleotide primers for qRT-PCR are provided in Table S4.

Statistical analyses

Statistical differences were determined with one-way analysis of variance (ANOVA) using OpenStat (http://statpages.info/miller/OpenStatMain.htm), or Student's *t*-test using Microsoft Excel. Prior statistical analyses, normal distribution of data was examined by Shapiro-Wilk test, and when data were deemed abnormally distributed, logarithmic transformations were used to improve normality before conducting statistical analyses.

Results

Comparison of internal and headspace volatiles in rice

Rice plants were treated with simulated herbivory, or remained untreated as controls, and then plants were subjected to volatile analyses. The internal (target leaf) and headspace (whole plant entrainment) VOCs were collected at designated time intervals from two independent sets of plants, grown in the laboratory or inserted in the collection chambers, respectively. About 80 volatile peaks could be detected in the combined analytical method, using *m/z* 40-300 range of detection in MS (Tables S1-S3). Overall, the rice volatile profiles showed a significant overlap between internal and headspace samples, however, some compounds appeared more specific to a single group of volatiles (Fig. 1). As expected, more volatile compounds with a shorter retention time in capillary GC showed a higher tendency to partition in the plant headspace, while later eluting compounds were usually recovered from internal leaf extracts. It confirms that volatility, in addition to changes in biosynthetic flux after simulated herbivory, is one of the important determinants in VOC distribution. This was exemplified by linalool which strongly induced by simulated herbivory, and it was therefore found both in the leaf and headspace 3-9 h after elicitation (Fig. 1). On the other hand, α-pinene was not induced by simulated herbivory stress, and it was headspace specific (Fig. 1); the later suggesting a presence of highly synchronized production and release mechanisms for this monoterpene. A strikingly irregular pattern, however, was found in case of yet another non-inducible monoterpene, D-limonene. This volatile appeared as one of the stable constituents in the rice leaves, whilst it was also released into rice headspace, where it followed a clear daytime-associated pattern of emission (Fig. 1).

The observed sesquiterpene distribution patterns were even more complex. A major rice sesquiterpene, β-caryophyllene, was retained in the leaves, as well as it was strongly fluctuating

during day/ and night periods in the headspace. Similarly, α-guaiene and β-elemene could be found in both compartments but γ-guaiene, β-sesquiphellandrene, and (*E*)-α-bergamotene were specific to headspace. In contrast, (*E*)-β-farnesene only appeared in the leaves. The same was true for nerolidol and geranyllinalool, two sesquiterpene and diterpene alcohols, respectively, which only accumulated inside of the rice leaves. Interestingly, nerolidol and geranyllinalool were strongly induced by simulated herbivory, and thus resembled to linalool, which is a monoterpene alcohol (Fig. 1). Although nerolidol and geranyllinalool have not been detected in the headspace of rice plants, they could, as discussed later, be serving as precursors of more volatile homoterpenes.

A specific release pattern was observed for GLVs. These compounds accumulated in relatively large quantities in the leaves, for example (*Z*)-3-hexenol and (*E*)-2-hexenal, but their presence in the headspace was restricted to small amounts and short time periods after wounding (Fig. 1). A representative of aromatics, methyl salicylate (MeSA), was somewhat increased in the headspace by simulated herbivory (significant in Fig. 3D), while it was also found in non-treated plants, both internally and externally (Fig. 1). This is consistent with our previous findings showing that 8-9 week-old rice plants accumulate significant amounts of constitutive MeSA (Mujiono et al., 2020).

Rice volatiles under natural conditions

While simulated herbivory can elicit robust volatile responses in laboratory plants, diurnal changes in volatiles are not as easily reproducible under artificial conditions, mainly because of transitional changes in natural light (quantity and quality), temperature, and humidity. In order to confirm laboratory results under more appropriate growth conditions, we carried out experiments with plants placed in the outdoor wire-mesh screen house that was used for protection of rice against accidental insect damage.

As in the laboratory, outdoor rice plants produced large amounts of linalool in leaves in response to simulated herbivory, while linalool levels remained low in untreated leaves of control plants (Fig. 2). In addition to basal levels of nerolidol and geranyllinalool detected in control untreated leaves, these compounds also rapidly accumulated in the leaves of outside rice treated with simulated herbivory (Fig. 2). β-caryophyllene and β-elemene accumulated more during day (Fig. 2, S3) but they did not show any immediate response to simulated herbivory (Fig. 2), which

was consistent with previous laboratory results (Fig. 1). Outdoor rice, however, showed an additional late increase of β-caryophyllene and β-elemene levels in the leaves treated with simulated herbivory (Fig. 2). GLV pools in rice leaves were generally less pronounced at night but increased during daytime. D-limonene and (*E*)-β-farnesene levels remained more stable in the leaves, showing no consistent response to simulated herbivory (Fig. 2). Overall, the results from the laboratory and outside experiments highlighted two common groups of terpenoids in rice. In the first group, represented by linalool (monoterpene), nerolidol (sesquiterpene) and geranyllinalool (diterpene), volatiles strongly responded to simulated herbivory, and thus should be considered as true HIPVs. In the second group, including β-caryophyllene and β-elemene, volatiles were mainly upregulated during daytime, showing some late responses to simulated herbivory when treated outside (see Fig. 2). While the first group pattern could be easily explained by plant response to high levels of jasmonates triggered by simulated herbivory (Fukumoto et al., 2013), later responses and daytime-dependent oscillations of β-caryophyllene and β-elemene should respond to other regulatory signals. Subsequently, experiments with JA deficient plants were conducted to corroborate the involvement of JA in the regulation of VOCs in rice.

