The role of hormonal signaling in rice defense against herbivores with focus on ethylene function

イネの植食性昆虫防御におけるエチレン機能に注目したホルモンシグナルの役割

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Dedication

Alhamdulillah,

I dedicate this work to my father Mr. Bonari and my mother Mrs. Sugimah,

for always loving and supporting me, and your whisper of a prayer.

To my beloved Nurlie Rahmadini, your love, support, and sacrifices made this journey bearable and possible.

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Abstract

Plants are autotrophs that serve as a nutrition for all other heterotrophic herbivorous organisms on this planet. As countermeasures against ubiquitous damage from insect herbivores, plants evolved various reactions collectively known as defense responses. Plant defense is based on production of various secondary metabolites that either accumulate in plants during lifetime (constitutive defense) or their accumulation is triggered by exposure to stress (induced defense). Defense metabolites can directly affect herbivores (direct defense); however, a subset of plant defense metabolites, known as volatile organic compounds (VOCs), is released to external environment, where they act as attractants of natural enemies of herbivores. This system is also called indirect plant defense and it is important part of so called tritrophic interactions between plants, herbivores, and natural enemies of herbivores. In my thesis, I investigated mechanisms that control VOC production in rice. The knowledge of rice indirect defense systems is then important for design of novel, environment-friendly protection mechanism in rice crop that is the most important food crop in Asia, including Japan and Indonesia. Such approaches are likely to be useful for replacing harmful use of pesticides in agriculture that is now main and often single option for farmers that try to protect their crops from herbivores. However, pesticides impose high cost to environment as many useful insects are killed by insecticides along with the pests of crops.

Rice plants (*Oryza sativa*) are already known to produce many volatile compounds in response to mechanical damage and insect attack. These compounds can be classified into three main metabolic groups, terpenoids, phenylpropanoids, and fatty acid derivatives but their accumulation and release from rice leaves remain poorly understood. In my PhD thesis, I therefore decided to investigate VOC accumulation and release, using the methods developed in Plant-Insect Interactions group, and advanced instruments available in the Institute of Plant Science and Resources (GC-MS, GC-FID, LC-MS/MS). In the first section of my thesis, I show that jasmonate signaling pathway is crucial for rice volatile production, which is demonstrated by impaired VOC levels in two independent jasmonate signaling mutants, *hebiba* (mutated in allene oxide cyclase gene, AOC) and *Osjar1* (mutated in jasmonate resistant 1 gene, JAR1). While scrutinizing the volatile products in rice, I realized that there are two types of VOCs, with linalool representing herbivory-induced volatiles, and caryophyllene, representing a group of diurnally regulated volatile compounds in rice. I could show that both types of volatiles are actually dependent on jasmonate pathway in rice.

After the establishment of the central role of jasmonates in volatile control in rice, next section of my thesis is then focused on the relationship between internal production of volatiles in rice leaves and their release to headspace of plants. I took the advantage of the current methodological progress achieved in the laboratory that allowed me to follow both volatile pools, internal compounds and those released from plants. Only the headspace VOCs are expected to work as signals for natural enemies of rice herbivores. In both cases, I employed new type of adsorbent materials, monolithic silica adsorbents

developed by GL Sciences, Ltd, yielding superb sensitivity and reproducibility of my measurements. I found that volatiles differ in their distributions, finding 3 basic patterns: rapid release of small size volatiles (e.g., α-pinene), equal distribution in headspace and internal leaf compartment (e.g., linalool), and retention of large size volatiles in leaf (e.g. nerolidol, geranyllinalool). In addition, many of volatiles showed diurnal regulation with low release at night and increased emissions in light periods. In order to get insight in various patterns of volatiles, I analyzed the hormone levels in rice leaves induced by simulated herbivory, and in control untreated plants, to show that induced jasmonate levels basically support induced volatiles, suggesting that herbivory triggers jasmonate production that activates expression of volatile biosynthesis genes that further triggers volatile production in the leaf. From size dependent release of volatiles, I hypothesized that small molecules may escape from rice leave by passive diffusion, but my results also predict existence of specific transporters for larger volatiles like caryophyllene.

Next, I asked if diurnal emissions and hormone control could be light-dependent, as suggested from diurnal patterns of emissions of major rice volatiles. I conducted series of experiments where rice plants were exposed to light during normal night period. This treatment was able to trigger jasmonate signaling, proposing a scenario that light-controlled jasmonates may be involved in control of volatile genes and volatile emissions in rice plants. This would be logical as natural enemies are active during the daytime, and therefore rice plants should emit volatiles and attract them during light periods.

The second main part of my thesis is focused on ethylene and its role in regulation of volatile production in rice. This part was submitted and already published in Journal of Experimental Botany. Because of complex patterns of volatiles in rice, I hypothesized that there should be more regulators that act in concert with jasmonates described in the first section of my thesis. As obvious candidate, I focused on ethylene that is a well-known regulator of plant responses to stress. It has been shown previously that ethylene is also involved in regulation of defense against herbivores, but mechanisms are not clear. For example, ethylene acts in different ways in case of attack by chewing-biting and sucking herbivores. In my approach, I first decided to investigate the ontogenetic aspects of rice volatile production. Interestingly, juvenile rice plants until 7 weeks produced low levels of volatiles in response to simulated herbivory, maximal levels at 8 weeks and then production dropped again. When I investigated jasmonate levels at each stage, the highest levels of JA-Ile were found in 8-week plants. These plants also featured lowest levels of ethylene, suggesting that jasmonates work as a positive regulators of rice volatile production, while ethylene may act in opposite way. Negative role of ethylene on rice VOCs was confirmed in experiments with exogenous ethylene application. As low as 1 ppm levels of ethylene could block linalool biosynthesis, and this level of ethylene was able to suppress transcription of linalool synthase gene in rice. The ethylene showed a broad negative effect on several other known genes involved in VOC production, such as SAMT in methyl salicylate, HPL3 in green leaf volatile, and CAS in caryophyllene productions. As these results were rather unexpected, I next engaged in finding examples of ecological situations where suppression of volatiles by ethylene would present advantage to rice plants. As young rice plants grow in water, and can be subjected to

occasional flooding, I speculated that shut down of volatile production in flooded leaves could be a preventive measure to over accumulation of volatiles that cannot be released from plants. Because flooding triggers ethylene that activates elongation of seedlings and escape from stress, I proposed that second function of ethylene would be the suppression of volatile production. Indeed, flooding suppressed volatiles in rice leaves subjected to simulated herbivory. Accordingly, VOC biosynthesis genes were suppressed in leaves under water, functionally connecting the ethylene-mediated transcriptional repression to actual volatile metabolic repression.

Furthermore, as mentioned above, volatile production was low in older 9-10-week-old plants, but ethylene was high at these stages. Therefore, in the final part of my thesis, I test the possibility that ethylene may work as natural suppressor of volatile production in maturing plants. This would be again ecologically relevant because older rice plants develop strong mechanical barriers and they may be less dependent on help of natural enemies, and therefore avoid volatile production on accounts of increased energy input to seed production. I used 1-metylcyclopropene (1-MCP), commercially available ethylene receptor blocker to show that simple repression of ethylene function can rescue volatile production in 9-week-old rice plants. Similar to flooding experiments, I could show that volatile production genes can be de-repressed by 1-MCP treatment, connecting the ethylene function to transcriptional regulation of volatile production in rice.

Overall, I conducted a deep study on mechanisms involved in the control of volatile production in rice. Such knowledge is particularly useful in design of natural protection measure for control of herbivore damage in crops. This can be achieved either by direct application of volatile compounds to rice crop in the field, although this approach may be too costly to be used in practice. Alternatively, it should be possible to construct rice plants with improved volatile profiles, emission mechanism that would more effectively attract natural enemies of herbivores, and therefore better protect rice from extensive damage.

Chapter 1 Introduction

1. General Background

1.1. Plant defense mechanism against herbivores

Autotrophic plants serve as nutrition for all other heterotrophic herbivorous organisms on the planet. As countermeasures against damage from insect herbivores, plants evolved various reactions collectively known as defense responses (War et al., 2012; Bruce, 2015; War et al., 2018). In fact, plants have been on Earth for millions of years, during which time they effectively adapted to complex environments imposing on them both favorable and adverse conditions. Plants therefore detect and respond actively to various stresses in the ecosystem, that can be either biotic or abiotic. In general, plant responses are regulated by a large set of small molecules, known as hormones in plants. Plant responses to necrotrophic pathogens and insects are mainly regulated by jasmonic acid, ethylene, and abscisic acid (Broekgaarden et al., 2015; Gimenez-Ibanez et al., 2016; Jamalian et al., 2013; Vos et al., 2015), while responses to biotrophic pathogens are mainly regulated by salicylic acid (Tanaka et al., 2015; Vos et al., 2015). In addition, together with gibberellin, ethylene is involved in controlling the adaptation of rice plants to flooding by promoting stem elongation (Jackson, 2008; Kuroha et al., 2018). Responses to drought and salinity stress are controlled by abscisic acid (Gray, 2004). In contrast, plant growth is mainly controlled by developmental hormones like auxin, cytokinin, gibberellin, brassinosteroids and others (Gray, 2004; Lorenzo et al., 2003). It is clear that hormones have many critical roles in plants.

As a primary energy producer, plants have always been under enormous pressure from consumers, i.e. herbivores, especially arthropods. In fact, insects are the main species that become herbivores. Insects feed on plants by various mechanisms, such as chewing-biting, cell-sucking, and sap-feeding (Dicke & Poecke, 2002). As countermeasures against such damages from insects, plants evolved various reactions collectively known as defense responses (War et al., 2012; Bruce, 2015; War et al., 2018). Plants have two major defense systems to cope with the attack of herbivores, so called direct and indirect defense (Chaudhary et al., 2018; Dicke & Poecke, 2002; Mello & Silva-Filho, 2002). These can be produced constitutively, or they become activated (induced) in response to herbivore

attack (Fig 1). As for their name, constitutive defense systems operate constantly in plants, regardless of herbivory incidence, such as cuticles, thorns, spikes, and toxic/anti-nutritive metabolites that accumulate in developmentally programmed fashion, which effectively reduces herbivore feeding and damage in plants (Wu & Baldwin, 2010). In contrast to constitutive defenses, induced defense responses are only triggered by herbivore damage in most plant species, which further inhibits herbivore consumption, growth, and survival on the host plants (Kessler, 2001).

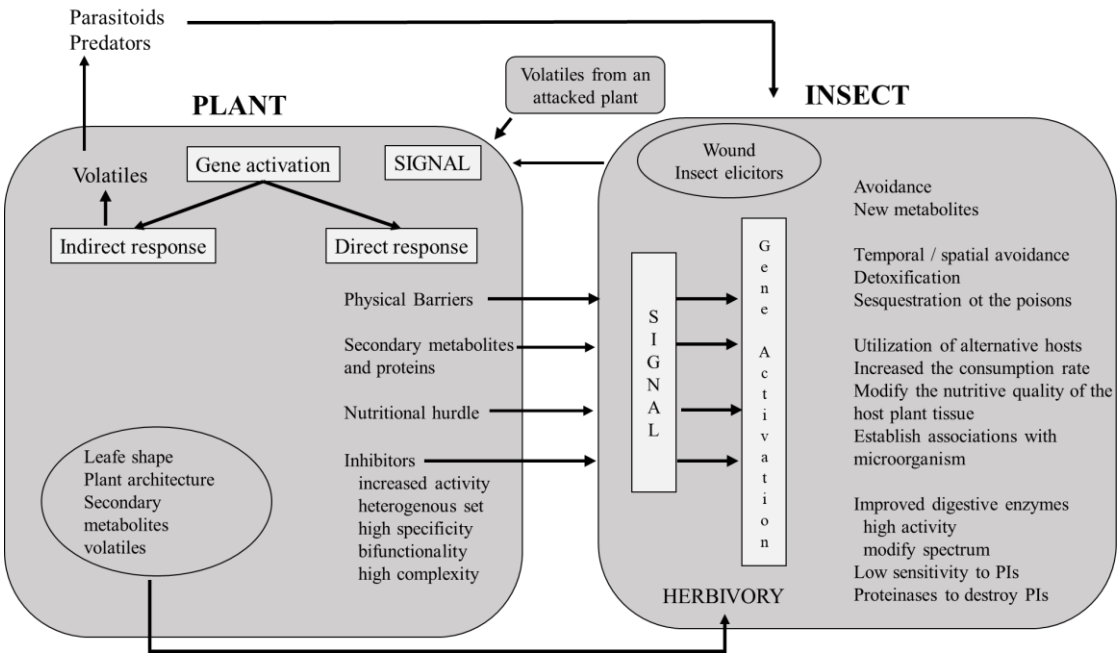

Fig. 1.1. Plant defense mechanism against herbivores (Mello & Silva-Filho, 2002). Scheme of direct and indirect defense mechanism in plants, mainly controlled by chemical defenses induced by insect elicitors or priming from neighbors. Constitutive defenses of both chemical and physical structures complement the plant's defense system against herbivores.

Direct defenses can be mediated by chemical and physical mechanisms. Chemical compounds such as diverse secondary metabolites directly influence the insect performance and feeding behavior (Dicke & Poecke, 2002; Sánchez-Sánchez & Morquecho-Contreras, 2017). Secondary metabolite compounds such as terpenoids, alkaloids, anthocyanins, phenols and quinones are known to interfere with insect development or kill herbivores (Handley et al., 2005). For instance, trypsin protease inhibitors in rice and benzoxazinoids in maize, have anti-digestive or toxic effects on insect herbivores (Qi et al., 2018). In addition to chemical defense compounds, direct defenses involve development of morphological structures that act as physical barriers, including trichomes, hairs, thorns, spines, prickles, and thick leaf cuticles (Dicke &

Poecke, 2002), or mechanical features such as thigmonasty, mimicry, and camouflage (Chaudhary et al., 2018)

Contrary to direct defense, indirect plant defense, which is the main topic of my research, cannot directly dispel or kill herbivores, but in a certain way, plants manipulate their micro-ecosystem to call for help from herbivore's natural enemies, such as parasitoids and predators. Indirect defense mechanisms are mediated by production of constitutive or induced blends of volatile organic compounds (VOCs) that are recognized by specific classes of natural enemies of herbivores to kill them, directly, or through parasitism (Aljbory & Chen, 2018; Arimura et al., 2005). Moreover, some plants could provide shelter for carnivores (*domatia*) or provide alternative food (nectar and extrafloral secretions), which can increase the chance of predation or parasitism on herbivores (Agrawal & Karban, 1997; Chaudhary et al., 2018; Dicke & Poecke, 2002).

2. The main thesis objectives

The main objective of this study was to decipher the role of hormonal signaling in rice defense against herbivores, particularly jasmonic acid and ethylene, and their crosstalk in the regulation of volatiles production in rice plants in response to biotic and abiotic stresses. In Chapter 3, I investigated how emission and production of volatile organic compounds are controlled at the molecular levels in rice, especially the role played by jasmonates. In Chapter 4, I focused on regulation of indirect defense in development, in response to herbivory, and under short flooding stress with special focus on role of ethylene as novel endogenous plant regulator in these processes.

Chapter 2 Materials and Methods

1. Plant growth conditions

Japonica rice *Oryza sativa* L. cv. Nipponbare was used in all experiments. Seeds were placed in germination trays with nutrient-rich soil pellets, Kumiai Ube Baido No.2 (MC Ferticom, Tokyo, Japan), and two weeks later, seedlings were transferred into larger individual pots with sterilized field soil mixed with nutrient-rich pellets at 4:1 (v/v) ratio. Plants were maintained in cultivation room under controlled growth conditions (14 h photoperiod, temperature 28 ± 3°C). Germinations were repeated every week to generate series of plants at developmental age of 5-11 weeks. In most experiments, youngest developed leaves of 6-10-week-old plants were used for treatments. In addition to wild type Nipponbare plants, JA-free mutant (*hebiba*; Nihonmasari genetic background) and JA-Ile associated mutant (*Osjar1*; Nipponbare background) were used to examine the jasmonic acid dependency of rice volatiles. Heterozygous seeds of *hebiba* mutant were obtained from Dr. Kazunori Okada, University of Tokyo (with permission of Dr. Michael Riemann, Karlsruhe Institute of Technology (KIT), Germany). OsJAR1 mutant was a TOS17 transposon mutant obtained from the National Institute of Agrobiological Sciences (NIAS), Japan. As homozygous plants are sterile (due to deficient jasmonate signaling), heterozygous seeds were planted and analyzed by genomic PCR, using specific primers for *hebiba* and *Osjar1*, to separate homozygotes, heterozygotes, and wild-type rice plants, subsequently used in experiments.

2. Plant treatments

2.1. Wounding and oral secretions (WOS)

Herbivory was simulated by wounding and oral secretion (WOS) treatment. WOS treatments were performed by making mechanical wounds, using a fabric pattern wheel, along both sides of the midvein of the youngest fully developed leaf on each plant, followed by application of 20 µL water-diluted 3:1 (v/v) oral secretions (OS), evenly spread over the wounded leaf surface. Treated and untreated plants were placed in the cultivation room for specified time period according to experiment and sampled as described. Insect oral secretions (OS) were collected from the fourth larval instar of

generalist chewing herbivore *Mythimna loreyi* (MYL) (Lepidoptera: Noctuidae) as described in Shinya et al., 2016.

2.2. Diurnal experiment

To uncover the oscillations of hormones and their role in VOC production in rice, we conducted 24-hour VOC, hormone, and gene expression analyses. For diurnal VOC oscillation analysis, two sets of six to seven-week old plants were placed in controlled room conditions (14 h photoperiod, temperature 28 ± 3°C). The WOS treatment was performed at 15:00 o'clock, and then leaf samples were collected every three hours (n= 3). Samples from untreated control plants were collected at the same time, so that all sampling could be completed at 15:00, 18:00, 21:00, 0:00, 3:00, 6:00, 9:00, 12:00, 15:00, 18:00, respectively. From the first set, leaf samples were used for internal VOC analysis, using internal VOC extraction method. In the second sets, VOC analysis was performed by headspace collection method to trap emitted volatiles from the rice plant.

For diurnal hormones analyses, a set of six to seven-weeks rice plants was placed in controlled room conditions (14 h photoperiod, temperature 28 ± 3°C). The youngest fully developed leaf on the plant was collected directly in 2 mL plastic screw tubes every hour started at 15:00 for 24 hours (n= 4 each time point), then immediately dipped into liquid nitrogen and stored at -80°C until hormone extractions. Same sample collection method was carried out to collect samples for genes expression analysis. However, for genes expression analysis, leaves samples were collected from both WOS-treated and control plants in 3 h intervals. At the first three hours, samples were collected at 0; 0.5; 1; 2; 3 hours after WOS treatment, then every 3 hours for completing the whole 24 h cycle (n=5).

2.3. Light experiment

Light manipulation setup was carried out to uncover whether light exposure during dark phase could trigger production of jasmonates in rice plants. The 7-week plants were placed in the dark growth room at night, and then at the specific time points, a group of plants were transferred into growth chamber with white light. The light exposure setup was repeated 3 times during night, first is from 22:00 to 0:00; then from 2:00 to 4:00; and the last is from 6:00 to 8:00 o’clock. Leaf sample were collected after one and two hours of light exposure to determine immediate hormone changes. Control plants were kept in

the room with normal regime that included daylight through glass window and turning "on" supportive fluorescent lights at 6 :00 o'clock. Samples were collected every hour from 21:00 to 8:00 to monitor basal hormone levels without strong light exposure.

2.4. Ethylene treatments for hormones and volatiles

To elucidate the role of ethylene in VOC biosynthesis, plants at five different growth stages (6-10 week after sowing) were treated with WOS, and each plant was immediately covered with transparent acrylic cylinder (10 cm diameter, 60 cm height), put in a small tray, and sealed with water at the base. Ethylene standard gas (GL Sciences Inc., Japan) was injected into each cylinder for concentration of ethylene in each cylinder to reach 1 ppm. Separate set of plants in cylinders were injected with equivalent amount of ambient air without ethylene, and used as controls. Four hours later, samples (treated leaves) for the leaf volatile measurements were cut and leaf weight was quickly determined before inserting each sample into a clean 16 cm glass tube enclosed with air-tight lid. All samples for internal leaf volatile analyses were stored at -80 ºC overnight, or until extraction of volatiles by heat distillation method. For hormone and gene expression analyses, leaves were collected directly in 2 mL plastic tubes before, and 0.5, 1, and 4 h after treatment, dipped into liquid nitrogen, and stored at -80 °C until hormone or RNA extractions.

2.5. Ethylene treatment for elongation test

To determine the effect of ethylene on rice elongation, plants were individually covered with water-sealed acrylic cylinders as described above and fumigated with ethylene gas at final 1 ppm air concentration. Internode length in each position of rice stem was measured before, and 24 h after ethylene application.

2.6. Ethylene inhibitor treatment

Ethylene perception inhibitor, 1-methylcyclopropene (1-MCP) was used to examine the effect of endogenous ethylene on VOC production in rice plants. The 9-10-week-old plants were pretreated for 24 h with a final concentration of 100 ppm 1-MCP in air, and then used for WOS treatment as before. Both 1-MCP-treated and control plants (n = 6-8) (without 1-MCP) were inserted inside of airtight acrylic glass cylinders (20 cm diameter, 85 cm height), and both sets were maintained under normal light and temperature conditions.

2.7. Flooding treatment

To elucidate VOC responses in flooded rice treated with WOS, 7-week-old plants were 90% flooded in water for 24 h using acrylic glass cylinder (20 cm diameter, 85 cm height). Plants were briefly removed from water, subjected to WOS treatment as described above, and returned into the water for continued flooding stress. Exactly 4 h later, plants were removed from water and treated leaves were harvested for further analyses (VOC analysis or genes expression). As control, non-flooded plants treated with WOS were kept in a separate room to avoid ethylene cross-exposure. Internode length was measured before, and 24 h after initiation of flooding stress.

3. Data collection and analysis

3.1. Headspace volatile method

Rice volatile organic compounds were collected following Sobhy et al. (2017) under optimized conditions with slight modifications using dynamic headspace collection method. Plants after treatment were carefully inserted into a 50 cm high and 15 cm diameter acrylic cylinder with an open base. The cylinder was equipped with an inlet port at 15 cm from the bottom and an outlet at the top. To seal the collection compartment, the base of the cylinder was placed into an aluminum tray filled with water. All the collection chambers were connected by tubing to a single pump ULVAC DAP-12S (ULVAC KIKO Inc., Japan). Outside air was purified with charcoal filter and pulled into the cylinder at approximately 10 L min^{-1} flow rate. After passing cylinder with plant, air was passed through a custom trap containing MonoTrap device (monolithic silica adsorbents; GL Sciences Inc., Japan). After trapping VOCs for time intervals depending on type of experiment, samples were eluted into 1.5 ml glass vials with dichlorometane (DCM) after spiking with 400 ng tetralin (1,2,3,4-tetrahydronaphthalene), which was later used as an internal standard.