Central role of oxylipin signalling in rice volatile production

The JA loss-of-function *hebiba* plants (cv. Nihonmasari genetic background) lack the oxylipins downstream of AOC, including *cis*-OPDA, JA and JA-Ile. Similar to other jasmonate mutants, *hebiba* plants remain infertile (Riemann et al., 2003). As expected, known HIPVs linalool and MeSA were severely compromised in the headspace of *hebiba* rice treated with simulated herbivory (Fig. 3A, D). It was noticeable that even constitutive levels of linalool in the headspace of laboratory-grown *hebiba* were less abundant compared to Nihonmasari wild-type. It was, however, surprising to see that even volatiles which did not show any immediate responses to simulated herbivory in our previous experiments (Fig. 1,2) were significantly downregulated in *hebiba* (Fig. 3A-C), showing only 50% or less headspace volatile contents over the 24 h trapping period [α-pinene, D-limonene, β-caryophyllene, (*E*)-α-bergamotene, (*E*)-β-farnesene]. The measurement of corresponding leaf transcript levels of *LINALOOL SYNTHASE* (*OsLIS*; Os02g0121700; Yuan et al. 2008), *SALICYLIC ACID METHYLTRANSFERASE (OsSAMT*; Os02g0719600; Zhao et al., 2010*)*, *CARYOPHYLLENE SYNTHASE* (*OsCAS*/*OsTPS3*; Os08g0139700; Cheng et al., 2007), and *HYDROPEROXIDE LYASE* (*OsHPL3*; Os02g0110200;

Tong et al., 2012) involved in *S*-linalool, MeSA, β-caryophyllene, and GLV production confirmed the volatile observations in *hebiba* headspace (Fig. 4). While levels of *LIS* and *SAMT* both elevated after simulated herbivory, consistent with increased linalool and MeSA levels, these genes were much less induced in *hebiba* compared to wild-type (Fig. 4). However, expression of *LIS* and *SAMT* returned to their basal levels 24 h later, and there was no apparent difference in expression between the wild-type and *hebiba*. The *CAS* and *HPL3* transcripts were not immediately induced by simulated herbivory but the transcripts were lower (significantly for *CAS*) in *hebiba* compared to wild-type (Fig. 4). The significant difference in *CAS* transcript levels in control plants was still observed in leaves collected 24 h later. Both constitutive and induced transcript levels of *MYELOCYTOMATOSIS ONCOGENE TRANSCRIPTION FACTOR 2* (*OsMYC2*; Os10g0575000; Ogawa et al., 2017) were reduced *in hebiba* relative to wild-type (Fig. 4), especially at the early 1 h time point, consistent with the function of MYC2 as central regulator in jasmonate signalling.

Because basal levels of linalool were reduced, and constitutive/diurnal volatiles were also lower in *hebiba* headspace relative to wild-type (Fig. 3), we speculated that different pools/forms of oxylipins could be involved in regulation of these volatiles. As one possibility, the rice plants may accumulate/maintain a low basal level of certain oxylipins, in addition to undergoing the jasmonate burst, which could be separately contributing to volatile emissions in rice. We therefore decided to monitor hormonal changes in detail, using simulated herbivory-treated as well as control (untreated) rice plants, and observed them over the whole 24 h period.

Hormonal changes and gene expression under natural conditions

Hormones were extracted from the leaves similar to those used in Fig. 2, including an additional sampling point at 30 min. The amounts of OPDA, JA, and JA-Ile increased sharply from their basal levels within 30 min after plants were treated with simulated herbivory (Fig. 5). Thus, we could confirm that these hormones precede the accumulation of HIPVs (Fig. 1 and Fig. 2). JA and JA-Ile attained their peak levels 1 to 3 h post treatment, and thereafter declined, however, hormones remained higher than in the untreated controls for the entire observation period (Fig. 5). A small amount of JA-Val conjugate and hydroxylation products of JA-Ile, OH-JA-Ile and COOH-JA-Ile were also detected in the simulated herbivory-treated rice leaves (Fig. 5). While basal levels of JA and JA-Ile remained very low in the plants without treatment, control OPDA levels started to rise in the evening and peaked around morning hours, thus preceding the observed daytime-

associated β-caryophyllene accumulation. This was consistent with another experiment conducted in the previous year (2019) with the untreated outdoor plants (Fig. S4). ABA levels were upregulated by simulated herbivory, possibly in response to wound-induced desiccation stress, and returned to basal levels 6 h later (Fig. 5). SA remained similar over the whole time of experiment, showing no significant changes associated with simulated herbivory (Fig. 5). It should be noted that basal levels of SA in rice, however, are extremely high, reaching as much as 20 μg hormone per gram fresh weight (Fig. 5,S4)