3.2. Analysis of the volatile organic compounds

The elutes were analyzed using a gas chromatography mass spectrometry (GC-MS) with Agilent 240 MS ion trap coupled to Agilent 7890A GC as described in Sobhy et al., 2017. Auto sampler Agilent 7693A was used to inject 1 µl of the sample into the injector port held at 230 °C in split mode that was adjusted to sample concentration (typically 3:1).

Helium was used as the carrier gas and ion trap was set at 260 °C. GC oven temperature was programmed at 40 °C for 3 minutes and then increased 5 °C per minute to 180 °C, then 20 °C per minute to 300 °C and held for 5 minutes before returning to initial conditions. Emission current of 30 µAmp was used to achieve the mass spectra ionization in the ion trap. Column flow of 1.000 mL/ minute was used with a 30 m long HP-5MS column of 0.25 mm inner diameter and 0.25 µm film thickness (Agilent Technologies). Mass spectra data within mass range of 40-300 m/z were collected in full scan mode. Spectra were tentatively compared with NIST 2011 mass spectral library and software, and finally confirmed by authentic standards of 23 compounds. Collected data were analyzed using Agilent Workstation Ver. 7.02 software.

3.3. Internal volatile extractions and analysis

Solid phase micro-extraction (SPME) was performed to collect volatiles from frozen rice leaf tissues with a customized procedure, following the original protocol developed by a Master course student, Tilisa Tohi. Briefly, frozen leaves in 16 mL glass tubes were removed from -80 ºC deep freezer, defrosted on ice, and then one MonoTrap (GL Sciences Inc., Japan) was suspended on a stainless steel pin inside of each PTFE (Polytetrafluoroethylene) lid containing a nitrile rubber packing. Each sample was spiked with 400 ng tetralin (1,2,3,4-tetrahydronaphthalene; FUJIFILM Wako Pure Chemical Corporation) in dichloromethane applied to bottom of each tube containing plant tissue. After tight closure, tubes with samples were inserted into a programmable heat block held at room temperature (aluminum block hole diameter 1.8 cm; depth 6.5 cm). Heat program was activated to bring temperature to 150 ºC, where it was held for 30 min before naturally cooling down heat block to next preset temperature at 60 ºC, and samples were incubated for additional 30 min at 60 ºC. Each MonoTrap was detached from its lid, dried with gentle stream of nitrogen gas, and eluted with 200 µL dichloromethane (DCM; FUJIFILM Wako Pure Chemical Corporation) using 1.5 mL GC vials supplied with 375 µL glass inserts (Tomsic Ltd., Japan). Whole assembly was sonicated in water bath sonicator for 5 min, three times, with 2 min pause in between each round of sonication to allow samples to cool down. Pins were carefully removed together with MonoTrap from each vial that was then tightly closed with lid and analyzed on GC-MS instrument.

Volatile compounds from eluted leaf samples in DCM were injected (1 µL) in a split mode (ratio 3:1) into an injector port of Agilent 7890A GC (HP-5MS capillary column, 30 m, 0.25 mm i.d., 0.25 µm film thickness, Agilent Technologies, USA) coupled to an ion trap Agilent 240 MS, following instrument setting and parameters described in Sobhy et al. (2017). MS data were analyzed with Agilent Workstation software ver. 7.02. Quantities of each reported volatile compound were calculated by comparison with the peak areas of a separately run authentic standards at concentration range 0.1-5 ng/µL, using split ratio for sample analysis (linalool, limonene, caryophyllene, E-β-farnesene, methyl salicylate, cis-3-hexenol; FUJIFILM Wako Pure Chemical Corporation).

3.4. Phythormone measurements

Phytohormone contents in collected leaf samples from WOS treatment and control at five plant stages (6-10 weeks) were measured by a triple quadrupole LC-MS/MS 6410 (Agilent Technologies, Santa Clara, CA, USA) equipped with a Zorbax SB-C18 column (2.1 mm id x 50 mm, (1.8 µm), Agilent Technologies). Phytohormone extraction method and analyses followed procedures developed in the laboratory and described in detail by Fukumoto et al., 2013.

3.5. Ethylene determination

Ethylene released from five different plant stages (6-10 weeks) or leaves from 7-week-old rice after flooding for 24 h (vs. air control), treated or untreated with WOS, was measured after incubating cut (i.e., wounded) leaves in closed glass containers for 24 h. The youngest fully developed leaf from each plant stage was cut, quickly measured for fresh weight, inserted into glass tube (12 cm height, 2 cm diameter, 50 mL volume), and then tightly sealed with a silicone plug. Leaves were incubated at normal light regime and temperature for 24 h, and 1 mL of inside gas sample was removed from each tube with 1 mL syringe after inserting sharp needle with side hole through a soft silicon plug. Ethylene content was determined by gas chromatograph (GC-2014, Shimadzu, Japan) fitted with packed column ShinCarbon ST 50/80 (2.0 m length, 3 mm i.d., Shimadzu), using standard flame ionization detector (FID) setup. Helium gas carrier was used at 25 mL/min, injection port was held at 200 ºC, and oven temperature was programed to remain isocratic at 200 ºC in each 5 min run.

3.6. Gene expression, quantitative RT-PCR

Gene expression was quantified with qRT-PCR essentially as described by Fukumoto et al. (2013). Total RNA was extracted from 100 mg leaf tissue using 1.2 mL Trizol reagent following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized from 1 µg of total RNA using PrimeScript reverse transcriptase (Takara Bio Inc., Japan) after completing standard DNase treatment and necessary cleanups of RNA samples. Transcript levels were quantified by THUNDERBIRD qPCR Mix (Toyobo, Osaka, Japan) on a CFX Connect ™ Real-Time System (Bio-Rad Laboratories, Inc, USA). OsEF1α housekeeping gene was used to normalize the relative transcript levels. Gene-specific primer sequences forward/reverse used for qRT-PCR are described in Supplementary Table 1.

3.7. Statistical analyses

Statistical differences as indicated in legends were conducted with one-way analysis of variance (ANOVA) followed by Fisher's LSD test ($P<0.05$), performed with an open source software OpenStat (http://statpages.info/miller/OpenStatMain.htm), or Student's t-test incorporated within the commercial version of Microsoft Excel (Microsoft Corporation, USA).

Chapter 3 General regulation mechanisms of VOCs in rice

1. Introduction

1.1. Background

Plant defense is essential for survival of plants in nature. In addition to mechanical defense systems, plants use an array of chemical weapons to counteract insect herbivores and other biotic stressors, such as pathogenic organisms. In this chapter, I focus on chemical defenses, and in particular volatile compounds produced by rice plants in response to mechanical damage and herbivory.

1.2. Volatile organic compounds in plants

Defense chemicals (Mithöfer & Boland, 2012), both volatile and non-volatile in nature, accumulate in plant cells, where they act as direct defense compounds (phytoanticipins and phytoalexins). In addition, a subset of defense-related volatile organic compounds (VOCs) is released to external environment, where they act as attractants of natural enemies of herbivores (Turlings & Tumlinson, 1992), danger signals for conspecifics, and even alert other plants in the close neighborhood (Karban et al., 2000; Erb, 2018). It has been reported that some volatiles can be absorbed by nearby plants, modified, and used as direct defense compounds against herbivores (Sugimoto et al., 2014). The VOCs released in response to herbivory, generally termed as herbivore-induced plant volatiles (HIPVs), can be released either rapidly (green leaf volatiles, GLVs) or gradually (terpenes, phenylpropanoids, and benzenoids) (Dicke et al., 2009; War et al., 2011). HIPVs are also produced and released systemically, i.e. in/from undamaged plant tissues, respectively (Paré & Tumlinson, 1999). Volatile synthesis and release, similar to accumulation of direct defense metabolites, is primarily triggered by herbivore feeding, oviposition behavior, or herbivore movement on the plant, and it is often amplified by chemical cues occurring in herbivore oral secretions, oviposition fluids, honeydew, and frass (Hilker & Meiners, 2010; Maffei et al., 2012).

The importance of plant volatiles is underlined by additional roles of these compounds in attraction of pollinators and frugivores (Rodríguez et al., 2013; Schiestl, 2015), function as antioxidants (Brilli et al., 2019), and antimicrobial agents (Junker & Tholl, 2013). Upon herbivory, HIPV production can be attributed to three main metabolic pathways, terpenoid, phenypropanoid, and fatty acid metabolism (Dudareva et al., 2006; Pichersky & Gershenzon, 2002). More than 2000 volatile compounds released in response to herbivory have already been identified from 900 plant families (War et al., 2011). Reflecting a high natural diversity of VOCs, blends of volatiles, rather than a single compound, are typically released from each plant in response to herbivory (Arimura et al., 2009). Discharge of some VOCs is tightly regulated and restricted to specific time of the day, showing a diurnal emission pattern controlled by circadian clock (Christensen et al., 2013; Zeng et al., 2017). In addition, volatile emissions from young and mature plants can differ significantly, with younger plants at vegetative stage usually releasing more volatiles, both constitutively and after elicitation by herbivory (Hare, 2010; Köllner et al., 2004; Rostás & Eggert, 2008; Shiojiri & Karban, 2006). Naturally, emission patterns of plant volatiles described above must be controlled, specifically and coordinately, by multiple regulators but molecular details remain mostly unknown.

1.3. Involvement of jasmonic acid in plant defense

Rapid defense responses against herbivores, including VOCs, are orchestrated by a network of dedicated plant hormones (Verhage et al., 2010). Specifically, plant defense responses against chewing herbivores and necrotrophic pathogens are controlled by jasmonic acid (JA), while piercing and sucking herbivores and biotrophic pathogens are controlled by salicylic acid (SA), and both signaling pathways are actively cross-linked with innate ethylene signaling (van Loon et al., 2006). Hormones may act individually, synergistically, or antagonistically, depending on stress factors and/or their combination (Verhage et al., 2010). Jasmonate signaling (Turner et al., 2002) is integrated with many other hormonal systems, including salicylic acid (SA), abscisic acid (ABA) and ethylene (ET) (Mello & Silva-Filho, 2002; Sánchez-Sánchez & Morquecho-Contreras, 2017; Vos et al., 2015). In addition to the biotic and abiotic stress responses, JAs also control plant growth and development (Larrieu & Vernoux, 2016). Jasmonates include many derivatives such as methyl jasmonate (MeJA), cis-jasmone, jasmonoyl-1-amino-1-

cyclopropane carboxylic acid (JA-ACC), a bioactive JA derivate (+)-7-iso-Jasmonoyl-L-isoleucine (JA-Ile), and coronatine (COR), which is the analogue of JA-Ile (Larrieu & Vernoux, 2016; Ruan et al., 2019; Yan et al., 2013). Notably, function of some derivatives remains unknown.

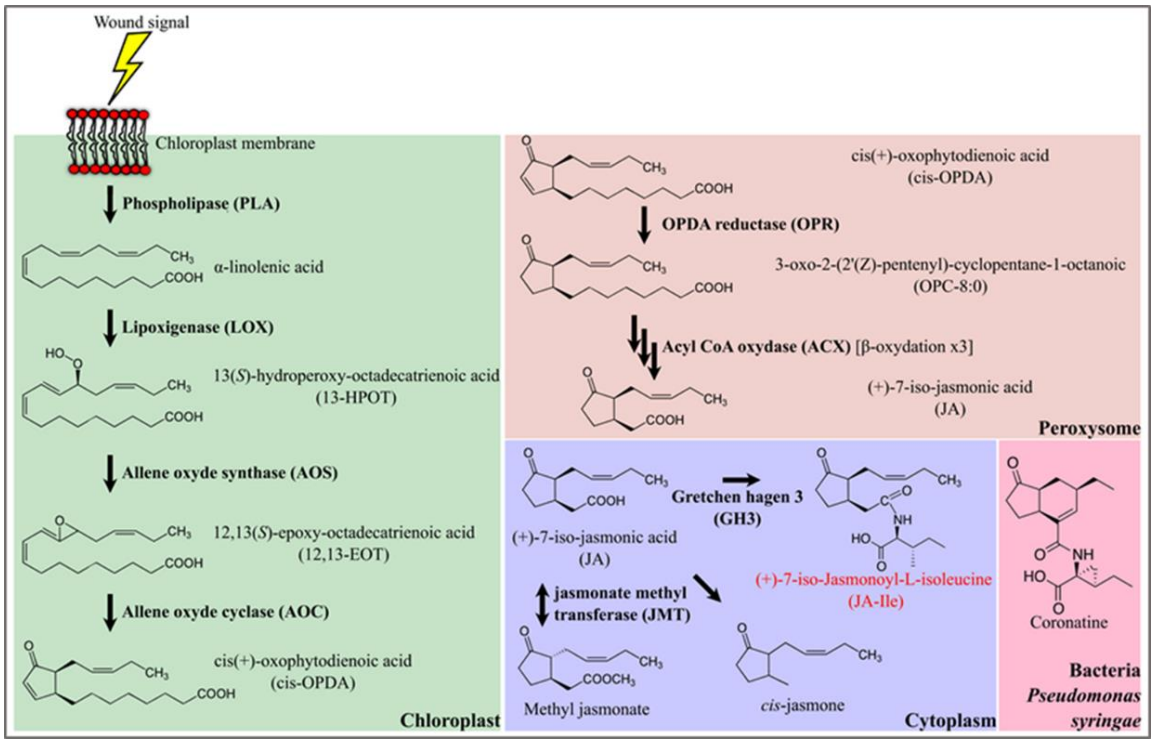

Fig. 3.1. Schematic figure of jasmonate biosynthesis in plants. In octadecanoid pathway, OPDA is generated from α-LeA, which takes place in chloroplasts. In peroxisomes, cis-OPDA is reduced by OPR to OPC then derived to JA by three steps of β-oxidation. JA is transferred to cytoplasm as source of JA conjugates that includes JA-Ile as bioactive hormone (from Larrieu & Vernoux, 2016).

Initiation of JA biosynthesis occurs in chloroplasts, where galactolipids contained in chloroplast membrane release the α-Linolenic acid (α-LeA) by lipase activity. α-LeA is converted into cis-(+)12-oxo-phytodienoic acid (cis-OPDA), under the sequential reactions of a series of chloroplast-localized enzymes, including lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). cis-OPDA is then transported to peroxisome and reduced by OPDA reductase (OPR) and then (+)-7-iso-jasmonic acid is formed by three subsequent rounds of beta oxidation using specific enzymes (Fig. 3.1) (Larrieu & Vernoux, 2016; Ruan et al., 2019; Turner et al., 2002; J. Wang et al., 2019).

Although JA can be converted into a number of derivatives and conjugates, JA-Ile is recognized as the most active JA form in plants (Fonseca et al., 2009; Woldemariam et al., 2014). JA-Ile conjugation is catalyzed by jasmonic acid resistant 1 (JAR1), group of Gretchen Hagen 3 (GH3) gene family. *OsJAR1*, *OsJAR2*, and *OsJAR3* members of GH3 protein family have been reported in rice genome, but only *OsJAR1* and *OsJAR2* were active in JA-Ile formation (Wakuta et al., 2011). Only *OsJAR1* contributed to stress-induced JA-Ile production in response to herbivory and blast fungus (Fukumoto et al., 2013; Shimizu et al., 2013); therefore contribution of OsJAR2 remains to be resolved. JA-Ile activates plant defense by binding F-box protein CORONATINE INSENSITIVE 1 (COI1) which increases the rate of JASMONATE ZIM DOMAIN (JAZ) protein degradation, relieving the transcriptional repression of both JA and JA/ET responsive defense genes (Schmelz, 2015). JAs accumulate within minutes after wounding and/or herbivory in affected plant tissues (A. J. K. Koo & Howe, 2009). Perception of JA-Ile by COI1-JAZ co-receptor complex and activation of defense genes (Wasternack & Hause, 2013) is known to include VOC biosynthetic genes in plants but it has not been directly investigated in rice.

1.4. Contribution of signals at plant-insect interface

Plant defense signaling induced by herbivores is potentiated by perception of insect-derived elicitors of plant defense. Insect herbivores produce oral secretions (OS) that contain a number of elicitors and effectors potentially recognized by plant receptors to initiate or modulate plant immune responses (Sobhy et al., 2017). Elicitors or effectors that are resealed by insects during feeding or oviposition are generally known as herbivore-associated molecular patterns (HAPMs). There is another class or elicitors derived from wounded host plants, called damage-associated molecular patterns (DAMPs). These insect elicitors are thought to help inducing (tailoring) direct and indirect defense cascades in plants, most likely by acting on the canonical wound signaling in plant tissues dependent on jasmonates (Wang et al., 2019; Wu & Baldwin, 2010).

1.5. Composition of volatile organic compounds in rice

Rice plants produce diverse secondary metabolites with diverse functions including anti-insect activity, at least 276 compounds have been identified in rice that belong to groups

of phenolic acids, flavonoids, terpenoids, steroids, alkaloids, and their derivatives (Wang et al., 2018). Furthermore, rice plants produce volatile organic compounds, either constitutively or induce them in response to herbivore attacks (HIPVs).

According to literature and our data, there are three main groups of volatile organic compounds in rice. The first group is green leaf volatiles (GLVs), including (*Z*)-3-hexenol, 2-heptanone, 2-heptanol, (*E*)-2-hexanal, and (*Z*)-3-hexenyl acetate. Second group is comprised of aromatic compounds, such as methyl salicylate, methyl benzoate, and indole. The largest group is terpene volatiles, such as monoterpenes and sesquiterpenes that are widely distributed in rice leaves. Monoterpenes include linalool; limonene; myrcene; α-pinene; β-pinene; α-thujene; sabinene; α-terpinene; (*E*)-β-ocimene; β-cymene; (*Z*)-linalool oxide; (*E*)-linalool oxide; 1,4-cineole; and fenchyl acetate. Reported sesquiterpenes consist of (*E*)-caryophyllene; trans-α-bergamotene; β-elemene; longifolene; (*E*)-β-farnesene; cyclosativene; α-copaene; (*E*)-nerolidol; α-curcumene; α-copeane; α-cadinene; β-bisabolene; α-humulene; β-gurjunen; and some others sesquiterpenes (Chumpolsri et al., 2015; Lee et al., 2016; Sobhy et al., 2017; Wang et al., 2018). Cheng et al., 2007, identified at least 25 sesquiterpene volatiles to be produced by *OsTPS3* gene induced by MeJA in recombinant protein assays. In the following chapter, most of these volatiles were detected in rice by methods developed in the Plant-Insect Interaction group, using a GC-MS ion trap system. In particular, the work of previous Master student Tilisa Tohi was instrumental in development of extraction/analytical methods, and identification of unknown peaks in rice volatile profiles. We identified some novel diterpenes to be induced by herbivory stress that mainly accumulated within the rice leaves. Although their function remains unknown, we speculate that these compounds may serve as precursors of smaller volatile compounds produced by rice cellular enzymes. The best example is a hypothetical formation of TMTT from geranyllinalool, identified as one of the novel herbivory induced rice products in this research.

Large diversity of other diterpenes is also reported in rice; however most of them are non-volatile compounds belonging to members of labdane-related super family compounds, including gibberellins (important signaling molecule) and diterpene phytoalexins, such as momilactones and phytocassanes (W. Wang et al., 2018). Although these compounds are also involved in anti-herbivory defense, these compounds were not a focus of this work.

2. Results

1.1. Jasmonic acid is required for VOC production in rice

Jasmonates control a broad spectrum of plant responses after elicitation by biotic stresses, including herbivores. One of the most important branches is the induction of terpenoid biosynthesis. To know the direct role of JA in volatile biosynthesis in rice plants, two JA deficient mutants, *hebiba* (mutated in OsAOC; Riemann et al., 2003) and *jar1* (mutated in OsJAR1; Riemann et al., 2008) were used to examined the emissions of VOCs. Plant responses were induced by artificial herbivory treatments, which is based on wounding and application of oral secretions (WOS) method, and plants were subjected to headspace volatile collections for released VOCs. In *hebiba* plants, which is complete JA deficient mutant, all of VOC emissions were low compared to wild type plants, including both herbivore induced plant volatiles (HIPVs) and un-induced "basal" volatiles. HIPVs from monoterpene group, such as linalool were extremely suppressed in *hebiba*, while limonene and α-pinene that are moderately induced in rice plants were also significantly reduced but still detectable in fair amounts in *hebiba* (Fig 3.2A). Group of sesquiterpenes, such as β-caryophyllene, (*E*)-β-farnesene, (*E*)-α-bergamotene were similarly reduced (Fig 3.2B), as well as aromatic compounds methyl salicylate, methyl benzoate, and indole (Fig 3.2C). The wound induced GLVs, (*Z*)-3-hexenol, 2-heptanol, and (*E*)-2-hexenal were also downregulated in *hebiba* (Fig 2.1D). These results demonstrate that jasmonate biosynthesis is necessary for indirect defense responses in rice plants, similar to other plant species (Schaller & Stintzi, 2008). The lack of VOC emissions in *hebiba* found both in WOS and control plants provides evidence that volatile compound production was tightly connected with JA biosynthesis, however, it is well known that large amounts of jasmonates can only be produced during stress responses, while basal levels of JA-Ile (active hormone) remain very low, or below detection in the intact non-stressed plants. It was therefore not clear at this point why basal volatiles in the non-induced plants are partly dependent on jasmonate pathway. Also, in the JASMONATE RESISTANT 1 mutant *Osjar1* with deactivated major JA-Ile synthase, emission of volatiles was much less affected by *OsJAR1* mutation, when comparing control and WOS treated plants. Only linalool, indole, and methyl benzoate were actually strongly suppressed in *Ojar1*plants

compared to controls (Fig 3.3A- Fig 3.3D). Many other volatiles did not change despite JA-Ile signal deficiency in *Osjar1* plants (Fukumoto et al., 2013).

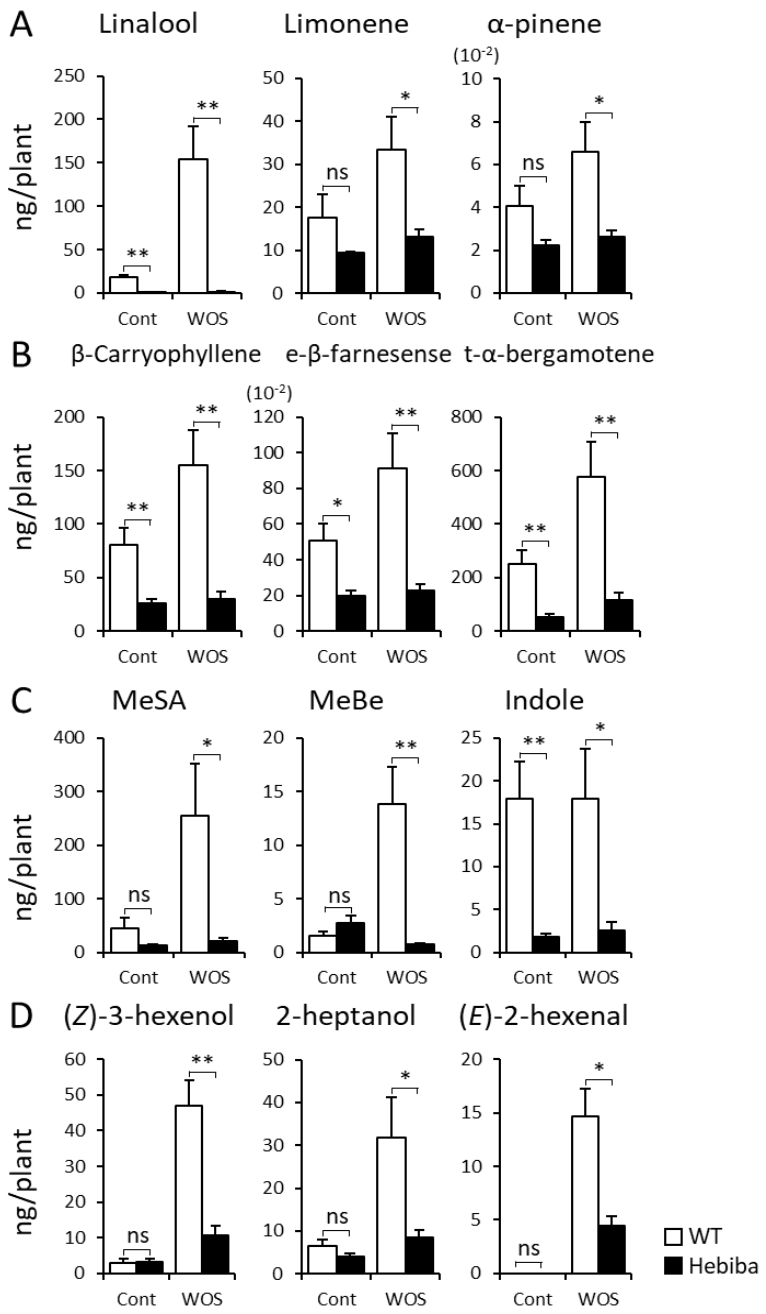

Fig. 3.2. Headspace volatiles in *hebiba* JA deficient mutant. Volatile organic compounds released from *hebiba* rice leaves before, and after WOS elicitation were determined by GC-MS. (A) Monoterpenes (linalool, limonene, and α-pinene), (B) sesquiterpenes (β-caryophyllene, (*E*)-β-farnesene, (*E*)-α-bergamotene), (C) Aromatics (methyl salicylate, methyl benzoate, indole) and (D) GLVs ((*Z*)-3-hexenol, 2-heptanol, (*E*)-2-hexenal). Data (n = 3) are shown as means ± SE; Cont, control; WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretions. Statistical differences between pairs of control (Cont) and WOS-treated plants were analyzed by Student's t-test (*P < 0.05; ** P < 0.01; ns, not significant).

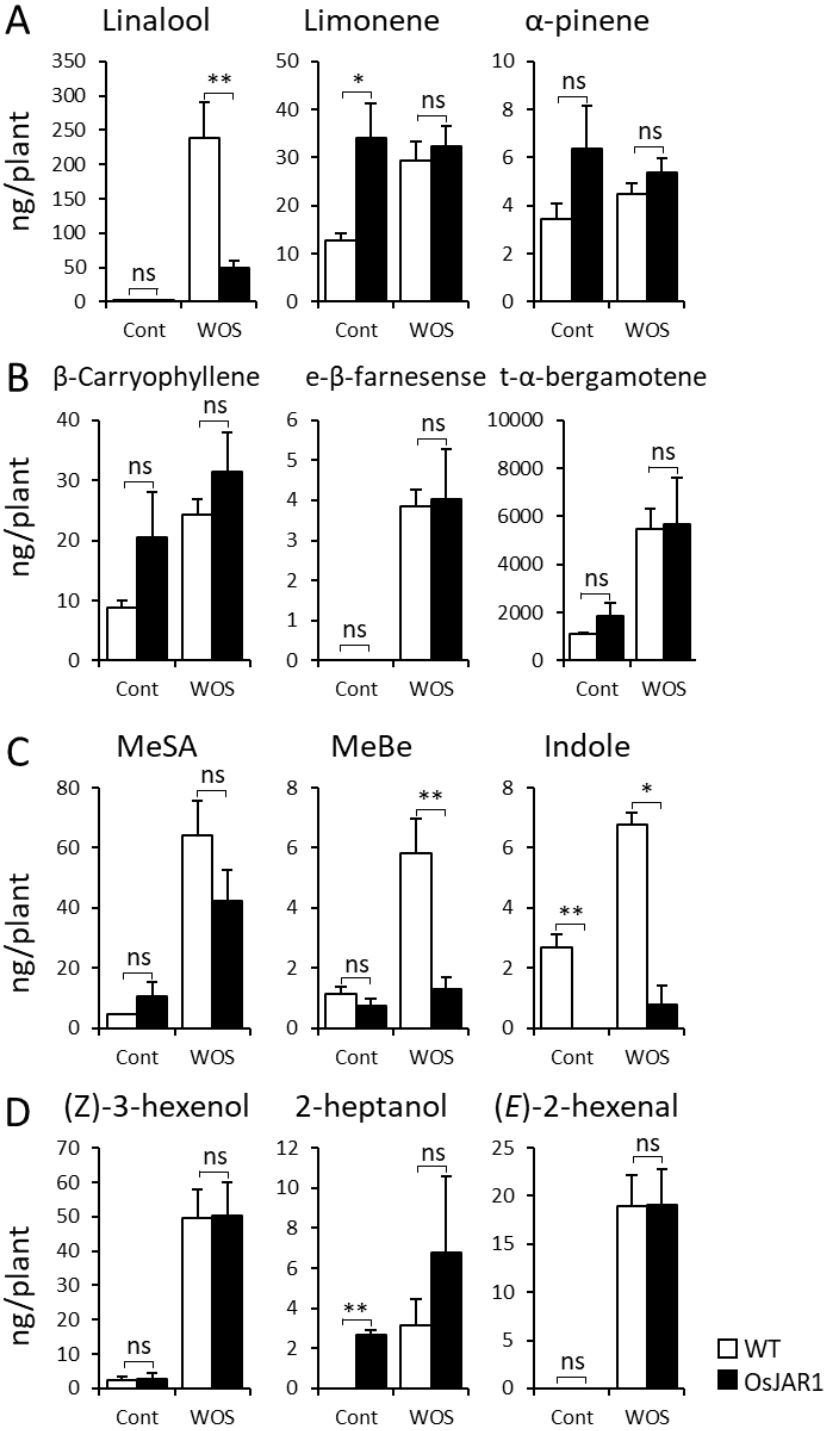

Fig. 3.3. Headspace volatiles in *Osjar1* mutant. Volatile organic compounds released from *Osjar1* rice leaves were determined by GC-MS before, and after WOS elicitation. (A) Monoterpenes (linalool, limonene, and α-pinene), (B) sesquiterpenes (β-caryophyllene (*E*)-β-farnesene, (*E*)-α-bergamotene), (C) Aromatics (methyl salicylate, methyl benzoate, indole) and (D) GLVs ((*Z*)-3-hexenol, 2-heptanol, (*E*)-2-hexenal). Data (n = 3) are shown as means ± SE; Cont, control; WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretions. Statistical differences between pairs of control (Cont) and WOS-treated plants were analyzed by Student's t-test (*P < 0.05; ** P < 0.01; ns, not significant).

1.2. Simple comparison of internal and headspace VOCs in rice

Recently, we developed a new method for analyzing the internal accumulation of volatile compounds in rice leaves, so it was possible for me to measure both the accumulated (reflecting production) and released (reflecting emission) volatile pools. This assumes that VOCs identified in plant tissue mirror VOC productions, especially in early time points after elicitation. Also, non-volatile products should be fully recovered in the leaf pool. Customized procedure of solid phase micro-extraction (SPME) was performed to collect volatiles products in leaf tissue, and at the same time, headspace volatiles were trapped in designated short time intervals (usually 3 h). Volatiles in the leaves are reported as internal VOCs (inVOCs), and headspace VOC are reported as hsVOCs in the following text. The data from both inVOCs and hsVOCs were normalized to leaf (in VOC) and plant (hsVOC), and synchronized, so that the values can be directly compared in presented graphs.

By simple comparison of inVOCs and hsVOCs profiles detected by GC-MS, it was clear that rice plants produced more volatiles than they emitted (Fig 3.4). At least 81 compounds were detected and monitored from the combined inVOC/hsVOC analytic method, which included easily to fairly volatile compounds that could be assessed by capillary gas chromatography, and belonged to *m/z* range 40- 300 used as general setting of ion trap MS. The main monoterpene linalool induced by herbivory was detected in both internal and headspace samples, but the amount was nearly fourfold higher in the headspace compared to inVOC (extracted ion traces *m/z* 93 in Fig 3.4A). The α-pinene was detected only in the headspace, while limonene was observed in both internal and headspace samples. Interestingly, nerolidol (a sesquiterpene alcohol), was detected in the inVOC analysis, but not in the headspace (Fig 3.4A). Nerolidol is a broad spectrum anti-herbivore compounds with antibacterial properties (Chen et al., 2020). Although nerolidol was not found in the headspace, or occurred only in traces, possibly due to low volatility, this compound is known to serve as a precursor for highly volatile homoterpene (*E*)-3,8-dimethyl-1,4,7-nonatriene (DMNT) in maize plants (Richter et al., 2016).

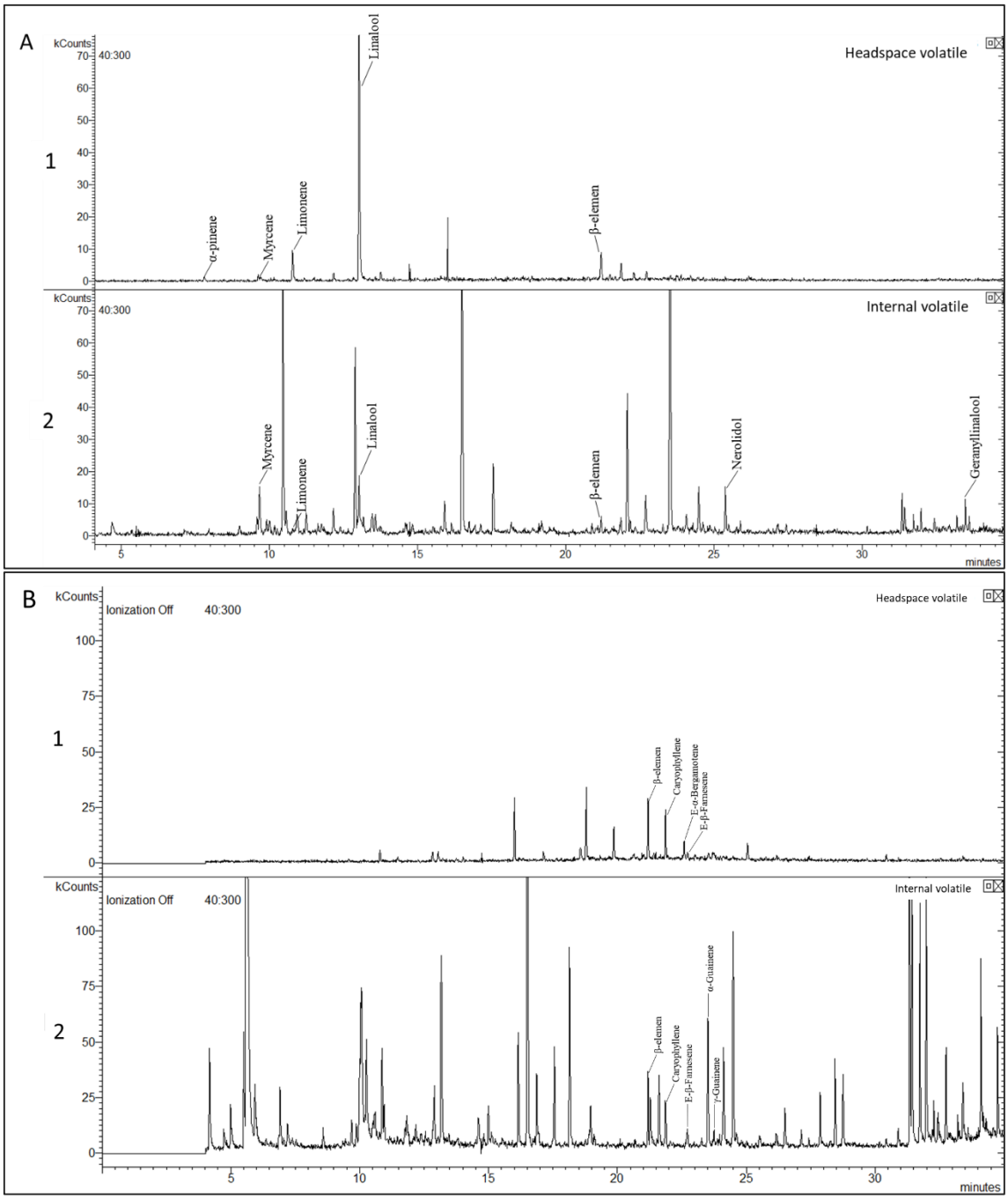

Fig. 3.4. The representative GC-MS chromatograms of volatile organic compounds in rice leaves. A representative of emission of rice plant volatile compounds from an intact plant collected by headspace method with monolithic silica (MonoTrap) adsorbents (1) and internal volatiles from rice leave tissue collected by customized solid phase micro-extraction (SPME) method with monolithic silica (MonoTrap) adsorbents (2). (A) extracted chromatogram to ion traces *m/z* 93 (B) extracted chromatogram to ion traces *m/z* 67. 1 µL sample was injected in GC-MC with a split mode ratio 1:3.

Similarly, internal VOCs contained some diterpenes, rather large compounds with 20 carbon or more, that eluted at late retention times (RT) from MS5 type of GC column, i.e., around RT=30 min. Interestingly, geranyllinalool which is a potential source of

another volatile homoterpene (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) was detected at 34 min, and it was strongly induced by WOS treatment (Fig 3.4A). These results show that rice plants may produce low volatile sesqui- and diterpenes, and use them as antibacterial compounds, or use them as precursors for more volatile products, such as DMNT and TMTT.

Striking differences existed in GLVs that occurred in large amount in the leaves, for example 3-hexenol, but their release to headspace was limited to minute amounts and short time periods after wounding (extracted ion traces *m/z* 67 in Fig 3.4B).

1.3. Evaluation of herbivory-induced and diurnal regulation of rice volatiles

We assumed from data in Figures 3.2. and 3.3. that while some VOCs are induced by simulated herbivory in rice, other volatiles are rather constitutive in their accumulation. Therefore, volatiles not only showed differential release properties, but they also responded differentially to herbivory cues. It posed an interesting question as to what is the role and regulation of these non-inducible volatiles?

To investigate the oscillation of volatile production and their emission patterns in rice plants at the same time, one set of rice plants at 8 weeks after sowing were treated by simulated herbivory, i.e., mechanical wounding followed by application of MYL oral secretions (WOS), while the same number of control plants in the second group were not treated, and used as corresponding controls. The initial WOS treatment was applied at 15:00 o'clock in the laboratory, and plants were immediately divided into two groups. The first group was set up in cylinders for headspace volatile trapping (n = 4) and the second group was used in parallel for inVOC leaf collections (n = 4). Sampling was done every three hours for 24 hours in a time-shifted manner, i.e., inVOC samples were sampled in the middle period of each hsVOC collection. Practically, hsVOCs from 18:00-21:00 collection is paired with inVOC leaf sample collected at 19:30, and so on.

As before, GLVs mainly appeared inside of rice leaves. The (*Z*-3)-hexenol and (E)-2-hexenal emissions were observed from treated plants only in first collection time period three hours after treatment (15:00-18:00). Furthermore, GLV emission was not detected at 21:00 onward. The (Z)-3-hexenal and (*Z*)-3-hexenyl acetate were not detected in the headspace at all (Fig 3.5), suggesting that their emission could be low and thus below

detection limit of our GC-MS method. Internally, GLV contents showed a relatively stable amounts over time. Actually, amount of GLV pools in the leaves were much higher compared to emitted levels found in the headspace. The stable accumulation of GLVs in leaves shows that GLVs are ready-to-use compounds that can be used quickly in the event of mechanical damage or insect attack. In this case, GLVs escape only via wounded cells that explains relatively small amounts of GLVs detected in the headspace of pattern wheel treated rice leaves. It seems GLVs literally stand in the first line of indirect plant defense.

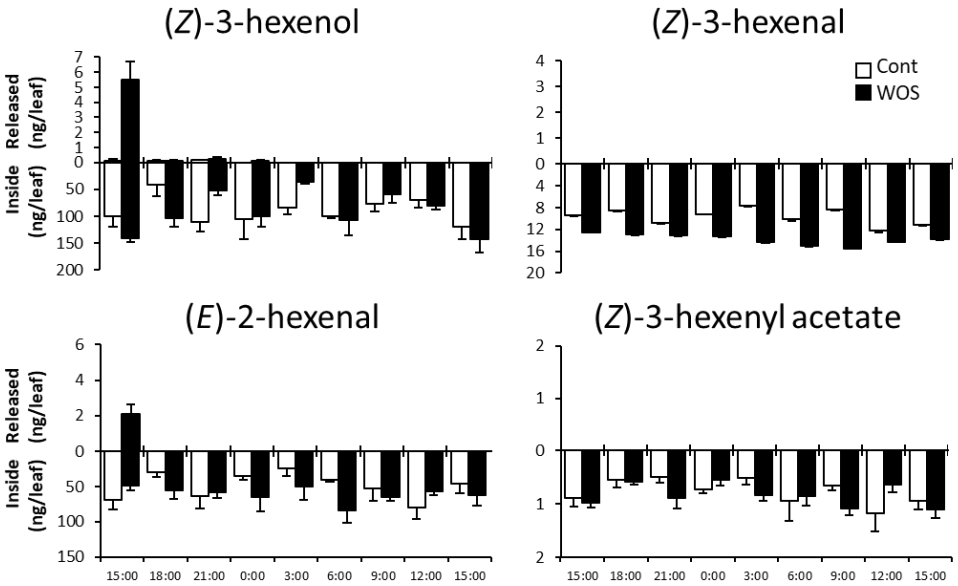

Fig. 3.5. The oscillation of wound released green leave volatiles (GLVs) emission and production pattern in rice. (*Z*)-3-hexenol, (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate were determined by GC-MS; released volatiles from intact and WOS-treated plants were collected by headspace method (n=4), and inside volatiles from leaf tissues were collected by SPME (n=4). Plants were induced at 3 pm, then samples were taken consecutively every three hours. Cont, control; WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretions.

Several volatile compounds from monoterpene group, including D-limonene, myrcene, and α-pinene were emitted diurnally, meaning that their emissions declined during the night and increased at daytime (Fig 3.6). Furthermore, D-limonene and myrcene were detected inside (inVOC) and outside (hsVOC) of leaves and their amounts were positively correlated with light presence. As shown above in the *hebiba* JA mutant, the production of these terpenes volatile was dependent on JA biosynthesis, even at non-induced plant state (Fig. 3.2). Here, we speculated for the first time that similar oscillation may exist in case of basal JA/JA-Ile contents, which may control these volatiles, as investigated in the later parts of this thesis. Another monoterpene, α-pinene, was only detected in the

headspace of rice plants (Fig 3.6), suggesting that this compound is produced in small amounts and it is rapidly released from the cells, so accumulation does not occur. Based on GC elution profile, α-pinene should be more volatile than limonene and myrcene.

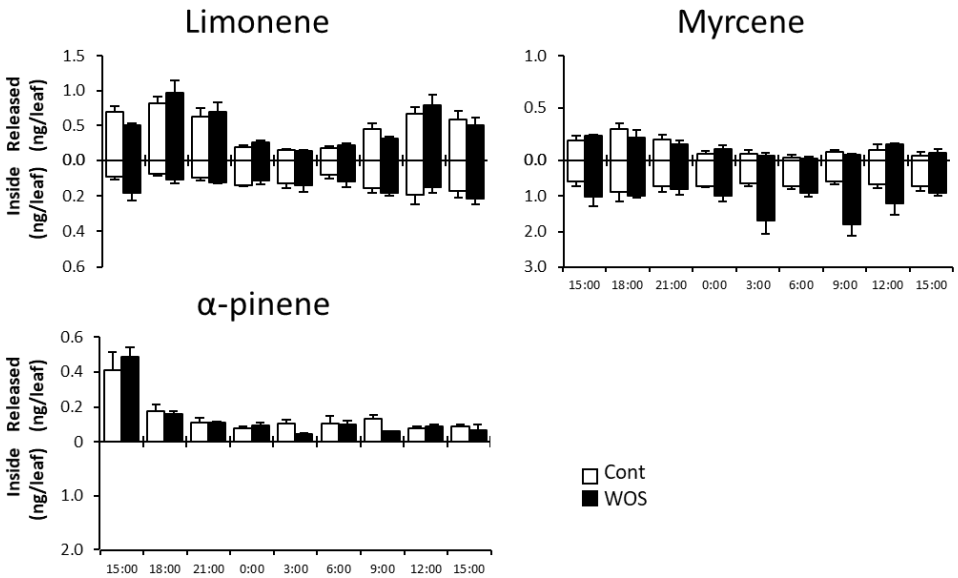

Fig. 3.6. The diurnal emission and production pattern of monoterpenes in rice. Monoterpenes limonene, myrcene, α-pinene were determined by GC-MS; released volatiles from intact plants were collected by headspace method (n=4) and inside volatiles from leaf tissues were collected by SPME (n=4). Plants were induced at 3 pm, then samples were taken consecutively every three hours. Cont, control; WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretions.

Similar to monoterpenes discussed above (Fig. 3.6), the oscillation of sesquiterpenes also showed a diurnal pattern, which was mainly apparent in the case of headspace samples (Fig. 3.7). The β-caryophyllene, β-elemene, and α-guainene sesquiterpenes were observed in both headspace and internal leaf space (Fig 3.7). Meanwhile, β-sesquiphellandrene, γ-guainene, and trans-α-bergamotene were only detected in the headspace. In contrast, *E*-β-farnesene was only detected in inVOC samples (Fig 3.7). This pattern is rather peculiar, showing differential distribution and release of sesquiterpene. Several explanations can be suggested, such as existence of specific transport mechanisms or specific physicochemical properties of these compounds, but all of these remain to be verified experimentally in the future.