The expression of genes in the oxylipin pathway and volatile biosynthesis was examined with *ACTIN 11* (*ACT11*) gene used as a housekeeping control (Jain et al., 2018) for normalization of transcripts. This gene showed notably less time-associated variation compared to OsEF1α which is normally used in the laboratory. In the core oxylipin pathway, *LIPOXYGENASE* (*LOX*) was strongly upregulated by simulated herbivory but remained low in controls that, however, showed a slight elevation around the next day noon. The transcripts of *LOX9*, also known as HI-LOX (Zhou et al., 2009), were less induced by rice treatment but this gene showed a relatively higher level of basal transcription, which tend to increase at night. Similarly, *AOC* transcripts only mildly responded to simulated herbivory while maintaining easily detectable expression levels in the control rice. Although basal *AOC* transcripts showed only a small elevation at night, clear peak of expression in dark occurred in the simulated herbivory treated leaves. The expression of *JAR1* for JA-Ile biosynthesis was promoted by simulated herbivory but another gene, *JAR2*, did not respond to stress treatment (Fig. 6). Instead, *JAR2* was mainly expressed in the dark period in both control and treated plants, showing a peak of expression prior to β-caryophyllene accumulation, and *CAS* transcript levels (Fig. 6). The central regulator *MYC2* not only transcriptionally responded to JA burst, which was expected, but it also showed an elevated transcript level at night, which declined just before dawn (Fig. 6). Jasmonate repressor proteins JAZ9 and JAZ11, whose genes are known to be strongly dependent on JA-Ile, were quickly induced by plant treatment but also showed an intriguing second peak of expression at night. Although control JAZ levels remained low, some kind of sensitization of the oxylipin pathways is likely to occur at night that putatively overlaps with the increased OPDA levels in outdoor rice, and night peaks of *CAS* and *JAR2* transcripts (Fig. 6).

In monoterpene biosynthesis, *DXS3* not only transcriptionally responded to simulated herbivory, consistent with linalool biosynthesis, but it also increased in controls at night (Fig. 6). Consistent with the strong linalool burst and prominent response of this volatile to herbivory, *LIS* transcripts were elevated in treated plants and remained at low levels in controls. In contrast, *CAS* for constitutive/diurnal β-caryophyllene gradually increased at night, showing a broad peak of expression and decline towards approaching dawn. Again, no direct *CAS* elevation by simulated herbivory was observed (Fig. 6), suggesting that endogenous jasmonates from internal hormone burst cannot upregulate this gene. Another gene, *SAMT* for MeSA biosynthesis elevated after simulated herbivory; interestingly, higher *SAMT* levels were also found in the control plants next morning. The *HPL3* for GLVs showed independent pattern of expression that mainly overlapped with the dark period, suggesting that rice plants may be replenishing their GLV pools at night and/or during early morning hours when light becomes available.

Discussion

In this study, we show that partitioning of terpenoids, aromatic compounds, and GLVs between internal pools in rice leaves and emitted headspace depends on the chemical structure of the volatiles, time of day, and also biotic stresses. Furthermore, experiments with the jasmonate-deficient rice show that both stress-induced and constitutive volatiles in rice are likely to be controlled by the oxylipin pathway. Although constitutive volatiles show no immediate response to JA-Ile burst naturally associated with the simulated herbivory treatment in rice, their emission was still suppressed in JA-mutant *hebiba* rice plants.

Unexpectedly wide role of oxylipins in control of rice volatiles

JA signaling is known to control the majority of defense responses in chewing herbivore-attacked plants (Wu et al., 2007). Accordingly, HIPVs could be induced by exogenous JA in rice (Cheng et al., 2007; Taniguchi et al., 2014). However, conclusive studies based on the loss-of-function mutant studies in the jasmonate pathway remain limited to a small number of model species such as Arabidopsis, maize, tomato, and tobacco (Attaran et al., 2008; Bosch et al., 2014; Christensen et al., 2013; Schuman et al., 2015). Silencing of OsPLDα4 and α5 decreased JA levels in rice, together with GLV and other volatile contents in the stripped stem borer-infested plants (Qi et al.,

2011). In contrast, silencing OsHI-LOX, another important enzyme in rice JA pathway, did not affect GLVs, despite significantly reduced JA levels found in transgenic plants (Zhou et al., 2009). In this study, we first dissect the role of jasmonates in rice HIPV control, taking the advantage of *hebiba* plants (Riemann et al., 2013). HIPVs represented by linalool dramatically declined in *hebiba* relative to WT (Fig. 3). Although *hebiba* shows some morphogenetic defects at seedlings stage, growth of juvenile and adult plants is comparable to WT. Therefore, reduced volatile levels, together with strongly reduced expression of HIPV-related genes in *hebiba* can decisively be attributed to transcriptional control of jasmonates. In support of this notion, silencing of the rice jasmonate-dependent defense switch, OsMYC2, strongly suppresses the *OsLIS* transcripts after JA application (Ogawa et al., 2017). Surprisingly, however, we found that even volatiles, and their associated genes, which do not immediately respond to the jasmonate burst are significantly lower in *hebiba*. This was best seen with β-caryophyllene emission and *OsCAS* transcripts, both of which declined in *hebiba* relative to WT (Figs. 3, 4). As transcripts of *OsCAS* also reduced in OsMYC2-knock-down plants (Ogawa et al., 2017), OsCAS is definitely controlled by oxylipins in rice, although, at this point, is not clear which compound might be involved in *OsCAS* regulation.

When we examined other stress hormone levels, as expected, neither ABA nor SA could associate with β-caryophyllene levels, or *OsCAS* expression patterns. In contrast, transcripts of *OsCAS* at night partly overlapped with the accumulation of another oxylipin lacking in *hebiba*, OPDA, especially in the control untreated leaves (Fig. 5 and Fig. S4). Transcripts of *OsAOC*, *OsJAR1*, *OsJAR2*, and *OsMYC2* also elevated in the control untreated leaves at night, suggestive of their possible linkage to OPDA function. OPDA signaling has been intensively studied in last decade, showing that *cis*-OPDA and its conjugates may work as functional hormones in plants, distinct from JA and JA-Ile (Dave & Graham, 2012; Han 2017; Wasternack & Hause, 2016). As biosynthesis of OPDA was reported in primitive mosses, *Physcomitrella patens* (Stumpe et al., 2010), *Selaginella martensii* (Ogorodnikova et al., 2015) and *Marchantia polymorpha* (Yamamoto et al., 2015), OPDA could still function as one of the ancient gate keepers for volatiles in higher plants.