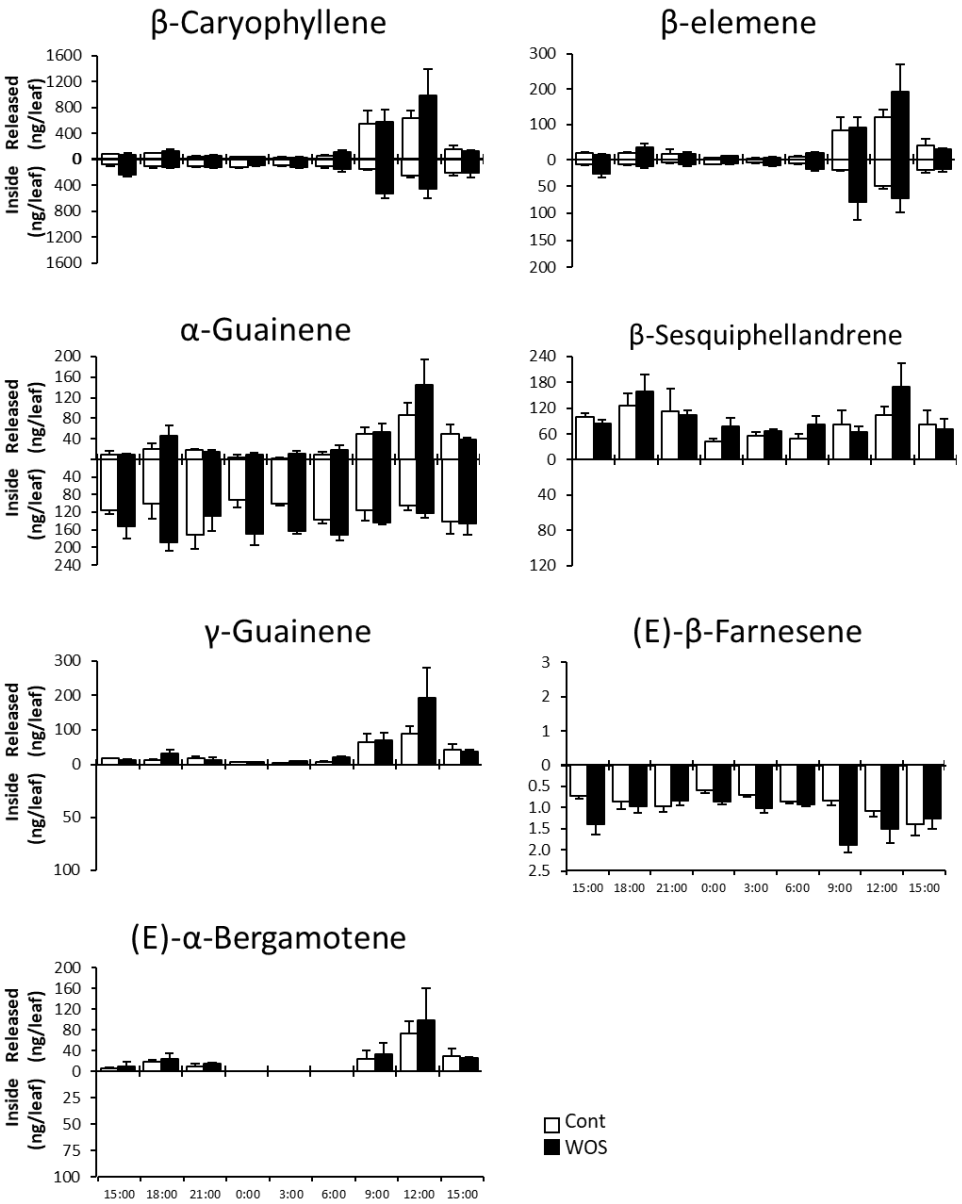

Fig. 3.7. The diurnal emission and production of sesquiterpenes in rice. β-caryophyllene, β-elemene, α-guainene, β-sesquiphellandrene, γ-guainene, *E-β*-farnesene, and *E*-α-bergamotene were determined by GC-MS; released volatiles from intact and WOS-treated plants were collected by headspace method (n=4), and inside volatiles from leaf tissues were collected by SPME (n=4). Plants were induced at 3 pm, then samples were taken consecutively every three hours. Cont, control; WOS, wounding followed by application of 20 μL diluted *M. loreyi* oral secretions.

Finally, the last group of rice volatiles of diverse biosynthetic origin was responsive to simulated herbivory, with basal levels showing diurnal patterns. These compounds can be called true "herbivore-induced plants volatiles (HIPV)" in rice, being significantly induced within first 3 hours after WOS (Fig. 3.8). In particular, linalool and methyl salicylate were detected in headspace, as well as they accumulated in the rice leaves. As

expected, linalool emission was strongly elevated at 6:00 pm and subsequently declined at 9:00 pm, then the emission was not significantly different between WOS-treated and control plants. Furthermore, linalool emissions of WOS-treated plants slightly increased in the morning (9:00 am to 12:00 pm). The inVOC production basically showed similar patterns as emitted hsVOCs. In a close magnification, we can see that basal linalool, although very low, still follows a basic diurnal patter, suggesting that besides being induced by herbivores, linalool is also diurnally regulated. Methyl salicylate emission was only induced at 6:00 pm (3 hours after induction) in treated plants. Thereafter, MeSA emissions remained steady during night and day. In contrast, the leaf internal methyl salicylate was variable, pending another repeat of experiments for making clear conclusions about this compound.

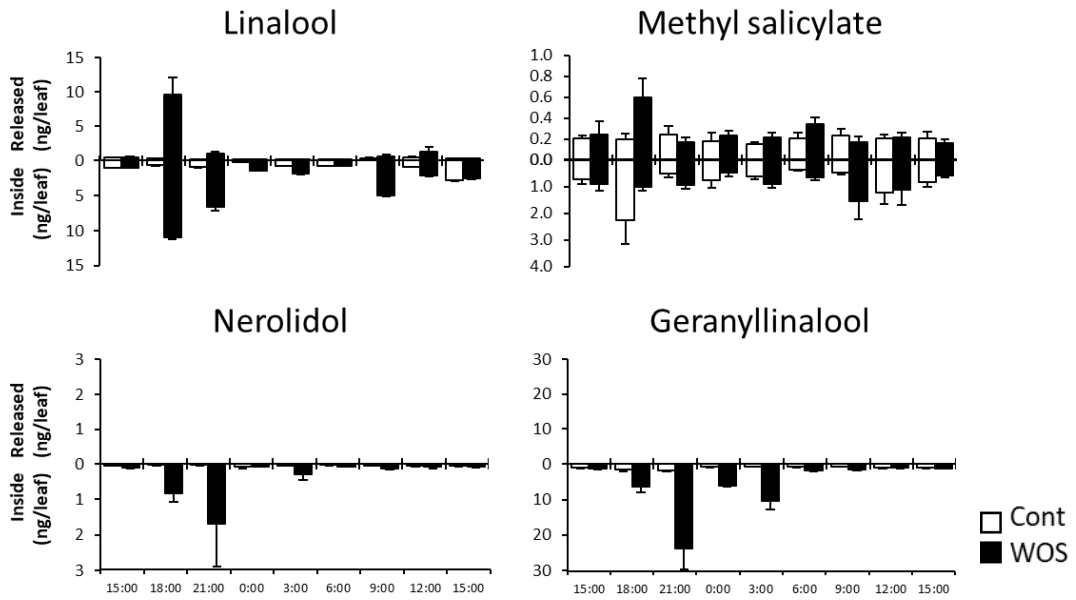

Fig. 3.8. The diurnal emission and production of herbivores-induced volatiles in rice. Linalool, methyl salicylate, nerolidol, geranyllinalool were determined by GC-MS; released volatiles from intact and WOS-treated plants were collected by headspace method (n=4), and inside volatiles from leaves tissues were collected by SPME (n=4). Plants were induced at 3 pm, then samples were taken consecutively every three hours. Cont, control; WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretions.

As probably most novel finding in this part, sesquiterpene alcohol nerolidol and diterpene alcohol geranyllinalool were successfully detected in the inVOC samples. The oscillation of these two compounds was identical, and the production was significantly induced within 3 hours (6:00 pm) after WOS treatments, reaching maximum peak at 9:00 pm, and then ramped down towards constitutive levels (Fig 3.8). Emissions of nerolidol and

geranyllinalool were not detected in the headspace, expectedly, as nerolidol and geranyllinalool are quite less volatile in GC, with RT=25 min and RT=34 min, respectively.

1.4. Hormonal regulation and VOC production in rice

1.4.1. Herbivore-induced phytohormones patterns

It is well known that jasmonates play diverse roles in plants, notably they serve as important signaling molecules in plant defense against insect herbivores and necrotrophic pathogens (Schaller & Stintzi, 2008). From the oscillation of rice plant volatiles, either the production or the emission, or both, should follow diurnal biological rhythms and/or occur in response to environmental stress, such as biotic stresses imposed by herbivory. Accordingly, these changes should be reflected in upstream signaling, in particular jasmonate levels that serve as positive regulators (Figs. 3.2 and 3.3) in VOC production of rice. Regarding the fact that JA-Ile is active hormone, the accumulation of JA-Ile and regulation of *OsJAR* genes are the most important parameters. Actually, OsJAR1 has been reported to affect phytochrome and JA signaling (Riemann et al., 2008; Wakuta et al., 2011). Furthermore, *hebiba* showed a reversal in the light responses (Sineshchekov et al., 2004). Based on results of VOC analysis in *hebiba* and *Osjar1* mutants, JA and JA-Ile are tightly associated with VOC biosynthesis in rice plants, that could possibly link to diurnal changes (day and night oscillation) in VOC emission. If this hypothesis is true, correlations between VOC accumulation and hormone content in rice tissues should exist. In test of this hypothesis, diurnal oscillations of stress hormones have been examined, including JAs, ABA, and SA. The jasmonates including jasmonic acid, OPDA, and JA-Ile significantly elevated within 30 minutes after plant induction by WOS (Fig 3.9), effectively preceding elevation of HIPVs (Fig. 3.8). JA elevation continued to increase and reached its maximum level (0.3 µg/mg FW) after 3 to 4 hours. Then JA levels declined to near constitutive levels. A slight increase occurred at 6 am that overlapped with the onset of light in the morning, indicating that light may be contributing to external factors that induce jasmonate biosynthesis.

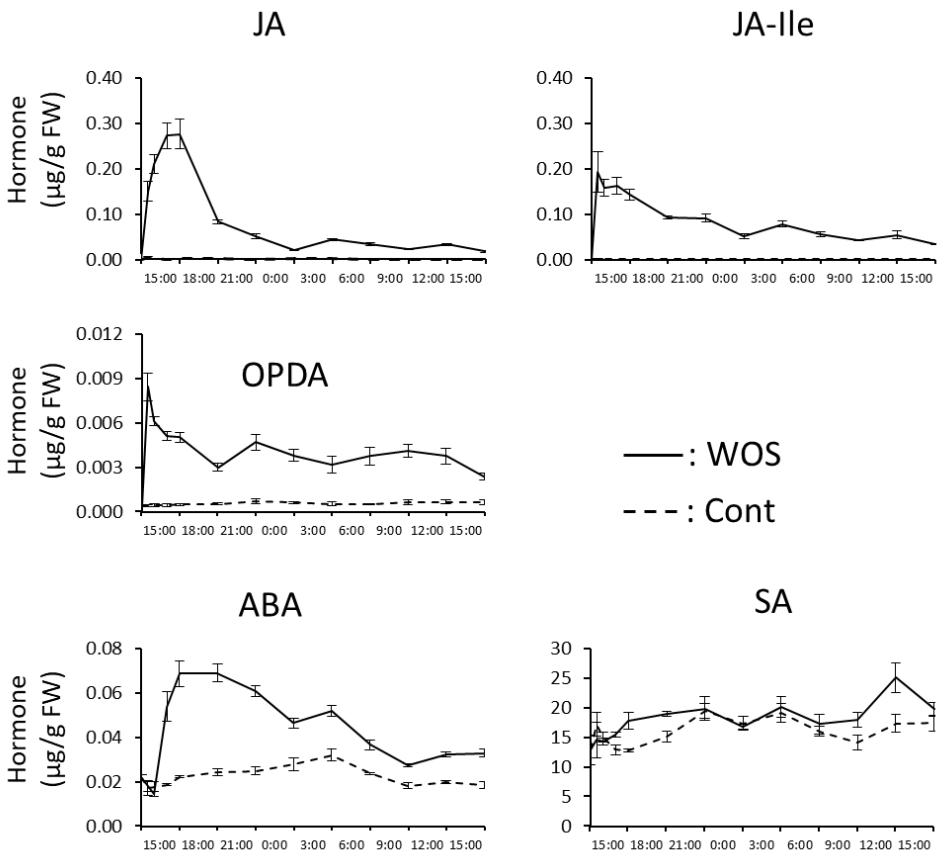

Fig. 3.9. The diurnal oscillation of phytohormones in rice plants. Jasmonic acid (JA), jasmonoyl-L-isoleucine (JA-Ile), 12-oxo-phytodienoic acid (OPDA), abscisic acid (ABA), and salicylic acid (SA) levels were determined by LC-MS/MS in rice leaves before (at 15:00 o'clock), and 30 minutes, 1 h, 2 h, 3 h after WOS elicitation and then sampled continuously every three hours for 27 hours. Data (n = 5) are shown as means ± SE. Cont, control; WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretions.

Similar to JA, OPDA and bioactive JA-Ile rapidly increased within 30 min in WOS treated plants but remained relatively high until following day. ABA levels also increased by WOS treatment, possibly as response to desiccation and drought stress experienced by wounded leaves (Fig 3.9). SA levels did not increase and stayed relative stable over the whole measured time period (Fig 3.9). Constitutive SA levels in rice are obviously high, posing a question how this hormone can regulate stress responses in rice. In experiment shown in Fig. 3.9, plants were treated by WOS and sampled in shorter intervals during the first 3 hours, whereas later, the samples were taken only once in every 3 hours. As this data did not show sufficient time resolution, it was not clear if low basal hormones follow diurnal regulation or not (Fig. 3.9). Therefore, these aspects had to be followed in the next experiment, after adjusting the experimental conditions.

1.4.2. Circadian and light-induced phyhormones regulations

It has been shown previously that photomorphogenesis in rice involves jasmonate regulation, and JA and JA-Ile levels increased in rice seedlings in response to blue light (Brendel et al., 2014). To examine light and diurnal regulation of phytohormone production in rice, particularly jasmonates, the 24 h levels of jasmonates, abscisic acid, and salicylic acid were again examined under controlled artificial light conditions in the laboratory but this time, we only used untreated rice and sampled every hour to get more detailed accumulation profile during one day. JA, JA-Ile, and OPDA all declined during dark period and immediately increased when the lights were turned on in the morning (Fig 3.10). ABA showed a similar diurnal pattern, where production decreased gradually in the afternoon and dropped in dark phase, and then increased slowly in the bright phase in the morning to afternoon (Fig 3.10). As expected, SA did not respond to changes in daylight and dark/night cycle (Fig 3.10), implying that SA production is not controlled by light.

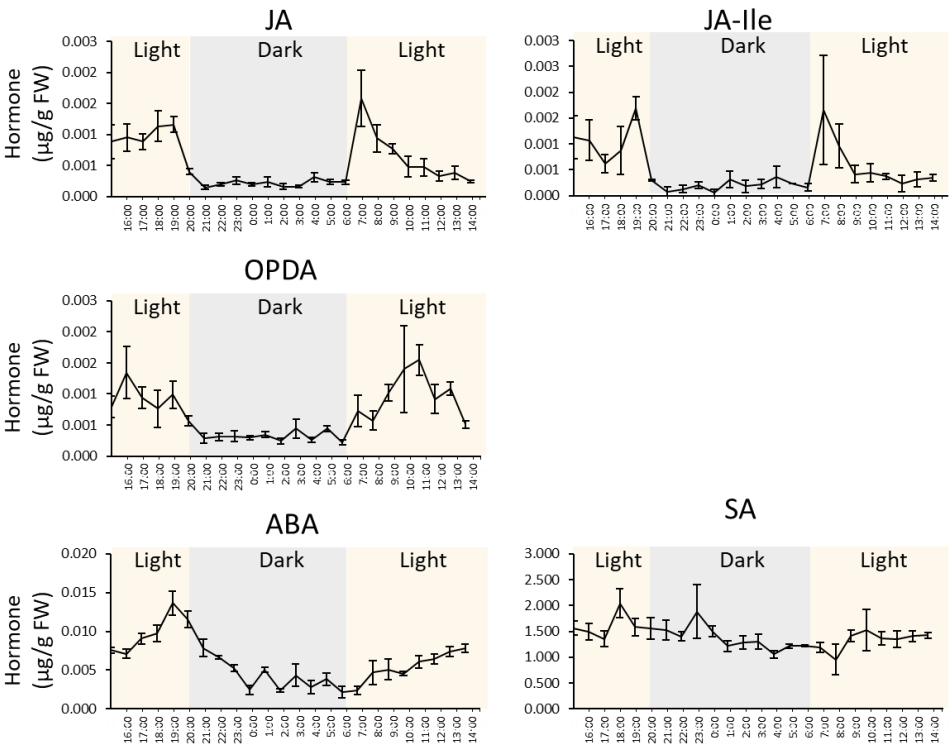

Fig. 3.10. The diurnal oscillation of phytohormones in rice plants under controlled light condition. Jasmonic acid (JA), jasmonoyl-L-isoleucine (JA-Ile), 12-oxo-phytodienoic acid (OPDA), abscisic acid (ABA), and salicylic acid (SA) levels were determined by LC-MS/MS in rice leaves under artificial controlled light conditions every hour for 24 hours starting from 3 pm. Data (n = 5) are shown as means ± SE. FW, fresh weight.

1.4.3. Light controlled jasmonate production

The diurnally oscillation of basal phytohormones in untreated rice plants, particularly jasmonates, suggested involvement of light or light change in triggering hormone biosynthesis. The low production of jasmonates at night could be due to absence of light. The question that arose was whether light exposure during dark phase could trigger production of jasmonates, and subsequently VOCs in rice leaves. To answer this question, phytohormone analysis was carried out in rice subjected to light manipulations during night.

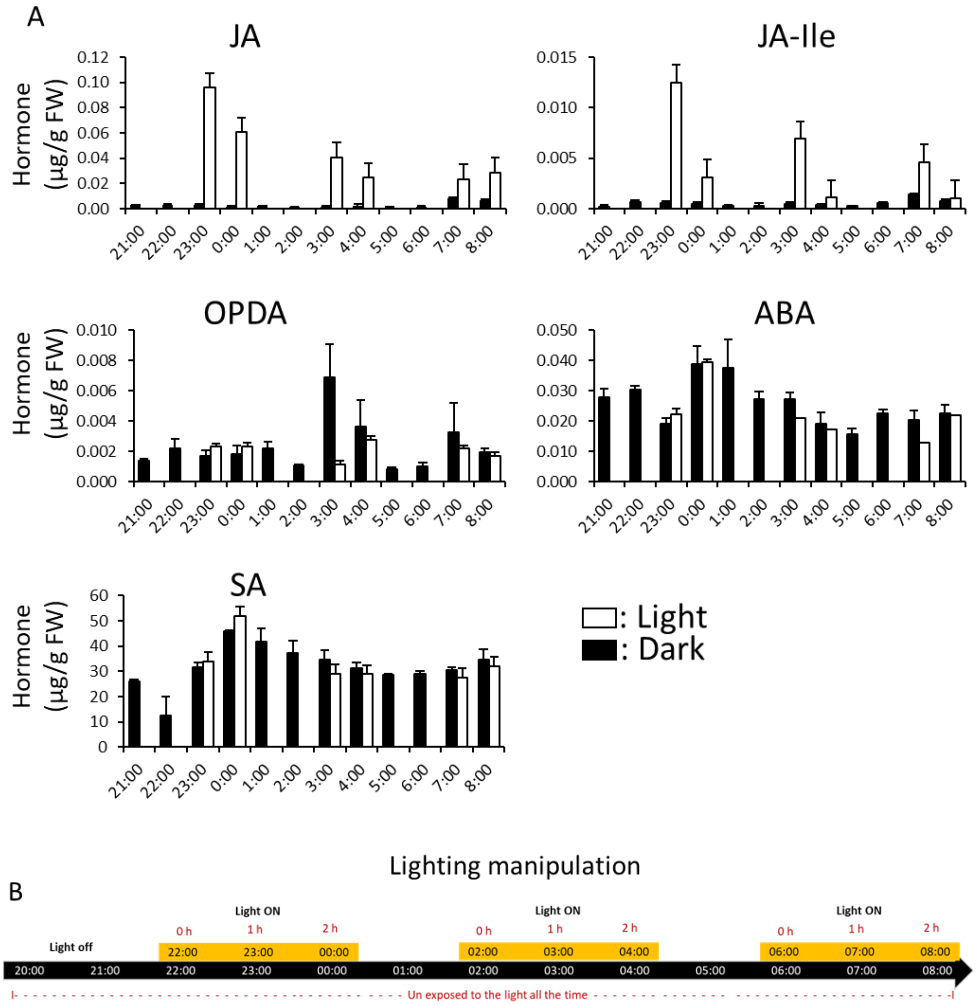

Fig. 3.11. The phytohormone production in rice plants under manipulated light conditions. Jasmonic acid (JA), jasmonoyl-L-isoleucine (JA-Ile), 12-oxo-phytodienoic acid (OPDA), abscisic acid (ABA), and salicylic acid (SA) levels were determined by LC-MS/MS in rice leaves (A) under manipulated artificial light condition. In the treated group of plants, the light was turned on for two hours in three different time intervals, 10 pm to 12 pm, 2 am to 4 am, and 6 am to 8 am. (B) Schematic illustration of light treatments. Data (n = 4) are shown as means ± SE. FW, fresh weight

From comparison between light exposed and basal levels of rice hormones at corresponding times, JA and JA-Ile productions were significantly increased in plants one and two hours after light exposure, showing maximum levels in the first hour. In control set, JA and JA-Ile remained but slightly increased at 7:00 am following the onset of bright light (Fig. 3.11). Interestingly, OPDA was not induced by light exposure. This experiment strengthens the evidence that light can induce JA and JA-Ile production, however molecular mechanisms of this induction remain to be determined. As expected, ABA and SA did not respond to light exposure.

1.5. Diurnal and herbivory regulation of VOC-related genes

Until now, many volatile products and phytohormones have been analyzed, but these should depend on enzymes encoded by respective genes from rice genome, and their expression during day/night cycle, and after exposure to stress conditions. Therefore, RT-qPCR was performed to analyze the transcriptional changes of volatile related genes, using leaves from 7-week-old plants. The *actin 11* (*ACT11*) house-keeping gene (Jain et al., 2018) was used to normalize transcript data, which showed less variation than OsEF1α normally used in the laboratory. Simultaneously, several genes involved in hormone biosynthesis, such as ethylene, jasmonic acid, gibberellin, and jasmonic acid signal transduction were analyzed to support hormonal changes described in the previous sections.

The WOS treatment was performed at 3 pm, and leaves sample was collected at 0 h, 30 minutes, 1 h, 2h, and 3 h after treatment, and then every three hours until following day (6 pm). This scheme was the same as used for hormone analyses in Figs. 3.9 and 3.10. Ethylene biosynthesis gene *OsACO2* (see Chapter 4) showed a low expression at night and it increased in the morning hours in control plants (Fig 3.12). Ethylene receptor *ETHYLENE RESPONSE 2* (*OsETR2*) transcript levels declined after WOS treatment, and in the morning (Fig 3.12). According to the RiceXPro gene expression profile database (*Rice Expression Profile Database: RXP_0002 Graph View*, n.d.) *Os04g0169100* similar to ethylene receptor *OsETR2* oscillation is strongly diurnal and upregulated at night. Ethylene response gene *EIN3-BINDING F-BOX PROTEIN 1* (*OsEBF1*) was induced in WOS treated plants, specifically at 30 minutes and 1 h post treatment. Similar to *OsETR2*,

OsEBF1, shows oscillatory pattern in treated and control plants. Ethylene responsive gene *EBF1*, same as *EBF2* (Binder et al., 2007), is involved in circadian ethylene responses (Kinmonth-Schultz et al., 2013). As ethylene accumulation and perception seem to oscillate during day, and after WOS treatments, it suggested an involvement of ethylene in VOC gene regulation. This notion was then investigated in detail in Chapter 4, showing that ethylene works as a negative regulator of volatile biosynthesis in rice.