Assessing rice volatile dynamics from observed volatile patterns

Previously, passive diffusion was proposed to be the main volatile release mechanism in plants (Widhalm et al., 2015; Mofikoya et al., 2019). Even if this may be largely true, volatiles with

different molecular weight and structure should obviously cross cell barriers with distinct efficiencies, suggesting that chemical composition in the leaves and plant headspace can differ dramatically. When we examined the rice terpenes, important attractants for natural enemies of herbivores, and herbivores themselves (Xiao et al., 2012; Wang et al., 2015), distribution of small monoterpenes was consistent with passive diffusion. For instance, monoterpene α-pinene was exclusively found in headspace, and other smaller volatiles were also partitioned between the leaf and headspace (linalool, β-myrcene). In contrast, higher molecular weight compounds, sesquiterpenes (nerolidol) and diterpenes (geranyllinalool), remained trapped in the rice leaves.

Against expectation, however, another sesquiterpene, β-caryophyllene, was abundant in the headspace, and furthermore, it showed a fluctuating light-dependent emission pattern. Also, low molecular weight monoterpene, D-limonene, was diurnally regulated in the headspace but endogenous levels of this compound in leaves remained stable, suggesting the existence of an additional release mechanism. The involvement of transporters in volatile emissions has already been proposed, however, molecular evidence remains limited to a few examples. The adenosine triphosphate–binding cassette (ABC) transporter, PhABCG1, is responsible for the release of phenylpropanoid and benzenoid volatiles in petunia flowers (Adebesin et al., 2017). The ABC transporter AtABCG29 functions as *p*-coumaryl alcohol transporter in lignin biosynthesis (Alejandro et al., 2012), and ABCG11/WBC11 and ABCG12/CER5 are associated with the transport of lipids from epidermis to cuticle (Pighin et al., 2004; McFarlane et al., 2010). In addition, passage of some lipophilic volatiles through hydrophilic cell walls is dependent on specific lipid transfer-like proteins (DeBono et al., 2009). Finally, composition of semi-volatile root exudates in tomato were altered after the genetic manipulation of ABC-C6 and ABC-G33 transporters (Cox et al. 2019). It remains an open question if specific transporters for plant terpenes also exist in plants, and if so, how they are participating in indirect plant defense. However, our data do provide a support for this hypothesis because of the differences in volatile profiles observed between internal leaf and external headspace samples.

Diurnal control of rice volatiles

Volatiles which have a diurnal release pattern are subject to multiple control mechanisms regulating their release (Kolosova et al., 2001; Gouinguene & Turlings 2002; Cheng et al., 2007; Christensen et al., 2013; Zeng et al., 2017; Joo et al., 2018; Joo et al., 2019). Apart from

hypothetical transporters as proposed above, emission of diurnal volatiles is often correlated with the light-driven stomatal opening (Niinemets et al., 2004). However, this would also mean an accumulation of volatiles in cells at night. This is likely to be prevented by secondary control of light over the energy inputs (photosynthesis) required for volatile biosynthesis (Dudareva et al., 2003; Dudareva et al., 2005; Owen and Penuelas, 2005; Dudareva et al., 2013, Pokhilko et al., 2015). In addition to light, rice volatiles are obviously controlled at transcriptional level. The endogenous transcripts of *OsCAS* increased at night (Fig. 6), just before the morning β-caryophyllene emissions (Fig. 1). Similarly, transcripts of *OsDXS3*, a key gene in rice methyl-D-erythritol 4-phosphate (MEP) pathway were strongly expressed at night, i.e., before monoterpene emission next day. This suggests that, while light and photosynthesis can metabolically restrict volatile emissions, the enzyme machinery in cells is likely to be prepared well before the sunrise. Consistent with these findings, basal transcripts of *OsTPS3* (*OsCAS*) were also diurnally regulated in Cheng et al. (2007). In this study, however, *OsTPS3* could be also induced by irrigation of rice seedlings with 250 μM methyl jasmonate, which contradicts our results on lack of *OsCAS* response to endogenous jasmonate burst from simulated herbivory (Figs. 5, 6). As levels of β-caryophyllene were also promoted by 100 μM JA in another independent study by Taniguchi et al. (2014), it remains to be determined if differences in plant age, hormone concentrations, cultivar, or application method account for these contrasting *OsCAS*/β-caryophyllene patterns.

Redefining a subset of *M. loreyi*-induced HIPVs in rice

It was rather surprising to find that only a small portion of rice VOCs could directly respond to simulated herbivory using *M. loreyi* OS. Apart from GLVs, known to escape from cells through open wounds, only four additional compounds in the study - MeSA, and terpenoids linalool, nerolidol and geranyllinalool – clearly increased after simulated herbivory. While MeSA and linalool appeared in the rice headspace, tertiary terpene alcohols, nerolidol (C_{15}), and geranyllinalool (C_{20}) were dominant in the leaves (Fig. 1). Previously, (*E*)-nerolidol was reported in the headspace of snapdragon (*Antirrhinum majus*; Dudareva et al., 2005) and kiwifruit flowers (*Actinidia chinensis*; Green et al., 2012), suggesting that a specific mechanism for (*E*)-nerolidol release may exist, however, it is not operational in rice. While the role of linalool as a HIPV is already well documented (Kessler & Baldwin, 2001; Raguso, 2016; Turlings & Tumlinson, 1992), molecular functions of similarly regulated nerolidol and geranyllinalool are less established. In maize, nerolidol and geranyllinalool are further converted to volatile homoterpenes (*E*)-3,8-