Plant response to light is regulated by several receptors and hormones, such as phytochromes, gibberellin, auxin, and ethylene (Facella et al., 2012). Therefore, gibberellin has a potential to be involved in the fine regulation of diurnal patterns of VOCs in rice plants. The *GIBBERELLIN-OXYGENASE 20* (*GA20ox*) (Pan et al., 2017) was downregulated 1 h after WOS treatment, then significantly increased after 3 hours. The regulation *GA20ox* in untreated plants was diurnal with low expression at night and high levels during the day, indicating a dependence on the dark and light phases. Transcription profiles of *OsLOX1* and *OsLOX9*, JA-biosynthesis related genes were highly dependent on WOS elicitation. Maximum transcription was reached within 1 h after elicitation, while the untreated plants remained low. The change was more significant in OsLOX1 >> OsLOX9, suggesting that OsLOX1 is a major gene for WOS-induced jasmonate accumulation. The JA response genes, *OsJAZ9* and *OsJAZ11* basically followed *OsLOX1* transcription and JA-Ile levels. Dynamic transcription patterns also appeared in *OsAOC* and *OsJAR1* transcription levels that were significantly induced at first hour after elicitation, similar to *OsLOX1*. However, *OsAOC*, and *OsJAR1* were also elevated in the morning, from 6 am and reached maximum level at the mid-day, which particularly occurred in the untreated plants. The elevation pattern in untreated plants could be associated with the diurnal production of jasmonates, however it still needs to be investigates in more detail in the future. *OsJAR2* was more variable, increased in the night and during day, but it remained lower in the morning and evening. This gene was little affected by WOS treatment, showing that OsJAR2 is mainly involved in regulation of developmental processes. It is also possible that night accumulation of OsJAR2 could be connected to morning JA-Ile formation from pool of enzyme that accumulates (get ready) at night.

The dynamics transcription of ethylene and jasmonate related genes suggest a possible effect on oscillation of volatiles biosynthesis genes, such as *OsHPL3*, *OsCAS*, *OsDXS3*,

OsLIS, *OsPAL*, and *OsSAMT* through *OsMYC2* that functions as a central regulator. For instance, monoterpene linalool biosynthesis gene *OsLIS* is strongly dependent on herbivory (WOS) elicitation, as also seen in some of the core JA biosynthesis gene. An expected *OsMYC2* transcript peak can be then observed after WOS elicitation, connecting JA signaling and volatile biosynthesis gene. Interestingly, some elevations in *OsMYC2* transcription also occurred in case of untreated plants, possibly reflecting the diurnal oscillation and regulation of volatiles in rice as shown in Fig 3.9 and 3.8.

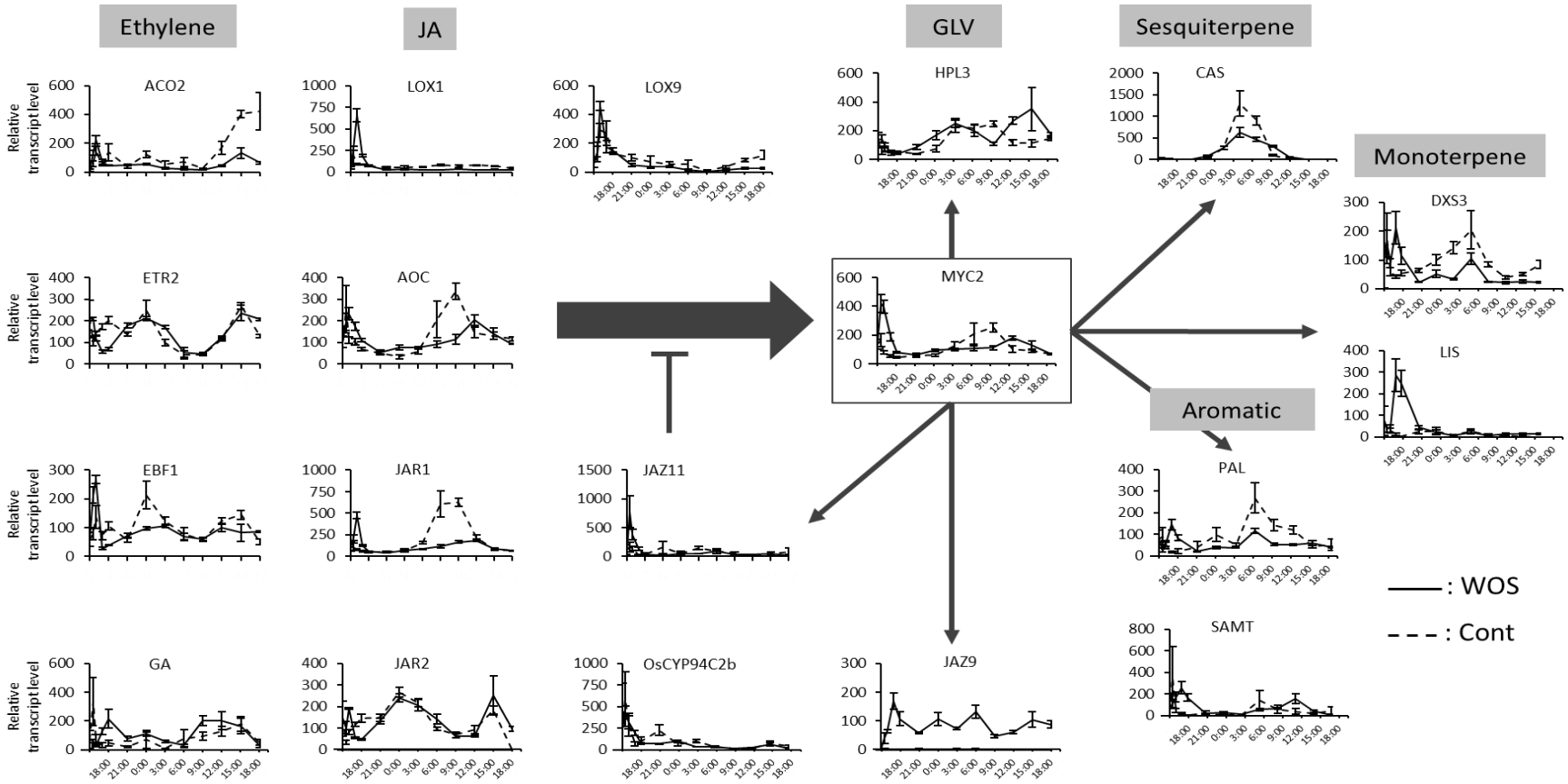

Fig. 3.12. The diurnal oscillation of relative transcript levels of phytohormone and VOC-related genes in rice plants. Gene expression was determined by qRT-PCR in rice leaves before (at 15:00 o'clock), and 30 minutes, 1 h, 2 h, 3 h after WOS elicitation and then consecutively every three hours for 27 hours. Data (n = 5) are shown as means ± SE. Cont, control; WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretions. Ethylene biosynthesis gene *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE 2* (*OsACO2*), ethylene responsive genes *ETHYLENE RESPONSE 2* (*ETR2*), *GIBBERELLIN-DIOXYGENASE* 20 (*GA20ox*), JA biosynthesis gene *LIPOXYGENASE 1* (*LOX1*) and 9 (*LOX9*), *ALLENE OXIDE CYCLASE* (*OsAOC*), JA-Ile conjugation gene *JASMONATE RESISTANT 1* (*OsJAR1*), 2 (*OsJAR2*), jasmonate-responsive genes *JASMONATE ZIM-DOMAIN 9* (*OsJAZ9*) 11 (*OsJAZ11*), JA-Ile hydroxylation *CYTOCHROME P450 ENZYME* (*OsCYP94C2b*), GLV biosynthesis gene *HYDOPEROXIDE LYASE 3* (*OsHPL3*), terpenoids biosynthesis related genes *OsDXS3*, *OsLIS*, *OsCAS*, methyl salicylate biosynthesis genes (*OsPAL*, *OsSAMT*).

3. Discussion

3.1. Jasmonic acid is required for VOC production in rice

In rice plants, jasmonic acid contributes to diverse plants responses, including environmental challenges (Liu et al., 2015). It serves as a key regulator in growth and development (Trang Nguyen et al., 2019). Various studies showed that jasmonates play various roles in response of plants to light (Sineshchekov et al., 2004), photomorphogenesis (H. J. Chen et al., 2018), salt stress tolerance (Hazman et al., 2015), blast fungus resistance (Shimizu et al., 2013; Wakuta et al., 2011), resistance to rice bacterial blight (TANIGUCHI et al., 2014). The role of jasmonic acid in the production of diverse volatile organic compounds in rice plants has been firmly established in this work. We show that production of VOCs, that work as essential cues in the indirect defense and various stresses responses, is clearly controlled by JA, which is most evident in case of *hebiba* JA-deficient rice plants (Fig 3.2A-Fig 3.2B). HIPVs, such as linalool, as well as some non-herbivore-induced volatile compound levels, have dropped significantly in *hebiba* compared to wild type plants. The *hebiba* plants are impaired in *OsAOC*, which is a single copy gene in the rice genome (Dhakarey et al., 2016; Trang Nguyen et al., 2019), resulting in an efficient disruption of all jasmonates, including precursor OPDA production. Even for GLVs, whose biosynthetic pathway (HPL3) is not directly induced by jasmonic acid, the production of compounds like (*Z*)-2-hexanol, 2-heptanol, (*E*)-2-hexenal, was also strongly compromised in *hebiba*, suggesting a possible presence of a negative feedback loop of linolenic acid on GLV production. The two pathways, JA and GLV crosslink at the level of linolenic acid, which serves as a common substrate in both metabolic branches. Further examinations are needed to investigate this hypothesis.

Although *hebiba* experiments showed conclusively the essential role of jasmonates in VOC production in rice, results of the second jasmonate mutant *Osjar1* suggest that role of JA is not as simple as it would be expected from *hebiba* results. JA itself is not active signaling compound. JA needs to be conjugates with another substrate, Ile, to form an active ligand for COI1 receptor. *OsJAR1* mediates conjugation of JA and isoleucine, to form JA-Ile, which is the most bioactive form. The JA-Ile function in plant responses to biotic stresses have been extensively studied. However, specificity of JA-Ile involvement

in the indirect defense against chewing herbivores remains questionable, as shown by some of our data. For instance, volatile production in *Osjar1* mutant has not decreased, except for highly inducible set of compounds: linalool, indole, and methyl benzoate (Fig 3.3A-Fig 3.3C). This may suggest that JA-Ile signalling specifically controls herbivore-induced volatiles, but it may be less important for regulation of basal (diurnal) volatile contents in rice. Another possibility is that OsJAR2 gene, also member of GH3 gene family, is involved in the biosynthesis of basal (diurnal) VOCs in rice plants, providing low but functional levels of JA-Ile in the intact (untreated) rice plants. Both OsJAR1 and OsJAR2 are known to be functional JA-Ile synthases in rice (Wakuta et al., 2011). Secondly, as we only monitored the released (headspace) volatiles; other mechanisms such as accumulation in the leaves and release mechanisms could be also involved in the unexpected differential patterns of volatiles that have been released from *hebiba* and *Osjar1* mutants. Therefore, in the next chapter, in order to understand intrinsic mechanisms involved in VOC release from rice, we attempted to examine the volatile leaf/head space dynamics in rice, as well as we examined jasmonate contents in rice in detail.

3.2. Volatiles production in rice plants

Volatiles are first produced in plant tissues, and then emitted to atmosphere, constitutively or induced by specific inducers, either mechanical or biological. These compounds can be classified into different groups with different molecular weight (mass), and thus differential volatility, from low mass compounds such as monoterpenes to those with longer carbon chains, such as diterpenes, triterpenes, or lipids. Whether the composition of volatile emissions in rice headspace is the same as the production in the rice tissues was unclear. The VOC profiles detected by GC-MS (Fig 3.4) showed that volatiles emitted from intact plant (hsVOCs) has distributed in temporally different patterns compared to the same compounds whose internal production was revealed for the first time, as inVOCs. In the WOS-treated plants, a number of emitted volatiles from intact plants was much less than volatiles detected in the rice leaf tissues. Presumably, plants produce many types of volatile compounds but not all of them are released either, constitutively, or after WOS induction. The question is how plants or cells can regulate selective release of these compounds. Possibly, assuming that passive diffusion is the

main mechanism of volatile release (Mofikoya et al., 2019; Widhalm et al., 2015), molecular size of those compounds could decide the selective emissions. On the other hand, other evidence shows that active transport of volatiles may exist in plants. The emission of volatiles from *Petunia hybrida* flowers was facilitated by an adenosine triphosphate–binding cassette (ABC) transporter (Adebesin et al., 2017). Therefore, further molecular investigations are needed to understand how selectivity of volatile emissions is achieved in plants like rice.

Emission of small size GLV compounds (C6 volatiles) was only detected in the first hour after WOS induction in the headspace, but these compounds were very abundant in inVOC fraction, which applied to all times of examination (Fig 3.5). In MS chromatograms (capillary column specification described in the method), GLV peaks appear in early retention times (RT), for example 5.5 minutes for (*Z*)-3-hexenol, showing that GLVs should be easily volatilized. As most GLVs are C_6 molecules (Scala et al., 2013), they are known to be quickly produced and released upon damage (Scala et al., 2013; ul Hassan et al., 2015); however, this seems to happen only through wounded surface of cells, suggesting existence of high retention capacity for GLVs in the leaves (or lack of transporters). Similarly, α-pinene is a C_{10} molecule (monoterpene) that was detected as early peak (RT 7.6 minutes) in the headspace sample of the rice plant, and it was not detected in the inVOC pools. It suggests that small monoterpenes may easily escape from the rice cells. Equal distribution was observed for linalool emissions in the headspace and retention inside of the leaf, recovered as inVOC fraction (Fig 3.8). The diurnal emission and production pattern of linalool clearly showed that this compound was induced by herbivory, supporting previous studies that linalool can be strongly induced by herbivore damage or herbivore oral secretions (Kessler, 2001; Raguso, 2016; Turlings & Tumlinson, 1992). Longer retention time was observed for larger size volatile sesquiterpene (C_{15}) like nerolidol, and diterpenes (C_{20}) like geranyllinalool, with retention times 25.2 and 35.4 minutes, respectively. Interestingly, nerolidol and geranyllinalool were only recovered in inVOC samples, and they were never detected in the headspace, despite of many experimental repetitions. The oscillation of these two compounds was very similar to linalool, and their production was strongly induced within 3 hours after WOS treatments (Fig 3.8). Although they are not emitted as indirect defense signals, we assume they are induced by herbivory to serve as precursors for other more volatile

homoterpenes, (*E*)-3,8-dimethyl-1,4,7-nonatriene (DMNT) and (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT). These terpenoids utilize geranylgeranyl diphosphate (GGPP), farnesyl diphosphate ((*E*,*E*)-FPP) and geranyl diphosphate (GPP) as substrates, which are formed through either the cytoplasmic mevalonate (MVA) or the plastidic 2-C-methyl-D-erythritol-4-phosphate (MEP) pathways (Su et al., 2017). Previously, geranyllinalool synthase (GLS) was induced in Solanaceae plant leaves by methyl jasmonate treatments (Falara et al., 2014), suggesting that core genes may also be subjected to jasmonate regulatory functions.

3.3. Regulatory mechanisms involved in VOC production in rice

To reveal whether diurnal light oscillations could influence hormonal levels and transcription of volatile-related genes, the 24 h oscillations of phytohormones (JA, JA-Ile, OPDA, ABA, and SA), and their associated genes were investigated in rice plants subjected to different light and dark conditions. Hormonal responses to day-night rhythm, and herbivores (WOS treatment), showed that JA, JA-Ile, OPDA, and ABA levels strongly increased only in WOS-treated plants (Fig 3.9), but high levels of jasmonates could not be found in control plants. Similarly, transcriptional levels of ethylene, jasmonic acids, and HIPVs related gene were strongly upregulated within 1 h after WOS treatment (Fig 3.12). In another separate experiment, basal level of JA, JA-Ile, OPDA declined at night phase, and increased in the morning, with onset of light. To uncover whether light induction could be a trigger for basal day hormone levels, or hormones just follow the internal biological clock, direct light manipulations at night were carried out. It was clear that light contributed to production of some but not all phytohormones, especially JA and JA-Ile (Fig 3.11). Plant response to environmental changes are controlled by hormonal signaling network among ABA, ET, JA, and SA (Nguyen et al., 2016), which is essential to synchronize plant growth and defense (Berens et al., 2017). The ability to harmonize the hormonal signals for routine life of plants, with those involved in sudden insect attack and other stresses, must have evolved in plants trough co-evolution. Rhythmic environmental changes, for instance, day-night cycles, cause plants to follow internal circadian clock, an intrinsic rhythm of gene expression in 24 h period (Jain et al., 2018). As an autotrophic organism, rice plants need light as energy resource for their growth and development, as well as for energy demanding adaptive responses, including defense

against herbivores. As mentioned above, the production of hormones, especially jasmonates, can be diurnally controlled by light. On the other hand, the same class of jasmonates can be strongly induced by herbivores (WOS treatment). Thus light and herbivore regulation pathways must involve active crosstalk to effectively control production of terpenes and other volatiles in the rice plants.

In rice plants treated with wounding and application of 20 µl *M. loreyi* oral secretion, monoterpene linalool production was significantly increased within three hours (19:30 o'clock) after elicitation (Supplementary Fig 1). This shows that elicitations by herbivores can still induce and accumulate HIPVs, regardless of onset of dark phase. However, this conclusion may not be exact as activation of the biosynthesis of volatile compounds has occurred still under light phase, same as increase in JA and JA-Ile that have already happened within one hour (16:00 o'clock) after WOS treatment (Fig 3.9). This was also the case of OsJAR1, and a central transcription factor, OsMYC2, which have been all upregulated earlier, and then followed by *OsLIS* gene for linalool production (Fig 3.12). In *Arabidopsis thaliana*, JA treatments partially restored the degradation of AtMYC2, AtMYC3, and AtMYC4 proteins occurring in dark phase periods, and light-activated phyB was required to stabilize AtMYC2 (Lockhart, 2014). It is therefore possible that a large amount of jasmonates produced by herbivore in attacked tissues are essential to actually override the negative regulatory mechanisms that cause lower plant responsiveness to herbivory at night. In our preliminary experiments, treatments of plants at night induced less jasmonates, and maximum was again restored in the next morning (unpublished data). However, in the untreated plants, many volatiles show diurnal patterns, with minimum production that is coincident with night. It is possible that these volatiles follow low level jasmonate changes, which are naturally more sensitive to repression, and therefore most of the VOCs are effectively switched off at night. The emission of linalool in *Hoya carfiosa* plant (Altenburger & Matile, 1990) and myrcene in *Antirrhinum majus* flower (Dudareva et al., 2003) was also under control of circadian rhythm. Several compounds like α-pinene, limonene, β-caryophyllene did not show a significant induction in WOS-treated rice plants. However, levels of α-pinene, limonene, and β-caryophyllene strongly decreased during dark phase, and reached lowest levels at 4:30 to 7:30, and then increased again during the day (Supplementary Fig 1). If our hypothesis is true, and volatiles can differentially follow low and high jasmonate changes,

it will be interesting to investigate mechanisms involved in the high induced jasmonate and low diurnal jasmonate regulation pathways. Previously, in *Artemisia annua* plants, the β-pinene, an isomer of α-pinene, was also under regulation of circadian clock (Lu et al., 2002), while methyl benzoate was less regulated by light phase and dark phase rhythm. However, in another plant, *Antirrhinum majus*, methyl benzoate was strongly regulated by light and dark phase rhythm, with high emission during light phase and low at dark phase (Kolosova et al., 2001).

These examples show that in plants, even the same compounds may have very different emission patterns in different species. It can be assumed that insects and other organisms respond to volatile blends, meaning that ratios in the same compounds in different blends encode different information, and characterize plant species or group of plants with similar properties. It is clear that although closely related rice and maize plants have similar types of volatiles, specialist insects like rice skipper (*Parnara guttata*) or brown planthoppers (*Nilaparvata lugens*) can recognize rice plants with high certainty. Clearly, volatile world can be very "colorful" for insect senses, and learning these hidden patterns may help people to design better protection strategies for plants, using very diverse and mostly harmless natural volatile products. The research described in this chapter then opens a new window for rice and manipulation of its volatile profiles based on the regulatory molecules and mechanisms reveled in this work.

Chapter 4 Ethylene functions as a suppressor of volatile production in rice

1. Introduction

1.1. Background

Despite a clear involvement of JA-Ile in control of VOCs, volatiles show complex patterns of emissions that presumes active participation of other hormones, such as ethylene, in regulatory circuits (Broekgaarden et al., 2015; Huang et al., 2005; Huffaker et al., 2013; Mewis et al., 2005; Paudel & Bede, 2015; Pierik et al., 2014; Rehrig et al., 2014; Schuman et al., 2018; Voelckel et al., 2001). With regard to volatiles, ethylene is already well-known for its positive role in aroma control of ripening fruits (Mitalo et al., 2019; Schaffer et al., 2007; Zhu et al., 2005). This chapter features and basically follows our recent publication in Journal of Experimental Botany by Mujiono et al. (2020).

1.2. Ethylene as a plant regulator

Ethylene is produced from 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC oxidase (ACO). The ACC synthase (ACS) and ACO genes are tightly controlled, both transcriptionally and post-transcriptionally, to regulate ethylene production in development and stress (Booker & DeLong, 2015; Larsen, 2015). Besides disease resistance, and modulation of plant defense responses to pathogens (Broekgaarden et al., 2015; Helliwell et al., 2016; Kusajima et al., 2018; van Loon et al., 2006), ethylene also affects plant-insect interactions, including emission of volatiles from herbivore attacked plants. For example, application of exogenous ethylene enhanced JA-mediated volatile production in lima bean (Horiuchi et al., 2001) and *Medicago truncatula* (Arimura et al., 2008). In corn (*Zea mays*) seedlings, volatile emissions triggered by feeding of beet armyworm caterpillars were suppressed by pretreatment of plants with an inhibitor of ethylene perception, 1-methylcyclopropene (1-MCP; Schmelz et al., 2003). As determined by highly sensitive photo-acoustic spectrometer, ethylene burst is induced by simulated herbivory in wild tobacco (*Nicotiana attenuata*) leaves within 1-2 h after treatment (Von Dahl et al., 2007). Negative effect of ethylene on plant resistance has also been reported, such as defective ethylene signaling increasing resistance of Arabidopsis

to the generalist Egyptian cotton worm (*Spodoptera littoralis*) (Stotz et al., 2000). Furthermore, ethylene perception was necessary for interference with parasitoid attraction to *Plutella xylostella*–infested Arabidopsis by simultaneous feeding of a phloem feeder, whitefly (*Bemisia tabaci*) and *P. xylostella* (Zhang et al., 2013). Thus, both positive and negative roles of ethylene should be considered when studying mechanisms of volatile emission in plant-insect interactions.