dimethyl-1,4,7-nonatriene (DMNT) and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) (Richter et al., 2016). As DMNT and TMTT have also been found in herbivore infested rice (Hu et al., 2020), and DMNT and TMTT contents increased after ectopic expression of lima bean PlTPS3 and PlTPS4 in *Oryza sativa* L. ssp. Japonica (variety Zhonghua 11) (Li et al., 2018), nerolidol and geranyllinalool could serve as endogenous rice precursors of DMNT and TMTT. However, the natural significance of DMNT and TMTT in rice, and conversion mechanisms from their proposed precursors, nerolidol and geranyllinalool, remains to be elucidated in the future.

Acknowledgments

We thank A. Miyake for help with development of GC-MS methods for headspace volatiles; Dr. H. Matsuura for providing deuterated jasmonate standards in LC-MS/MS analyses; Drs. K. Okada, M. Riemann and P. Nick for kindly providing *hebiba* seeds; and Dr. T. Bruce for manuscript inputs and English editing. This work was supported by Ohara Foundation and Grants-in-Aid for Scientific Research (No. 24570026 and 16K08143 to I.G., No. 18K05558 to T.S.). T.T. was supported by Pacific-LEADS program from JICA; I.S.S. by the JSPS postdoctoral fellowship P13379; and K.M. by IsDB 4 in 1 Project by Ministry of Research, Technology, and Higher Education, Republic of Indonesia.

Data availability statement:

The data that supports the findings of this study are available in the supplementary material of this article.

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Figure legends

Fig. 1

Accumulation patterns of headspace and internal leaf volatiles in rice. Simulated herbivory (Wounding, oral secretion = WOS) elicitations were conducted on plants in the cultivation room at 15:00 and volatiles were collected using headspace collection periods (15:00-16:30; 16:30-19:30; 19:30-22:30; 22:30-1:30; 1:30-4:30; 4:30-7:30; 7:30-10:30; 10:30-13:30; 13:30-16:30) and internal leaf volatiles collection time points (15:00; 18:00; 21:00; 0:00; 3:00; 6:00; 9:00; 12:00; 15:00). Headspace (bars above *x*-axis; n = 3) and internal leaf volatiles (bars below *x*-axis; n = 3) were plotted in mirrored graphs for direct comparison of internal leaf volatile pools and emitted headspace volatiles. Headspace volatiles from dynamic trapping setup of entire plants are sums of volatiles released over 3 h period (or 1.5 h for first collection period); internal leaf volatiles from static trapping of cut leaves are total volatile contents found in each leaf at examined time point. Data are shown as means ±SE. Cont, untreated controls (white bars); WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretion (black bars); RT, retention time of volatile peak in GC chromatograms; compounds marked # are shown as 10^{-6} downscaled relative peak areas normalized by tetraline IS. Statistical differences between Cont and WOS sample pairs at each time point were analyzed by Student's *t*-test (*$p < 0.05$; ** $p < 0.01$; no label, not significant). Pairs of samples suggesting marginal significance are labeled with *p*-values.

Fig. 2

Accumulation of internal leaf VOCs in rice under natural conditions. Simulated herbivory (Wounding, oral secretion = WOS) elicitation was conducted on 7 week-old rice plants in the wire-mesh screenhouse at 15:00 and leaves were collected in 1 h intervals for 25 h. Control plants remained untreated. Volatile compounds were extracted from leaves and examined by GC-MS. Data (n = 4) are shown as means ± SE. Cont, untreated controls (dashed line); WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretion (solid line); compounds marked # are shown as relative peak areas normalized by tetraline IS.

Fig. 3

Comparison of headspace VOCs in wild-type and JA-deficient *hebiba* mutant plants. VOCs released from the wild-type (open bars) and *hebiba* (black bars) rice leaves before and after WOS elicitation in 24 h time period were determined by GC-MS. (A) Monoterpenes (linalool, D-limonene, and *α*-pinene); (B) sesquiterpenes (β-caryophyllene, (*E*)-β-farnesene, (*E*)-α-bergamotene); (C) GLVs ((*Z*)-3-hexenol, 2-heptanol, (*E*)-2-hexenal); (D) aromatics (MeSA,

methyl salicylate). Data (n = 6) are shown as means ± SE; Cont, untreated controls; WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretion. Different letters indicate significant differences between treatments ($p \leq 0.05$) determined one-way ANOVA followed by Fisher's LSD test. Compound marked # is shown as relative peak area normalized by tetraline IS.

Fig. 4

Relative transcript levels of VOC-related genes in wild-type and JA-deficient *hebiba* mutant plants. Leaf samples were collected before, 1 h and 24 h after WOS treatment. Relative transcript levels of rice *HYDROPEROXIDE LYASE* (*OsHPL3*), *LINALOOL SYNTHASE* (*OsLIS*), *SALICYLIC ACID METHYLTRANSFERASE (OsSAMT)*, *CARRYOPHYLLENE SYNTHASE* (*OsCAS*), and JA signaling-related *OsMYC2* were determined by quantitative RT-PCR. Data (n = 3) are shown as means ± SE; Cont, untreated controls; WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretion. Statistical differences between pairs of wild-type (WT) and mutant (hebiba) were analyzed by Student's *t*-test (*$p < 0.05$; ** $p < 0.01$; no label, not significant). Pairs of samples suggesting marginal significance are labeled with *p*-values.

Fig. 5

Phytohormone levels in control and WOS-treated rice under natural conditions. The levels of 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA), jasmonoyl-L-Isoleucine (JA-Ile), jasmonoyl-L-Valine (JA-Val), hydroxy-jasmonoyl-L-Isoleucine (JA-Ile-OH), carboxy-jasmonoyl-L-Isoleucine (JA-Ile-COOH), abscisic acid (ABA), and salicylic acid (SA) were determined by LC-MS/MS in WOS-treated and control untreated leaves. Samples at time zero (15:00) were from untreated plants; samples from 30 min, 1h, and then every for 25 hours were collected in parallel from the treated and untreated plant sets maintained in the wire-mesh screenhouse. Data (n = 4) are shown as means ± SE. Cont., untreated controls (dashed line); WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretion (solid line).

Fig. 6

Relative transcript levels of phytohormone and VOC-related genes in rice under natural conditions. JA biosynthesis genes *LIPOXYGENASE* (*LOX*), *LIPOXYGENASE 9* (*LOX9*), *ALLENE OXIDE CYCLASE* (*OsAOC*); JA-Ile conjugating *JASMONATE RESISTANT 1* (*OsJAR1*),

2 (*OsJAR2*); JA signaling pathway (*OsMYC2*), jasmonate-responsive *JASMONATE ZIM-DOMAIN 9* (*OsJAZ9*), *11* (*OsJAZ11*); terpenoids biosynthesis-related genes *OsDXS3*, *OsLIS*, *OsCAS*; methyl salicylate biosynthesis gene (*OsSAMT*); and GLV biosynthesis gene *HYDOPEROXIDE LYASE 3* (*OsHPL3*) transcript levels were determined by quantitative RT-PCR. Gene expression was determined in samples at time zero (15:00; untreated plants), samples from 30 min, 1h, and then every hour until 25 h from parallel sets of treated and untreated plants maintained in the wire-mesh screenhouse. Data (n = 4) are shown as means ± SE. Cont, untreated controls (dashed line); WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretion (solid line).

SUPPORTING INFORMATION

Supplemental Figures

Fig. S1

Environmental conditions during experiment in wire-mesh screenhouse in Figs. 2, 5, and 6. The experiment was conducted in summer season on 22-23 June, 2020. 7-week-old plants, previously acclimated for 3 weeks in the wire-mesh screenhouse, were treated with simulated herbivory at 15:00, or remained untreated, and sampling was conducted in 3 h intervals until 18:00 next day. Light intensity measured by TOPCON IM-3 Luxmeter (*lux*; Tokyo Optical Co. Ltd, Japan) was converted to Photosynthetic Photon Flux Density (*PPFD*) (µmol m^{-2} s^{-1}) using empirical conversion factor for natural sunlight (ppfd = lux/54) according to https://www.apogeeinstruments.com/conversion-ppfd-to-lux/.

Fig. S2

Environmental conditions during experiment in wire-mesh screenhouse in Supplemental Fig. S3. The experiment was conducted in summer season on 19-20 June, 2019. Untreated 7-week-old plants, previously acclimated in the wire-mesh screenhouse for 3 weeks, were first sampled at 15:00 and sampling was then conducted in 1 h intervals for 24 h. Light conversion *lux* to *PPFD* was as described in Fig. S1.

Fig. S3

Internal leaf volatiles contents in outside cultivated rice. Plants were acclimated in the outside wire-mesh screenhouse for 3-weeks prior sample collection on summer day in 2019. Youngest developed leaves were sampled in one hour intervals for 24 h, extracted for internal VOC and measured by GC-MS to show daytime-dependent changes of rice leaf volatiles under natural light and temperature conditions. Data (n = 4) are shown as means ± SE. Compound marked # is shown as relative peak area normalized by tetraline IS.

Fig. S4

Basal phytohormone levels in plants from outside experiment shown in Supplemental Fig. S3. Leaves from untreated plants were sampled every hour from 15:00 for 24 h, extracted and measured by LC-MS/MS to obtain detailed information about hormonal changes of 12-oxo-phytodienoic acid (OPDA); jasmonic acid (JA); jasmonoyl-L-Isoleucine (JA-Ile); abscisic acid (ABA); salicylic acid (SA) in rice leaves. Data (n = 4) are shown as means ± SE.

Supplemental Tables

Table S1

List of all detected headspace (headspace VOC - S1a) and internal leaf volatile compounds (internal VOC - S1b) reported in Fig. 1 (inclusive of tentatively identified and unknown compounds). Data (n = 3) are shown as averages ± SE.

Table S2

List of all detected internal leaf volatile compounds (internal VOC) reported in Fig. 2 (inclusive of tentatively identified and unknown compounds). Data (n = 3) are shown as averages ± SE.

Table S3

List of all detected internal leaf volatile compounds (internal VOC) reported in Supplemental Fig. S3 (inclusive of tentatively identified and unknown compounds). Data (n = 4) are shown as averages ± SE.