1.3. Examples of ethylene functions rice

Among land plants, rice (*Oryza sativa*), which is the most important food crop in Asia, shows rapid elongation in response to water submergence. As this stress response is known to be controlled by ethylene (Iwamoto et al., 2010; Kuroha et al., 2018), we hypothesized that crosstalk of ethylene with other stress pathways might be specifically tailored in rice, and in this study, we examined in detail a crosstalk of ethylene with innate production of VOCs in rice. In particular, we focused on developmental- and stress-triggered accumulation of VOCs in young rice leaf to investigated basic layers of JA-ethylene crosstalk, using ethylene treated plants, and those impaired in perception of ethylene by pretreatment with 1-MCP. We show that both, stress-induced and constitutive, VOCs are under strong control of ethylene signaling in rice plants. Our data suggest that ethylene works as a negative regulator of VOCs that can be, at least in part, attributed to existence of putative protective measures against detrimental accumulation of high levels of VOCs in the young rice seedlings, or cost saving strategy in mature rice plants that already possess robust mechanical defense against chewing herbivores.

2. Results

2.1. Developmental regulation of VOCs in rice

As accumulation of VOCs was highly variable in field experiments with rice plants of different age, we hypothesized that VOC production might be developmentally programed in rice. We therefore decided to check VOC contents in the leaves of synchronized six to ten-week-old rice plants in the laboratory. To asses multiple VOCs, the youngest fully developed leaf was first wounded with a fabric pattern wheel, and wounds were immediately treated with 20 µL of diluted oral secretions (OS) isolated from *Mythimna loreyi* larvae (referred afterwards as simulated herbivory; or abbreviated as WOS in text and figures). Monoterpene linalool accumulation, measured 4 h after treatment, was strongly stimulated in the leaves at all growth stages, compared to control leaves without treatment, but induced volatile levels were significantly higher in 8-week-old plants. Another monoterpene, limonene, accumulated constitutively in the leaves, and it only significantly increased by simulated herbivory in 8-week-old plants (Fig. 4.1A). Among sesquiterpenes, β-caryophyllene accumulated at all stages without substantially responding to simulated herbivory, while (*E*)-β-farnesene showed induction profile most similar to linalool (Fig. 4.1B). Although methyl salicylate was promoted by simulated herbivory at 6-8 weeks, older plants accumulated much high levels of methyl salicylate (MeSA), even without treatment (Fig. 4.1C). (*Z*)-3-hexenol, a representative of green leaf volatiles (GLVs), accumulated in young leaves but strongly declined in older plants, and it was somewhat depleted 4 h after simulated herbivory (Fig. 4.1C), consistent with the expected rapid escape of GLVs from the open wounds (Dicke et al., 2009). Overall, multiple compound-specific accumulation patterns among rice VOCs were observed, particularly revealing a transitional change in rice occurring around eight weeks after germination, when the plants are kept under controlled laboratory conditions. We assumed that such patterns could be due to differential ability of plants at different age to produce signaling molecules, namely phytohormones, in response to external stress.

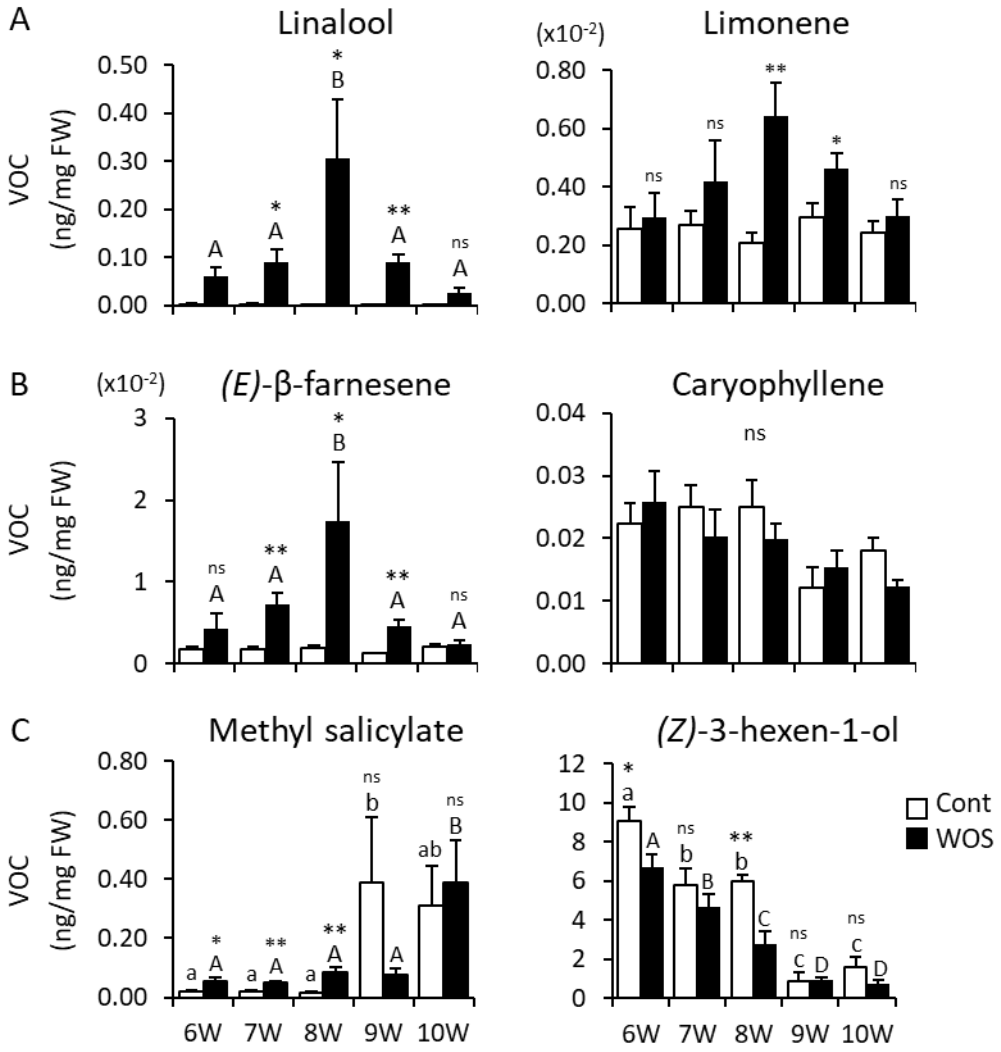

Fig. 4.1. Ontogeny- and WOS-regulated accumulation of VOCs in rice leaves. Volatile compounds were determined by GC-MS in rice leaves of 6-10-week-old plants before, and 4 h after WOS elicitation. (A) Monoterpenes (linalool, limonene), (B) sesquiterpenes ((*E*)-β-farnesene, caryophyllene), (C) methyl salicylate, and GLV ((*Z*)-3-hexenol). Data (n = 5) are shown as means ± SE; Cont, control; WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretions. Statistical differences between pairs of control (Cont) and WOS-treated plants at each age were analyzed by Student's t-test (*P < 0.05; ** P < 0.01; ns, not significant). Different letters (a-c, or A-D) indicate significant differences ($p \leq 0.05$) between different plant age determined separately for each group (Cont or WOS) by one-way ANOVA followed by Fisher's LSD test. W, weeks-old; FW, fresh weight.

2.2. Hormonal responses in vegetative rice development

To address this possibility, phytohormone levels were determined in comparable set of rice plants at age 6-10 weeks after exposure to simulated herbivory. Leaves in similar position as used for VOC measurement were treated with WOS, and samples for hormone determination were collected 1 h later, to precede volatile biosynthesis measured 4 h post

elicitation (Fig. 4.1). Compared to control leaves, JA strongly accumulated in treated leaves, but the levels were not particularly different among developmental stages (Fig. 4.2A). In contrast, highest level of JA-Ile, known to be predominant active form of jasmonates, accumulated at highest level in leaves of 8-week-old plants (Fig. 4.2A), showing an accumulation profile similar to linalool and (*E*)-β-farnesene (Fig. 1A, B). Abscisic acid (ABA) levels were not particularly induced by simulated herbivory at 1 h, showing only a slightly higher contents in 7- and 8-week-old plants (Fig. 4.2A). Interestingly, SA increased gradually in development, reaching the highest levels in 9-10-week-old plants (Fig. 4.2A), providing a potential ground for the formation of high levels of MeSA in older plants (Fig. 4.1). Finally, ethylene gas in headspace was measured after incubating cut rice leaves in 50 mL glass containers for 24 h. Ethylene profile was inverse when compared to that of JA-Ile, with lowest ethylene being produced from 8-week-old rice leaves (Fig. 4.2B), and particular increase was detected towards 9-10 weeks of age. As hormone profiles suggested (1) a positive role of JA-Ile, and (2) a negative role of ethylene in developmentally-regulated VOC production in rice, JA and ethylene signaling, as well as transcript levels of VOC biosynthesis-related genes were investigated.

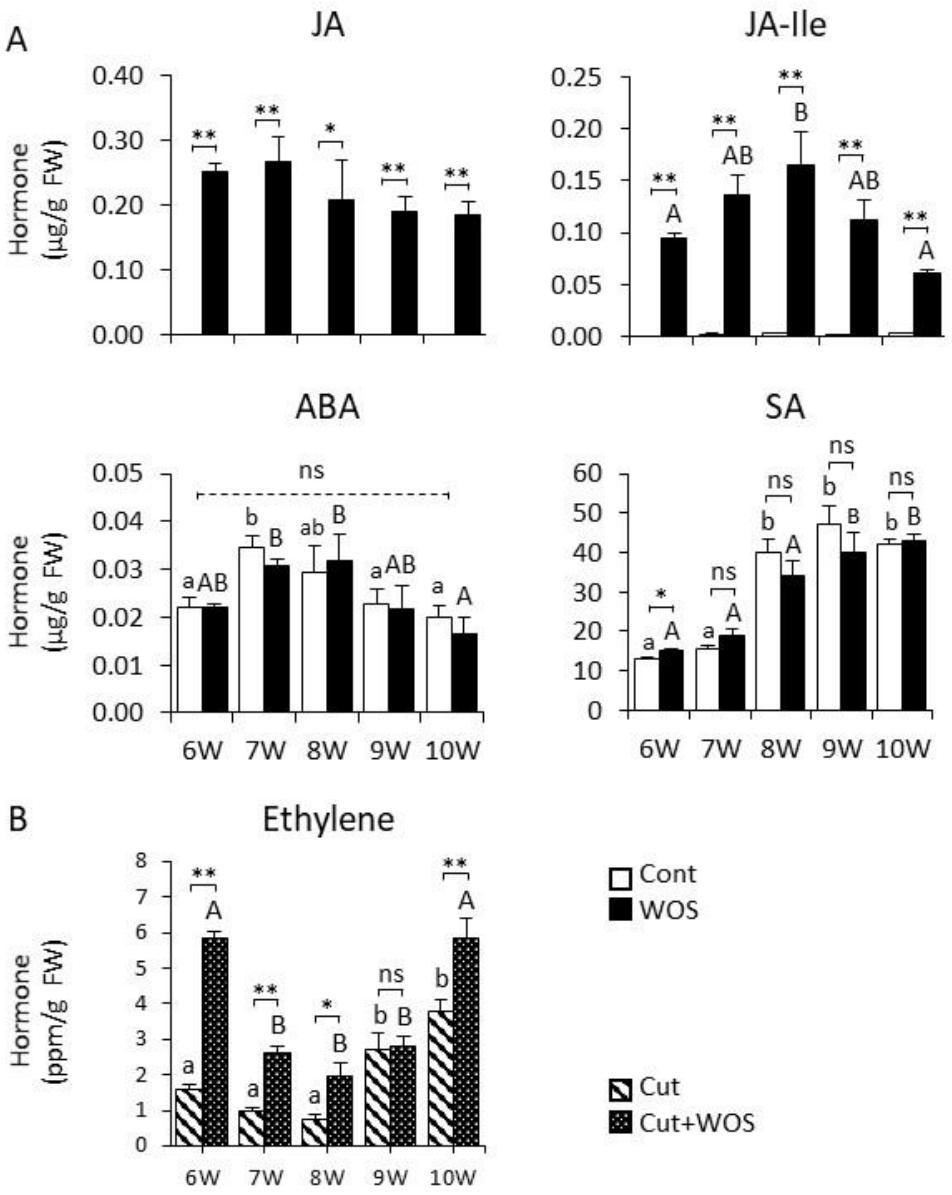

Fig. 4.2. Ontogeny- and WOS-regulated accumulation of phytohormones in rice leaves. (A) Jasmonic acid (JA), jasmonoyl-L-isoleucine (JA-Ile), abscisic acid (ABA), and salicylic acid (SA) levels were determined by LC-MS/MS in rice leaves of 6-10-week-old rice before, and 1 h after WOS elicitation. Data (n = 5) are shown as means ± SE. Cont, control; WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretions. Statistical differences between pairs of control (Cont) and WOS-treated plants at each age were analyzed by Student's t-test (*P < 0.05; ** P < 0.01; ns, not significant). Different letters (a, b or A, B) indicate significant differences (p ≤ 0.05) between different plant age determined separately for each group (Cont or WOS) by one-way ANOVA followed by Fisher's LSD test. (B) Ethylene levels produced by cut rice leaves (Cut) and cut WOS-treated leaves (Cut+WOS) of 6-10-week-old plants determined by GC-FID after 24 h accumulation period. Data (n = 3) are shown as means ± SE. Different small letters indicate significant differences (p ≤ 0.05) between plant ages determined by one-way ANOVA followed by Fisher's LSD test. W, weeks-old; FW, fresh weight.

2.3. Transcriptional regulation of VOC production in rice leaves

Defense responses in plants depend on hormone-mediated transcriptional reprograming of stress exposed plants, including genes involved in jasmonate/ethylene signaling, and VOC production. In 6-10 week-old plants, transcripts of *ALLENE OXIDE CYCLASE* (*OsAOC*), a single copy gene in JA biosynthesis pathway in rice (Michael Riemann et al., 2013), were already abundant in untreated plants, and the levels further increased after simulated herbivory (Fig. 4.3A), showing a profile similar to induced JA levels (Fig. 4.2A). Similar transcriptional profiles were also found for other JA pathway committed genes, *LIPOXYGENASE 1* (*OsLOX1*), *ALLENE OXIDE SYNTHASE 1* (*OsAOS1*), *OXO-PHYTODIENOATE REDUCTASE 7* (*OsOPR7*), and *OsCYP94C2b* (for hydroxylation of JA-Ile) (Supplementary Fig. S2A). The *OsLOX9* and *OsAOS2* transcripts showed rather different regulation, being low in younger plants but gradually increased in development (Supplementary Fig. S2A). The chloroplast-localized *PHOSPHOLIPASE Dα4* (*OsPLDα4*), herbivory- and wound-induced gene implicated in release of linolenic acid from cell membranes (Qi et al., 2011), was highly upregulated by WOS, and reached maximum expression in 8-week-old rice (Supplementary Fig. S2A). Although we expected the *JASMONATE RESISTANT 1* (*OsJAR1*) profile to basically follow the JA-Ile levels (Fig. 4.2A), as this Gretchen Hagen 3.5 (GH3.5) gene family protein is responsible for JA and Ile conjugation in rice JA-Ile biosynthesis (Fukumoto et al., 2013), induced *OsJAR1* transcripts were highest in the leaves of 6-week-old rice, and gradually declined in development (Fig. 4.3A), showing a lack of substantial correlation between transcript levels and JA-Ile contents. The central regulator of jasmonate pathway, *MYELOCYTOMATOSIS ONCOGENE TRANSCRIPTION FACTOR 2* (*OsMYC2*) transcripts were upregulated by WOS in way similar to *OsJAR1* (Fig. 4.3A) but profiles of two jasmonate-responsive genes encoding JAZ repressors, *JASMONATE ZIM-DOMAIN 9* (*OsJAZ9*) and *OsJAZ11*(Ye et al., 2009) were more similar to JA-Ile (Fig. 4.3B). Among two SA- and JA-induced defense regulators in rice, *OsWRKY45* (Shimono et al., 2007) and *JA-REGULATED MYB TRANSCRIPTION FACTOR* (*OsJAMYB*) (M. W. Lee et al., 2001), respectively, only the second gene was clearly induced by simulated herbivory (Supplementary Fig. S2B).

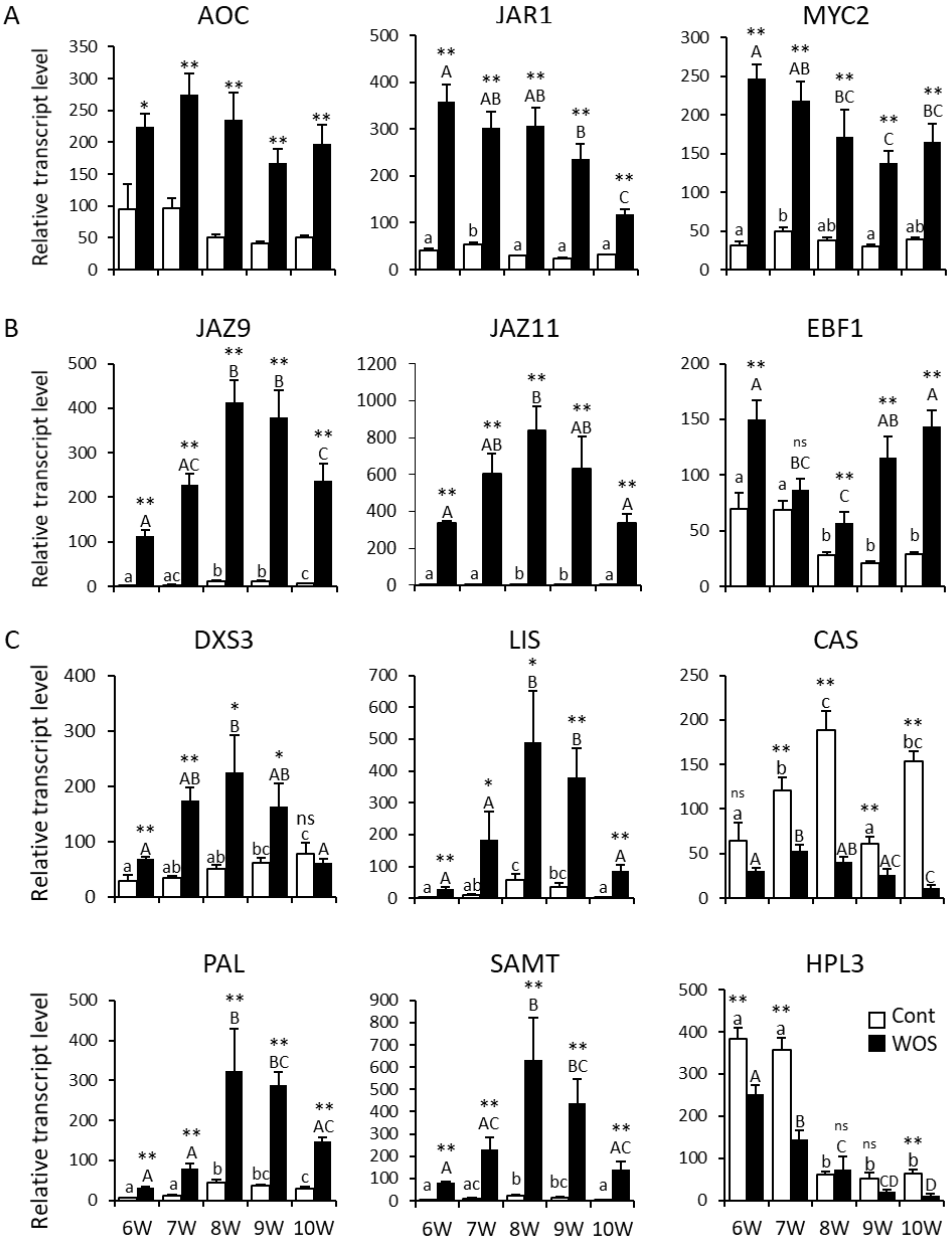

Fig. 4.3. Relative transcript levels of phytohormone and VOC-related genes in rice plants at different developmental stages. Gene expression was determined by qRT-PCR in rice leaves of 6-10-week-old plants before, and 1 h after WOS elicitation. (A) Induction of JA biosynthesis (*OsAOC*, *OsJAR1*) and signaling–related genes (*OsMYC2*). (B) JA (*OsJAZ9*, *OsJAZ11*) and ET (*OsEBF1*) response genes. (C) VOC biosynthesis related genes: Terpenoids (*OsDXS3*, *OsLIS*, *OsCAS*), methyl salicylate (*OsPAL*, *OsSAMT*), and GLVs (*OsHPL3*). Data (n = 5) are shown as means ± SE; Cont, control; WOS, wounding followed by application of 20 µL diluted *M. loreyi* oral secretions. Statistical differences between pairs of control (Cont) and WOS-treated plants at each age were analyzed by Student's t-test (*P < 0.05; ** P < 0.01; ns, not significant). Different letters (a-c, or A-D) indicate significant differences ($p \leq 0.05$) between different plant age determined separately for each group (Cont or WOS) by one-way ANOVA followed by Fisher's LSD test. W, weeks old.

As we already suspected the involvement of ethylene in developmental regulation of rice VOCs, transcript levels of ethylene-responsive *EIN3-BINDING F-BOX PROTEIN 1* gene (*OsEBF1*) (Ma et al., 2019) were measured along with the JA markers. As expected from ethylene data in Fig. 4.2B, lowest levels of *OsEBF1* transcripts (Ma et al., 2019) were detected in 8-week-old plants (Fig. 4.3B), further supporting the occurrence of differential ethylene responses in rice ontogeny. *ETHYLENE RESPONSE 2* (*OsETR2*), receptor for ethylene (Watanabe et al., 2004), and four other genes putatively involved in ethylene biosynthesis, were also investigated (Supplementary Fig. S3A). Highest *OsETR2* transcript levels in untreated plants were found in 6-7-week-old rice, declined at 8-9 weeks, and then slightly increased again at 10 weeks, consistent with the *OsEBF1* levels. In contrast to *OsEBF1* induction (Fig. 4.3B), simulated herbivory downregulated *OsETR2* transcripts, suggesting a negative feed-back regulation of the receptor's transcription. Ethylene biosynthesis genes, *1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 1* (*OsACS1*), *OsACS3*, *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE 1* (*OsACO1*), and *OsACO2* were generally higher, and more induced by WOS in younger plants, showing minimum levels of expression in leaves of 8-9-week-old rice (Supplementary Fig. S3B). The positive regulatory gene in ethylene signaling, *ETHYLENE INSENSITIVE3-LIKE1* (*OsEIL1*) (Mao et al., 2006) was moderately induced by simulated herbivory, showing lowest levels of expression in 8-9-week-old of plants (Supplementary Fig. S3C).

In VOC biosynthesis, transcripts of *1-DEOXY-D-XYLULOSE 5-PHOSPHATE SYNTHASE 3* (*OsDXS3*) and *LINALOOL SYNTHASE* (*OsLIS*) closely followed linalool accumulation (Fig. 4.3C), suggesting that linalool production is controlled at transcriptional level of both, input core methylerythritol 4-phosphate (MEP) pathway, represented by *OsDXS3*, and terminal terpene synthase, *OsLIS*. Similar regulation was found for *PHENYLALANINE AMMONIA-LYASE* (*OsPAL*) and *S-ADENOSYL-L-METHIONINE: SALICYLIC ACID CARBOXYL METHYLTRANSFERASE* (*OsSAMT*) involved in MeSA production (Y. J. Koo et al., 2007), which was consistent with MeSA levels found in the young plants (6-7 weeks) but discrepant with 8-10 week stages of development (Fig. 4.1C). It suggests that in an older plant, another late development-associated methyltransferase and/or PAL might be additionally involved in MeSA production. The *CARYOPHYLLENE SYNTHASE* (*OsCAS*) transcripts were more

constitutive across development, and suppressed by simulated herbivory at 1 h after treatment (Fig. 4.3C). Finally, *HYDOPEROXIDE LYASE 3* (*OsHPL3*) gene closely followed the (*Z*)-3-hexenol (GLV) levels in the leaves, and showed partial suppression by simulated herbivory at some time points (Fig 4.3C). In summary, transcript levels of most rice VOC biosynthesis genes showed good correlation with VOC levels in the leaves, suggesting that their expression reflects tissue capacity for the production of corresponding volatiles. As transcriptional patterns of induced VOC genes followed positively JA-Ile, and negatively ethylene hormonal trends (supported by ethylene makers, receptor, and biosynthesis genes), we further investigated a direct involvement of ethylene in VOC production in rice.

2.4. Ethylene treatment transcriptionally inhibits volatile production in rice

Rice plants were exposed to exogenous ethylene (1 ppm) and leaves were collected 4 h later for VOC analysis. Ethylene clearly inhibited the accumulation of linalool, especially in younger plants (Fig. 4.4A). (*E*)-β-farnesene was also inhibited in young plant stages but accumulation of other volatiles was only slightly, or not affected by ethylene (Fig. 4A-C). As before, MeSA levels were constitutively higher in older plants, and although MeSA was suppressed at some stages by ethylene, the resulting differences were not statistically significant. In general, rice VOCs dependent on inducible transcriptional regulation were more affected by ethylene, suggesting that ethylene is specifically blocking some mechanisms in gene activation, with the effective concentration of ethylene less than (or equal to) 0.1 ppm (Fig. 4.4D). A time course experiment was then conducted with 7-week-old plants treated with ethylene (1 ppm), and leaves were sampled before, 0.5, 1, and 4 h post treatment in the presence or absence of ethylene. Consistent with previously reported dose-dependent responsiveness of OsEBF1 to ethylene (Ma et al. 2020), transcript levels of this marker gene were clearly elevated by ethylene 1 h post treatment (Fig. 4.5C). Ethylene receptor *OsETR2* and signal transduction gene *OsEIL1* were also transcriptionally stimulated by ethylene (Supplementary Fig. S7).

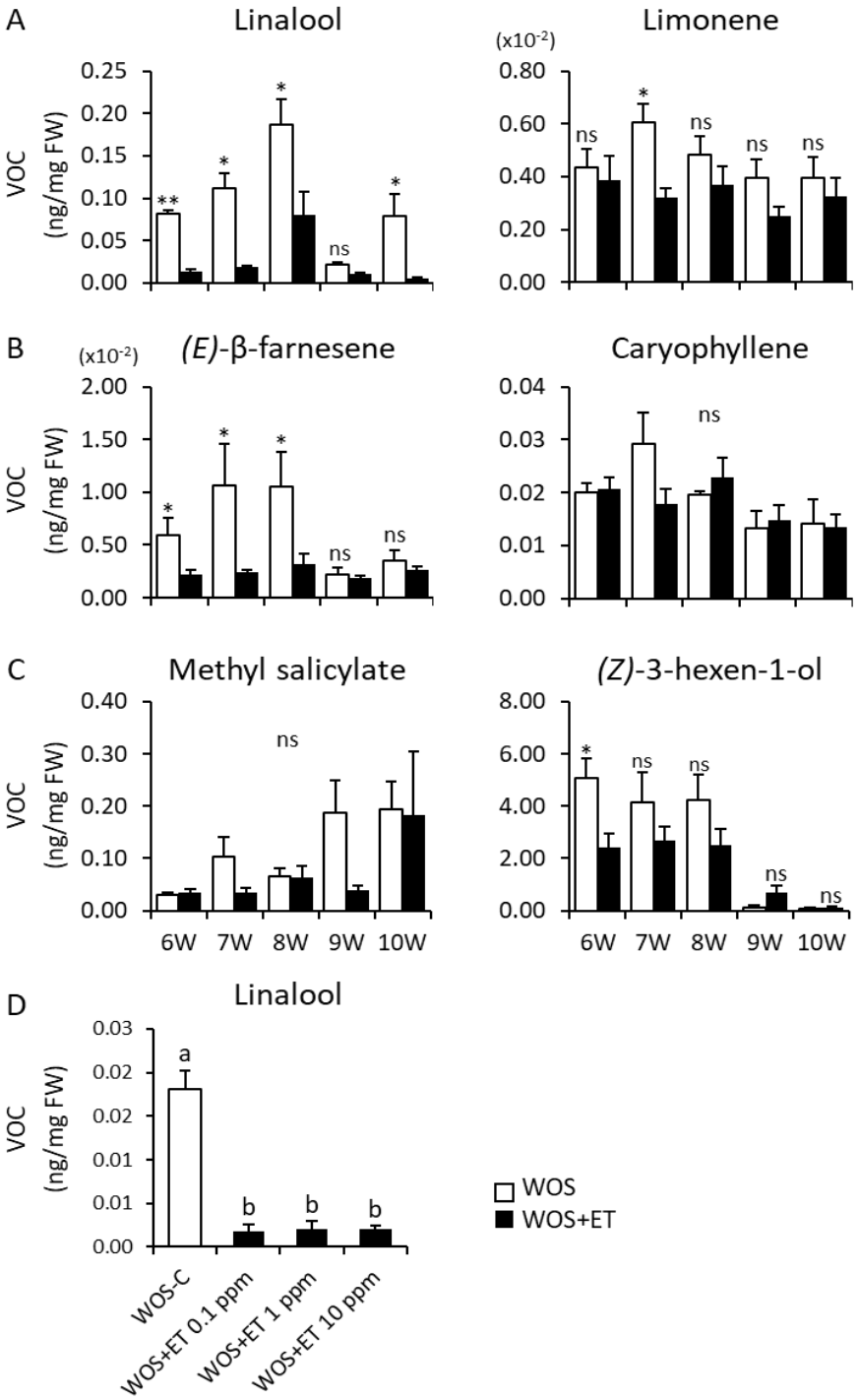

Fig. 4.4. Effect of ethylene on ontogeny-dependent VOC accumulation in rice leaves. VOC levels were determined in leaves of 6-10-week-old plants treated with WOS, and inserted in closed containers containing ambient air (WOS), or in containers with 1 ppm ethylene containing air (WOS+ET). Leaves were collected after 4 h incubation and analyzed by GC-MS. (A) Monoterpenes (linalool, limonene), (B) sesquiterpenes ((E)-β-farnesene, caryophyllene), (C) methyl salicylate, and GLV ((Z)-3-hexenol). Data (n= 5) are shown as means ± SE. (D) Effect of different concentrations of ethylene on production of monoterpene linalool (n = 3). In (A-C), statistical differences between pairs of WOS and WOS+ET treated plants at each age were separately analyzed by Student's t-test (*P < 0.05; ** P < 0.01; ns, not significant). In (D), different letters (a, b) indicate significant differences between treatments ($p \leq 0.05$) determined one-way ANOVA followed by Fisher's LSD test.

When we determined the JA and JA-Ile levels in ethylene-treated leaves, JA accumulation was partially reduced but ethylene did not affect the early accumulated JA-Ile (Fig 4.5A). At gene level, *OsAOC* transcripts were partially suppressed by ethylene at 1 h post treatment, which trend was also found in *OsJAR1* (Fig. 4.5B). The *OsMYC2* transcript levels were induced early, showing a slight but significant reduction by ethylene at 1 and 4 h post treatment (Fig. 4.5B), same as *OsJAZ11* (Fig 4.5C). Interestingly, *OsJAZ9* transcripts peaked later, and showed much larger suppression by ethylene (Fig. 4.5C), suggesting that ethylene is mainly affecting genes with later (~1 h) response but largely avoids primary JA response genes, such as *OsJAZ11* and *OsMYC2*. Among other JA-related genes, *OsPLDα4* and *OsAOS1* were suppressed by ethylene but *OsLOX9* and *OsJAMYB* showed induced transcriptional patterns (Supplementary Fig. S6).

Finally, *OsDXS3*, *OsLIS*, *OsPAL*, and *OsSAMT* were all highly suppressed by ethylene at 1 h post treatment, and except for OsPAL, their transcripts retained almost undetectable levels in 4 h ethylene-treated leaves (Fig. 4.5D). Surprisingly, *OsCAS* and *OsHPL3* transcripts were also suppressed by ethylene, becoming significantly low 4 h after ethylene application (Fig. 4.5D). It shows that while transcriptional repression of ethylene immediately affected major herbivory-inducible VOCs, like linalool, it may also shut down the other volatiles, like caryophyllene and GLVs. However, previous transcript levels and/or protein seem to delay the visible ethylene impact, and sustain VOC levels in the leaves constant for at least 4 h, as shown in Fig. 4B, C. The complete loss of *OsCAS* and *OsHPL3* transcripts in 4 h ethylene-treated rice leaves in 6-10-week-old rice leaves was further confirmed in another experiment shown in Supplementary Fig. S5. Upregulation of ethylene marker gene *OsEBF1* across every developmental stage (Supplementary Fig. S5) was also confirmed.

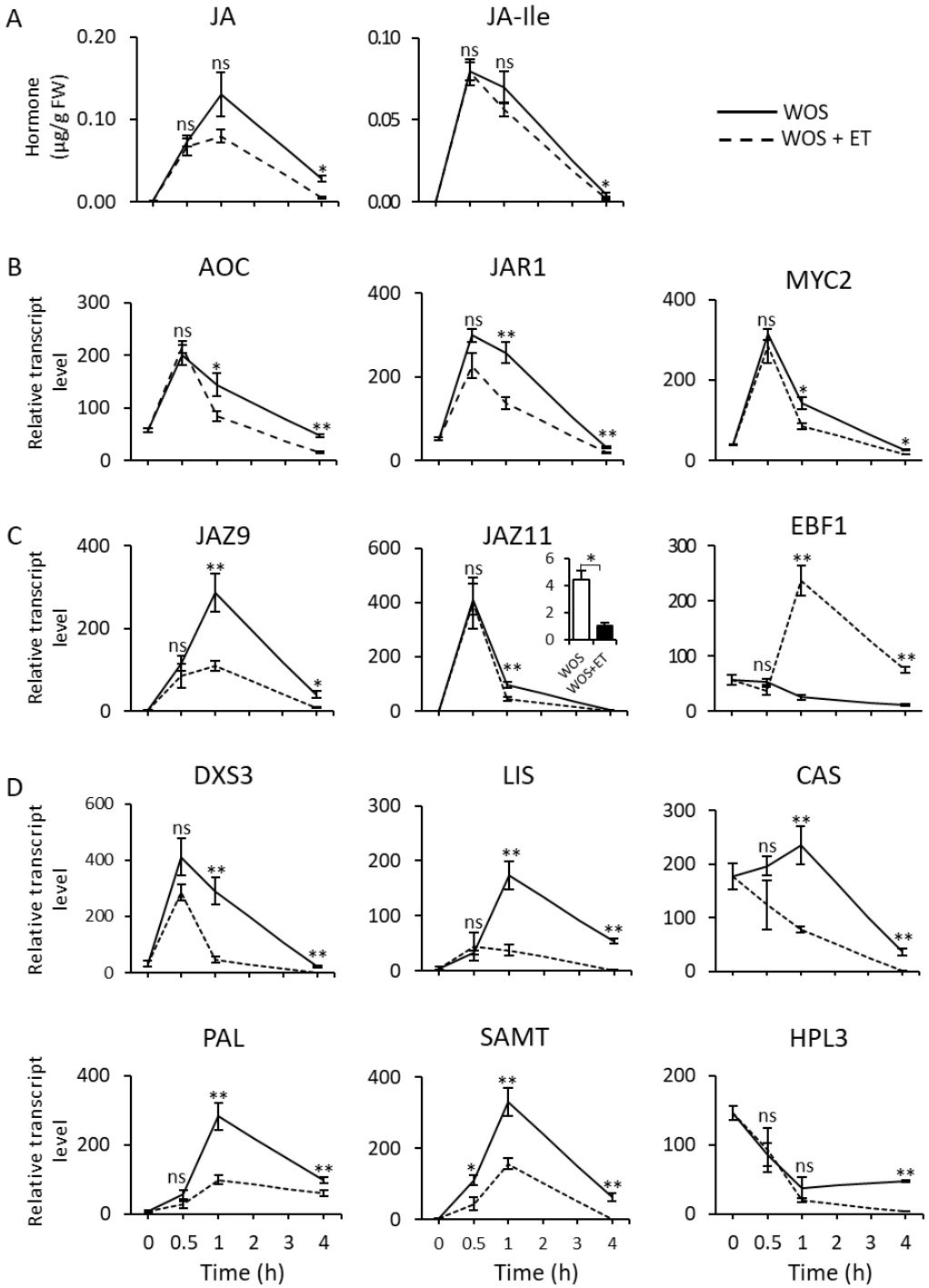

Fig. 4.5. Effect of ethylene on phytohormone levels, signaling and VOC-related transcription in rice. Leaf samples were collected before and 0.5, 1, and 4 h after WOS treatment from plants kept in the presence (WOS+ET, dashed line) and absence (WOS, solid line) of ethylene (1 ppm). (A) JA and JA-Ile levels, (B) relative transcript levels of JA biosynthesis (*OsAOC*, *OsJAR1*) and signaling (*OsMYC2*)-related genes, (C) relative transcript levels of JA (*OsJAZ9*, *OsJAZ11*), and ET (*OsEBF1*) response genes, (D) relative transcript levels of terpenoid (*OsDXS3*, *OsLIS*, *OsCAS*), methyl salicylate (*OsPAL*, *OsSAMT*), and GLVs (*OsHPL3*) related genes. Data (n = 4) are shown as means ± SE. Statistical differences between treatments were analyzed at each time point by Student's t-test (*$P < 0.05$; ** $P < 0.01$; ns, not significant).

2.5. Examination of VOC-related physio-ecological functions of ethylene

One of the best studied stress functions of ethylene in rice includes its role in flood-induced internode elongation. When 7-week-old rice was submerged in water, ethylene increased, and last newly developed internode elongated (Fig. 4.6A, C). Flood-induced ethylene was further promoted by simulated herbivory (Fig. 4.6C). As expected, exogenous ethylene (1 ppm) caused faster elongation of rice plants but only at younger stages, i.e., before 8 weeks (Fig. 4.6B), collinear with the urgency of smaller plants to escape from submergence and anoxia stress.

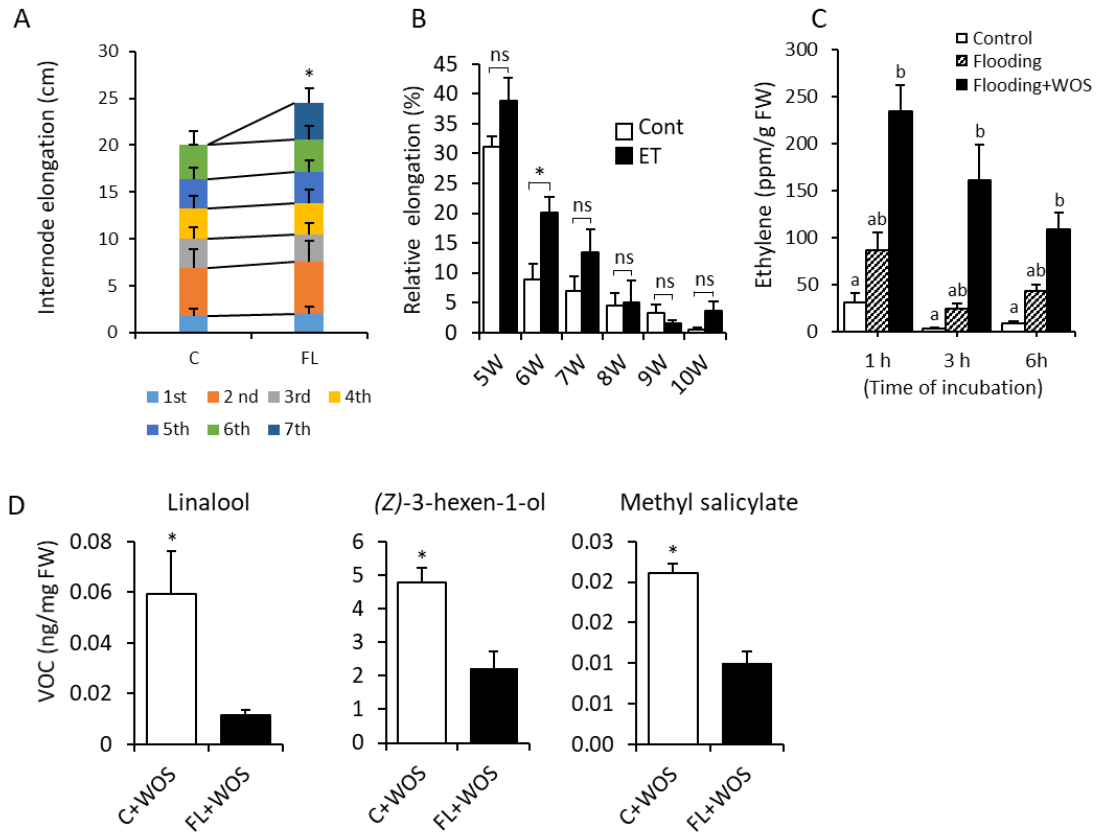

Fig. 4.6. Ethylene function in submergence response in rice. Rice plants were submerged in water for 24 h prior WOS treatment or measurement of internode/stem length. (A) Individual internode lengths before (Cont) and after flooding (FL) in 7-week-old rice. (B) Effect of 1 ppm ethylene applied for 24 h on relative elongation rate in rice determined as % elongation of stem relative to length before treatment. (C) Ethylene emissions from leaves pre-treated with flooding stress for 24 h. (D) VOC levels in WOS-treated leaves from 24 h water-submerged (FL+WOS), and open air (C+WOS) grown rice. Sample were collected and analyzed 4 h after WOS treatment. Data (n= 5) are shown as means ± SE. Statistical differences between internode length (A), stem length at each age (B), and pairs of treatments (D) were analyzed by Student's t-test (*$P < 0.05$). Different letters in (C) indicate significant differences between treatments ($p \leq 0.05$) at each time point determined one-way ANOVA followed by Fisher's LSD test. W, weeks-old; FW, fresh weight.

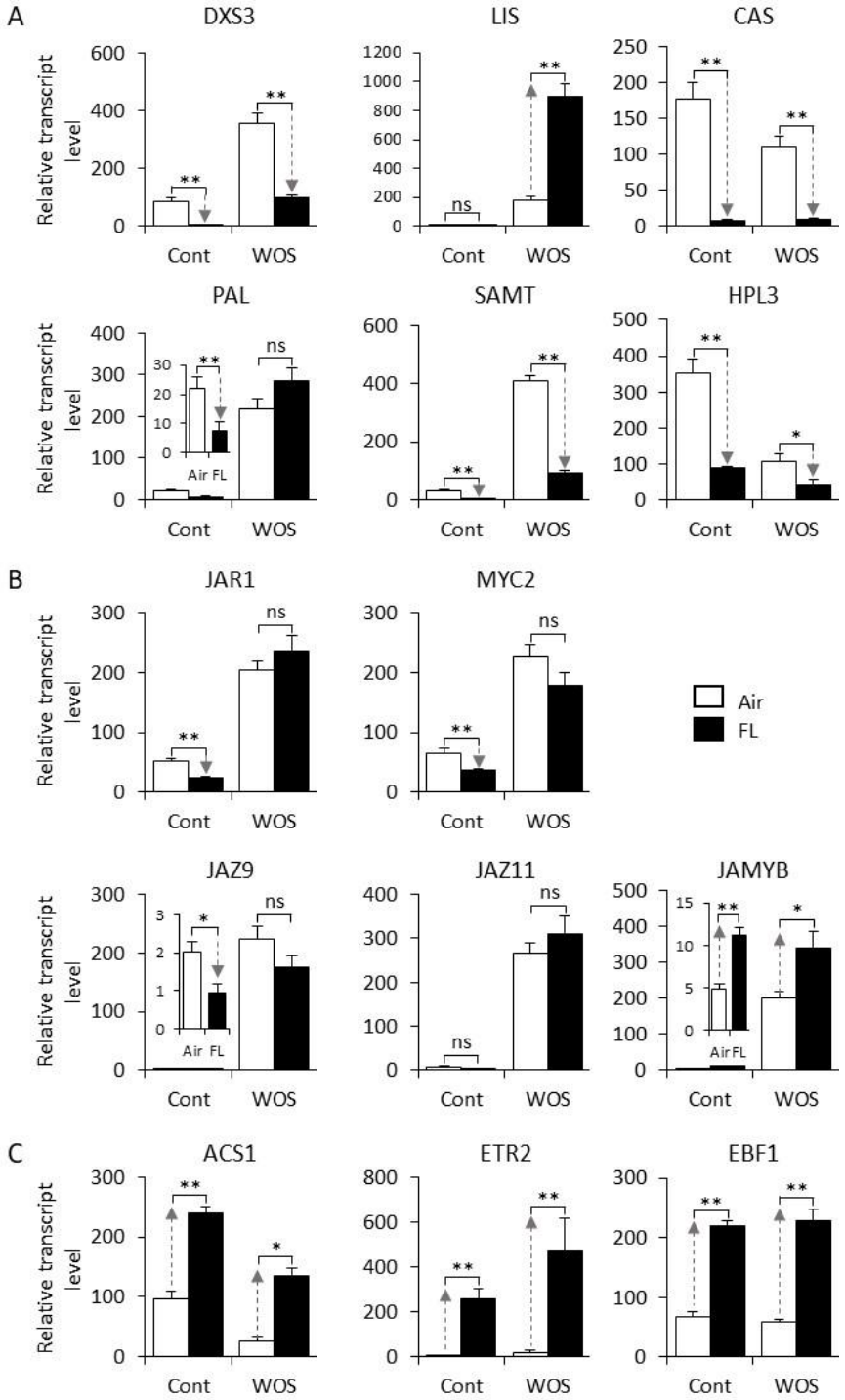

Fig. 4.7. Effect of water submergence on expression of JA/ethylene signaling and VOC biosynthesis genes. Rice plants at 7 weeks were submerged in water for 24 h (FL), or kept without water (Air), and subsequently treated with WOS, or left untreated (Cont). Samples were collected 1 h after last treatment and analyzed by qRT-PCR. (A) VOC biosynthesis-related genes (*OsDXS3*, *OsLIS*, *OsCAS*, *OsPAL*, *OsSAMT*, *OsHPL3*), (B) JA biosynthesis/signaling–related genes (*OsJAR1*, *OsMYC2*, *OsJAZ9*, *OsJAZ11*, *OsJAMYB*) and ethylene biosynthesis/signaling genes (*OsACS1*, *OsETR2*, *OsEBF1*). Data (n = 6) are shown as means ± SE. Statistical differences between pairs of flooded and air samples were analyzed by Student's t-test (*$P < 0.05$, ** $P < 0.01$; ns, not significant). Arrows show suppression or induction of transcripts by flooding stress.