Table S4

Primers and gene IDs used in this study

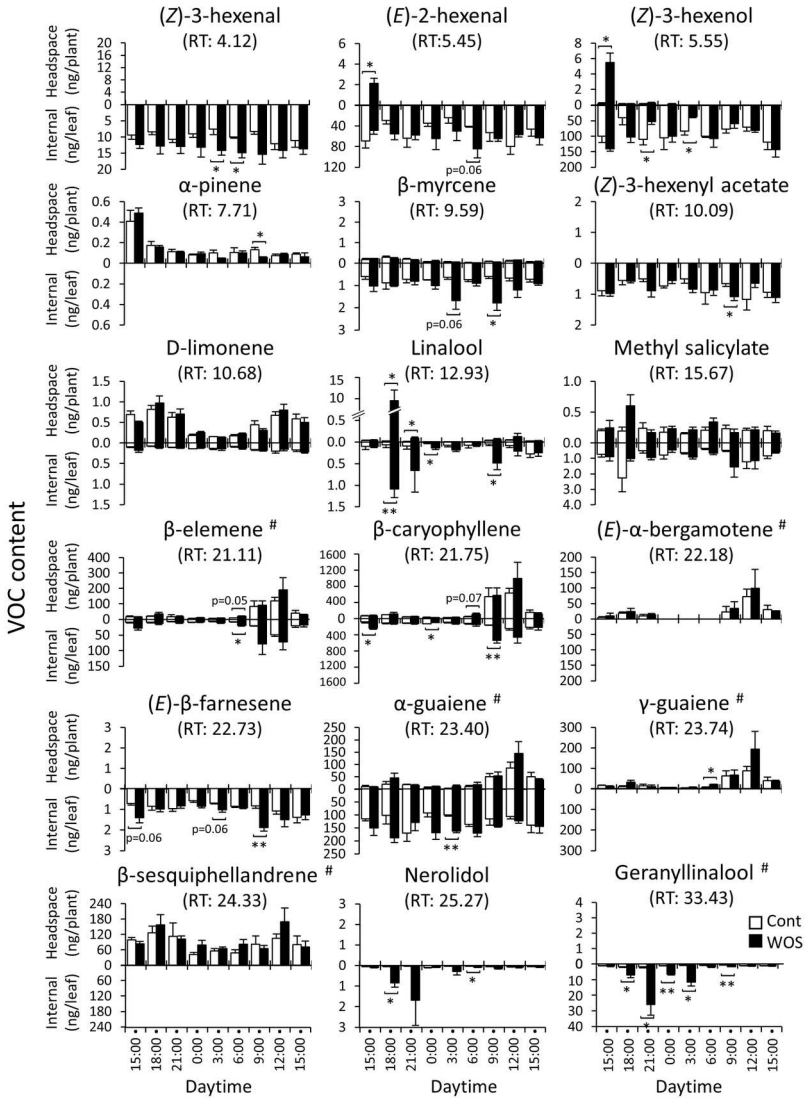

Fig. 1

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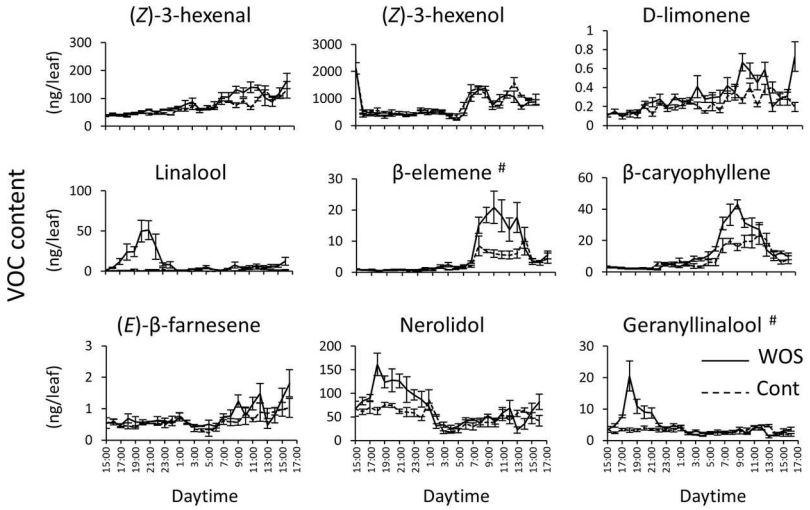

Fig. 2

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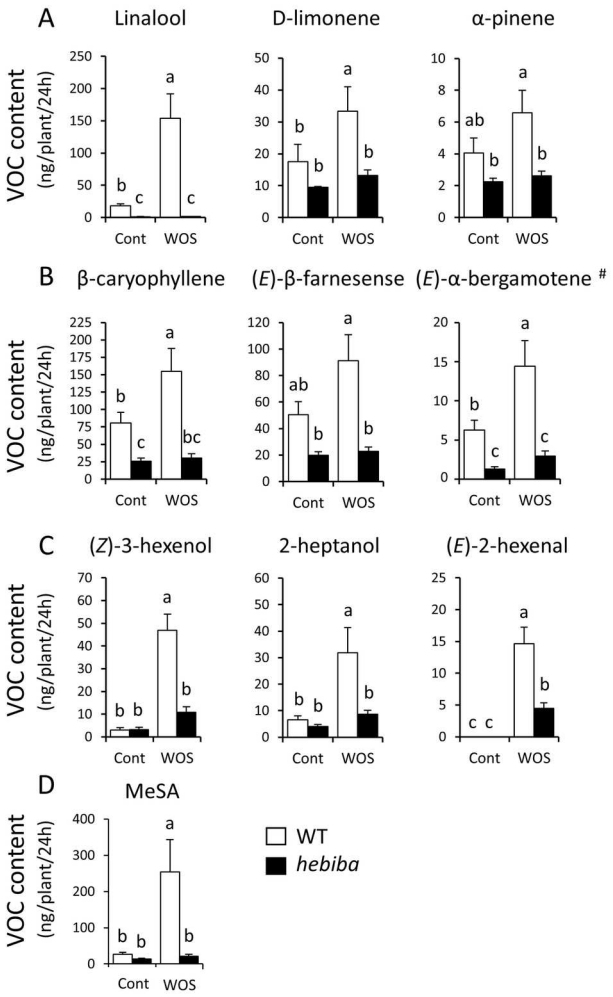

Fig. 3

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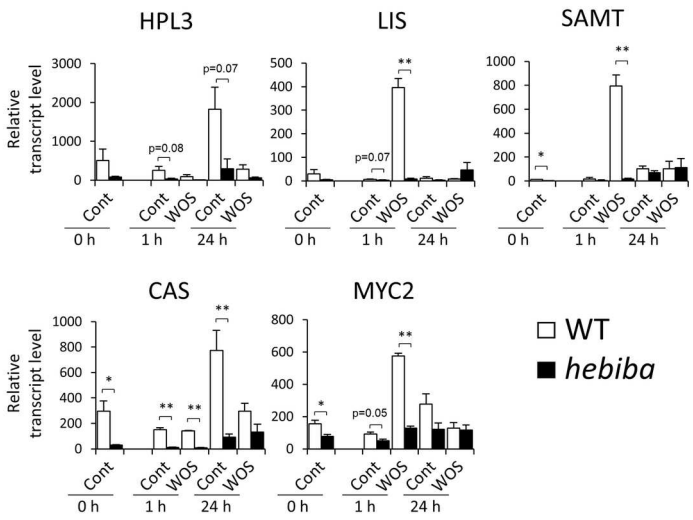

Fig. 4

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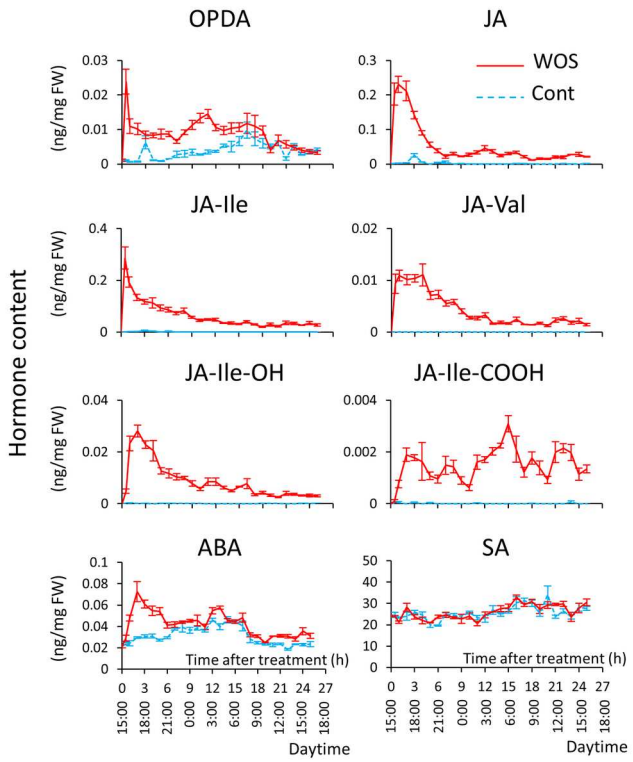

Fig. 5

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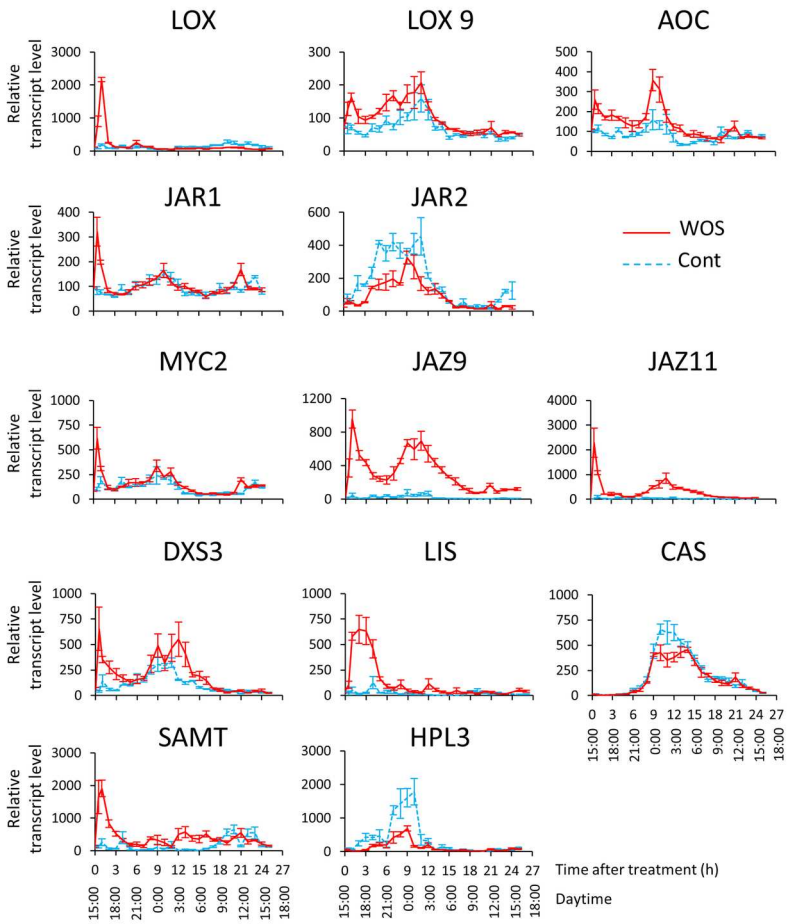

Fig. 6

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