To prevent high VOC accumulation and, potentially, cellular damage, submerged plants may quickly block their VOC production by means of submergence-induced ethylene (Fig. 4.6C). Indeed, linalool, (*Z*)-3-hexenol, and MeSA levels in submerged leaves were all significantly reduced by flooding stress (Fig. 4.6D). At transcriptional level, constitutive and simulated herbivory-treated *OsSAMT* and *OsHPL3* transcripts were significantly reduced by flooding (Fig. 4.7A), supporting the lower MeSA and (*Z*)-3-hexenol levels in submerged leaves (Fig. 4.6D). While levels of *OsSAMT* were effectively suppressed by submergence of plants, associated *OsPAL* transcription was not affected under these conditions (Fig. 4.7A), suggesting that PAL may be exempted from ethylene control, possibly due to PAL requirement in other functions, such as accelerated growth under water.

Consistent with the reduced levels of linalool in submerged leaves, transcription of *OsDXS3* gene in core MEP pathway was suppressed by flooding of leaves (Fig. 4.7A). However, unexpectedly, submerged leaves treated with simulated herbivory showed much higher level of *OsLIS* transcripts compared to WOS-treated leaves kept under aerated conditions (Fig. 4.7A). These results reveal an existence of yet unknown regulatory mechanism(s) in *OsLIS* expression, possibly including posttranslational modifications, and/or direct regulatory function of linalool (or its metabolite) in control of *OsLIS* transcription. Previously, function of some monoterpenes as competitive inhibitors of ethylene perception was proposed (Grichko et al., 2003), implying that linalool (or other monoterpene) could be directly interfering with ethylene signaling in submerged plant tissues. It should be noticed that higher transcript levels of *OsLIS* did not support linalool production (Fig. 4.6D), most likely due to submergence-induced repression of multiple genes in MEP pathway, including input level *OsDXS3* gene (Fig. 4.7A; Supplementary Fig. S8). Other genes, including *OsJAR1*, *OsMYC2*, *OsJAZ9*, *OsJAZ11*, *OsJAMYB*, *OsACS1*, *OsETR2*, and *OsEBF1* (Fig. 4. 7B, C) showed expression patterns consistent with previous exogenous ethylene treatments (Fig. 4.5; Supplementary Fig. S6, 7), further supporting the presence and function of endogenous ethylene in flood-induced leaves, and modulation of VOC biosynthesis.

As VOC levels substantially decreased in 9-10-week-old plants, despite JA-Ile accumulation, we speculated that ethylene, accumulating preferentially in older plants (Fig. 4. 2B), might be responsible for this reduction. To test this possibility, 9-week-old plants were

pretreated with 1-methylcyclopropene (1-MCP) for 24 h, and then leaves were treated with simulated herbivory. Samples were collected from both, WOS-treated and untreated plants, 4 h after the last treatment. As predicted, even control plants solely exposed to 1-MCP for 24 h produced higher levels of mono- (linalool, limonene) and sesquiterpenes ((*E*)-β-farnesene and caryophyllene), and these levels could be further enhanced by simulated herbivory (Fig. 4.8A, B). Consistent with our previous data, MeSA accumulation was not promoted by 1-MCP in mature plants (Fig. 4.8C), further suggesting involvement of yet another, most likely ethylene-insensitive methyltransferase in MeSA production in mature stages of rice development. Finally, accumulation of (*Z*)-3-hexenol was also promoted by 1-MCP (Fig. 4.8C), suggesting that natural decline in GLV content found in adult rice (Fig. 4.1C) could be due to an increased level/sensitivity of/to ethylene in these plants. Although there are many other functions controlled by ethylene in rice, two selected examples above provide the first clear evidence that ethylene acts as an effective mobile transcriptional repressor of VOC production in rice, contributing to variable VOC blends emitted from rice plants.

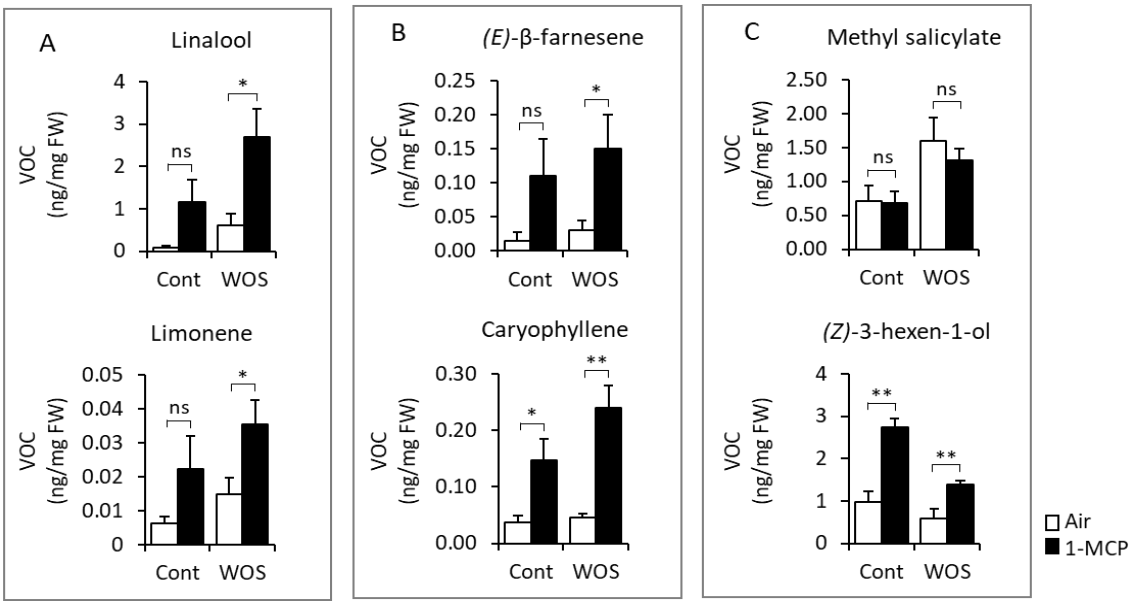

Fig. 4.8. Effect of ethylene inhibitor 1-MCP on VOC production in rice. Rice plants at 9 weeks were pretreated with 20 ppm 1-MCP-containing air for 24 h (1-MCP), or kept in container with ambient air without ethylene inhibitor (Air), and subsequently treated with WOS, or left untreated (Cont). Samples were collected 4 h after last treatment and analyzed by GC-MS. (A) Monoterpenes (linalool, limonene), (B) sesquiterpenes ((*E*)-β farnesene, caryophyllene), (C) methyl salicylate, and GLV ((*Z*)-3-hexenol). Data (n = 6) are shown as means ± SE. Statistical differences between pairs of 1-MCP-treated and air samples were analyzed by Student's t-test (*$P < 0.05$, ** $P < 0.01$; ns, not significant). FW, fresh weight.

3. Discussion

Plants evolved multiple stress response pathways to cope with variable environmental conditions in their lifetime. Typically, stress exposure is causing rapid accumulation of secondary messengers, reactive oxygen species (ROS) and phytohormones, that trigger stress-specific transcriptional reprograming for increased resistance and/or tolerance of plants. Although regulatory mechanisms in plants responding to single stress, such as pathogens, herbivores, salinity, or drought are well studied, much less is known about coordination of plant responses to simultaneously occurring stress conditions that presumes active crosstalk among individual regulatory pathways. Here we addressed the role of ethylene and show that it serves as a context-dependent modulator of well-known stress- and ontogeny-related VOCs in rice plants.

3.1. Volatile production in rice is developmentally regulated

Volatile emissions from similarly treated rice in the field can greatly vary depending on developmental stage. In particular, young rice produced less VOCs that gradually increased until heading stage, followed by a drop in VOC production at grain filling and senescence stages (Andama et al., 2020). When similar experiment was conducted under controlled laboratory conditions, rice leaves produced maximal amount of VOCs around eight weeks of age, and then VOC levels decreased, suggesting that VOC production is linked to ontogeny program in rice. Similarly, HIPV production in *Datura wrightii* was constrained by plant ontogeny to vegetative stages of growth, which could be partially restored by rejuvenation (Hare, 2010). In sagebrush, young plants were more effective emitters of volatiles as well as they better perceived volatile cues (Shiojiri & Karban, 2006). Vegetative stage soybean plants emitted 10-fold more volatiles per biomass than reproductive plants, and young leaves emitted about three times more volatiles than their older counterparts (Rostás & Eggert, 2008). Similarly, volatile production in maize not only dramatically declined in maturity but older plants also produced a different blend of volatiles (Köllner et al., 2004). Production of extra floral nectar (EFN) for attraction of natural enemies of herbivores can be also developmentally regulated in some plants (Quintero et al., 2013). It has been proposed that such ontogenetic shifts are related to internal conditions of plants, such as allocation costs, anatomical constraints, and external factors, such as seasonal variance in occurrence of herbivores and their natural enemies

(Quintero et al., 2013). From simple overlap of VOCs in Fig. 4.1, it is clear that volatile blends in rice may greatly differ between young (6-week) and older (10-week) plants, with high and low portion of GLVs, respectively, complemented by variable levels of methyl salicylate. Natural enemies could even distinguish the 8-week stage of rice, based on the high linalool fraction in the herbivory-induced volatile blend (Fig. 4.1). This might be of a great ecological significance, as larvae of parasitoid wasps develop together with their host animal, and remaining physiological time of the host plant can indicate herbivores with sufficient life potential to support the entire natural enemy's larval cycle.

3.2. JA–ethylene crosstalk in rice

The VOCs, and especially HIPVs, are predominantly controlled by jasmonate signaling in plants (Dicke & Baldwin, 2010; Joo et al., 2019). In order to understand differential accumulation of VOCs in rice ontogeny (Fig. 4.1), we first examined phytohormones using young developed leaf. Our data show that maximal JA-Ile was reached in 8-week-old rice (Fig. 4.2). In *N. attenuata* plants, herbivory-induced ethylene levels (and jasmonates) were significantly suppressed after flowering (Diezel et al., 2011). In rice, however, ethylene and expression of several ethylene response and biosynthetic genes attained minimal levels in 8-week stage, suggesting that JA-Ile and ethylene may work in oppose ways, as positive and negative regulator of VOCs, respectively. This was confirmed by exogenous application of ethylene to rice, which triggered rapid transcriptional repression of several genes. Interestingly, primary response genes in JA pathway, i.e., those induced by simulated herbivory within 30 min, such as *OsMYC2*, were not substantially affected by co-application of ethylene. However, other genes that peaked later (1 h) were significantly down-regulated by ethylene, which included major herbivory-inducible VOC genes in rice. Interestingly, even genes for constitutive VOCs, which primarily did not respond to WOS treatment, such as *OsCAS* and *OsHPL3* (Fig. 4.3C), were eventually suppressed by ethylene 4 h post treatment (Fig. 4.5D). It suggests that even these volatile products may need jasmonate signaling to maintain their transcription, and volatile levels. In circadian genes, like *OsCAS* (Cheng et al., 2007), ethylene could be also contributing to diurnal oscillations, together with rhythmicity of basal jasmonate levels already shown in Arabidopsis plants (Goodspeed et al., 2012). It is worth pointing out that two JAZ repressor genes examined in our study showed

differential response to ethylene. While induced expression of *OsJAZ11* basically followed the primary response regulator OsMYC2, the elicited *OsJAZ9* transcripts peaked later, and they were highly suppressed by ethylene. The example of differential regulation of JAZ repressors provides yet another support for specialized functions of individual JAZ proteins well demonstrated in *N. attenuata* plants (Li et al., 2017; Oh et al., 2012). It also shows that while ethylene may regulate some of the JA-Ile-mediated responses, other JA signaling branches in defense may remain independent of ethylene. It will be important to systemically examine the response of all other JAZ genes to reveal the extent of ethylene control over the jasmonate pathway in rice, and *vice versa*.

3.3. Ethylene in rice defense against herbivores

Initially, it has been reported that ethylene signaling positively contributes to VOC emissions from rice brown planthopper (BPH) (*Nilaparvata lugens*) infested rice (Y. Lu et al., 2006). Later, however, ethylene signaling was shown to have contrasting effects on rice defense against chewing and piercing-sucking insects, striped stem borer (SSB) (*Chilo supresalis*), and BPH, respectively. While silencing of ethylene biosynthetic gene *OsACS2* reduced resistance of rice to SSB, and suppressed SSB-induced volatiles, reduced ethylene levels promoted BPH performance and increased VOC emissions from OsACS2-silenced rice plants (J. Lu et al., 2014). Such BPH-specific responses could be mediated by BPH-inducible F-box protein OsEBF1 that targets the OsEIL1 regulator in ethylene signaling for proteasome degradation (Ma et al., 2019). In a functional test, overexpression of OsEBF1 and OsEIL1 suppressed and enhanced BPH performance in transgenic rice, respectively. As OsEIL1 acts on defense via promoting *OsLOX9* gene transcription, JAs supposedly works as negative regulators of BPH resistance (Ma et al., 2019). Accordingly, BPH resistance was promoted in another independent study using OsHI-LOX-silenced rice plants with reduced JA levels (Zhou et al., 2009).

However, our data show that *OsLOX9* transcription may not be directly linked to JA/JA-Ile accumulation in chewing herbivore elicited plants (Supplementary Fig. S2A), although its expression was promoted by ethylene (Supplementary Fig. S6A), and thus consistent with the role of OsEIL1 and ethylene as positive regulators of OsLOX9 in rice. Overall, it seems that ethylene regulates VOC production, both positively and negatively, depending on feeding guild of attacking herbivore. Our study also reveals that

experimental conditions, and especially plant age must be considered when studying the JA-ethylene crosstalk in rice (Fig. 4.2). Furthermore, rice plants contain multiple ACS (5) and ACO (6) isoforms (Thi et al., 2012), and two differentially localized/expressed ETR receptors, OsERF2 and OsERS1 (Yu et al., 2017) with variable expression patterns in rice (Iwai et al., 2006). Therefore, response to chewing and sucking insects could also involve different spatiotemporal activation of enzymes in ethylene pathway, resulting in complex patterns, and stress-adjustable plant responses. We recently found that rice plants react defensively to bacterial symbionts in BPH honeydew that is deposited on leaves during feeding (Wari et al., 2019). Thus, BPH feeding may trigger pathogen-related genes in ethylene biosynthesis (Yang et al., 2017), such as pathogen-inducible *OsACS1* and *OsACO1* (Iwai et al., 2006), and further modulate SA/JA signaling in rice during BPH attack.

3.4. Ethylene functions as context-dependent regulator of VOCs in rice

While involvement of ethylene signaling in anti-herbivore responses remains complex, we focused on basal rice physiology to emphasize importance of ethylene in rice. For example, the over-accumulation of certain VOCs caused internal damage in petunia flower cells, when the release was blocked by mutation in a specific ABC transporter, PhABCG1 (Adebesin et al., 2017). Similar scenario could occur in submerged rice leaves that, presumably, cannot release hydrophobic hydrocarbons (e.g., terpenes) to environment. Additionally, VOC attractants of natural enemies, and natural enemies themselves, cannot function in submerged rice, thus making the production and release of VOCs undesirable/irrelevant. Along with this hypothesis, production of rice VOCs was blocked by flooding stress, presumably as a result of flood-induced ethylene (Kende et al., 1998; Kuroha et al., 2018; Fig. 4.6C). In contrast to rice, partial flooding with the waterline 1 cm over soil level caused elevation of SA biosynthesis in maize seedlings that increased production of the anti-insect C-glycosyl flavones (maysins) and herbivore-induced volatile phenolics, benzyl acetate, and phenethyl acetate under combined stress, flooding and herbivory (Block et al., 2019). In another example of physiologically meaningful roles, ethylene blocked production of VOCs in rice plants older than 8 weeks. It has been shown that leaves of mature rice are rarely attacked by chewing herbivores in the field, which was, at least in part, due to silicon-impregnated non-glandular trichomes

abundantly present on rice leaves (Andama et al., 2020). Therefore, rice plants can fend-off most of the chewing herbivores by mechanical defense at mature stage, and therefore, may be conserving all affordable resources for reproduction and seed development, on account of defensive VOC production and release, which becomes repressed by ethylene.

Chapter 5 General discussion

1. Discussion

Rice plants are C3 crops capable of photosynthesis (Karki et al., 2013) and production of primary metabolites. Diverse secondary metabolite compounds are also produced as by-products of photosynthesis (Wang et al., 2018). Secondary metabolites play important roles in plant growth and development, especially they function in biotic and abiotic stress responses, known as plant immunity (Erb, 2018) and defense against herbivores (Andama et al., 2020). More than 276 secondary metabolites have been identified in rice plant (Wang et al., 2018), including VOCs. One of the major functions of VOC in defense against herbivores is their role in indirect defense, by recruiting natural enemies of herbivores. The production of VOCs in rice plants is regulated by jasmonic acid, and in *hebiba* (*OsAOC* mutated plant), production of VOCs including GLV was almost completely suppressed. It proves that jasmonic acid hormone is required for chemical plant defense that includes volatile compounds. Furthermore, HIPV were specifically dependent on JA-Ile, as showed in *Osjar1* mutant, and production of induced volatiles was significantly suppressed in this mutant.

Headspace volatile (hsVOC) and internal volatile (inVOC) analysis revealed at least 81 volatiles compounds that could be successfully detected from leaves of rice plants. Among them, HIPVs compounds such as linalool, nerolidol, and geranyllinalool, were synchronously induced by WOS (20 µl *M. loreyi* oral secretion) treatment. Linalool is a monoterpene volatile known to be highly attractive to parasitic wasps (Yuan et al., 2008), fig wasps, fungus gnats and moths (Raguso, 2016), and increase egg predation rate (Dicke & Baldwin, 2010). Linalool was found to be released from treated plants in the hsVOC analysis, but it was equally stored in the leaf tissue, as observed in the inVOC analysis. Sesquiterpene nerolidol and diterpene geranyllinalool, although production was induced by WOS treatment, these two compounds were not released by rice plants. Accumulation of these two induced compounds only occurred inside of the leaf tissue. Compared to linalool ($C_{10}H_{18}O$), nerolidol ($C_{15}H_{26}O$) and geranyllinalool ($C_{20}H_{34}O$) have much higher molecular weight, and it can be predicted that are not easily released. According to Arimura et al., (2005), biosynthesis of anti-herbivore (E)-4,8-dimethyl-1,3,7-nonatriene

(DMNT) and TMTT proceeds via oxidative degradation by P450 enzymes of sesquiterpene (E)- nerolidol and the diterpene geranyllinalool as precursors. In addition to HIPVs, GLVs were induced by WOS and released immediately after wounding. GLVs are indeed known as active signals in plant defense, and GLVs are also rapidly released upon mechanical wounding. Other compounds detected in hsVOC and inVOC analysis were diurnal compounds. The emission of these compounds was not dependent on JA-Ile, and emission pattern or production followed the rhythm of light. These compounds are composed of several monoterpenes, and most are sesquiterpenes or diterpenes. Oscillation of these compounds is generally high in the light phase and decreases in the dark phase. At gene level, qPCR analysis of ethylene and jasmonic acid biosynthesis/signaling genes suggested that the production of VOC in rice plants is a manifestation of complex jasmonic acid-ethylene crosstalk, involving diverse genes. Therefore, further investigations are needed to reveal how each volatile is produced and released by plants.

As described above, production of volatiles in rice plants is under control of complex crosstalk between jasmonic acid and ethylene. Ethylene is a well-known regulator of plant responses to stress. From the investigation of ontogenetic aspects of rice volatile production, using 6 to 10 week-old plants, HIPVs production was low in the young stage at 6 weeks, gradually increased and reach maximum level at 8 weeks, and then decreased in the maturity stage. Interestingly, JA-Ile levels were similar to HIPVs, with maximum production at 8-week plants. Conversely, lowest production of ethylene was observed in 8-week plants and it was high at 10 weeks. It suggested that jasmonates work as positive regulators of rice volatile production, while ethylene may act as suppressor. The exogenous ethylene application showed that production of HIPVs was reduced, confirming the initial hypothesis that ethylene negatively regulates HIPV production. In rice plants, ethylene is known as plant growth regulator, especially promoting elongation in young seedling in order to respond to flooding stress. Investigation to this function confirmed that 1 ppm ethylene application can promote internode elongation at 6 weeks within 24 h. As low as 1 ppm levels of ethylene could then block linalool biosynthesis, as well as this level of ethylene was able to suppress transcription of linalool synthase gene in rice. The ethylene showed a broad negative effect on several other known genes involved in VOC production, such as SAMT in methyl salicylate, HPL3 in green leaf

volatiles, and CAS in caryophyllene production. As these results were rather unexpected, we searched for specific ecological situations where ethylene suppression of volatiles would represent an advantage to rice plants. As young rice plants grow in water, and can be subjected to occasional flooding, we proposed that shut down of volatile production in flooded leaves could be a preventive measure against over accumulation of volatiles that cannot be released from rice plants. Because flooding triggers ethylene that activates elongation od seedlings, and escape from stress, the second function of ethylene could be suppression of volatile production. Indeed, flooding was able to suppress volatiles in rice leaves subjected to simulated herbivory. Accordingly, VOC biosynthesis genes were suppressed in the leaves under water, functionally connecting the ethylene-mediated transcriptional repression and actual volatile metabolic responses.

Overall, jasmonates and ethylene play important regulatory roles in control and production of volatile organic compounds in rice. However, different classes of volatiles show various patterns of regulation, emission and accumulation, showing true complexity in plant regulation, and need for further research to fully understand the role volatile compounds in plants.

2. Conclusion

A comprehensive study on mechanisms involved in control of volatile production in rice was conducted. Such knowledge will be particularly useful in the future design of natural protection measures for control of herbivore damage in crops, based on environment-friendly principles of integrated pest management (IPM) approach.

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Supplemental Figures

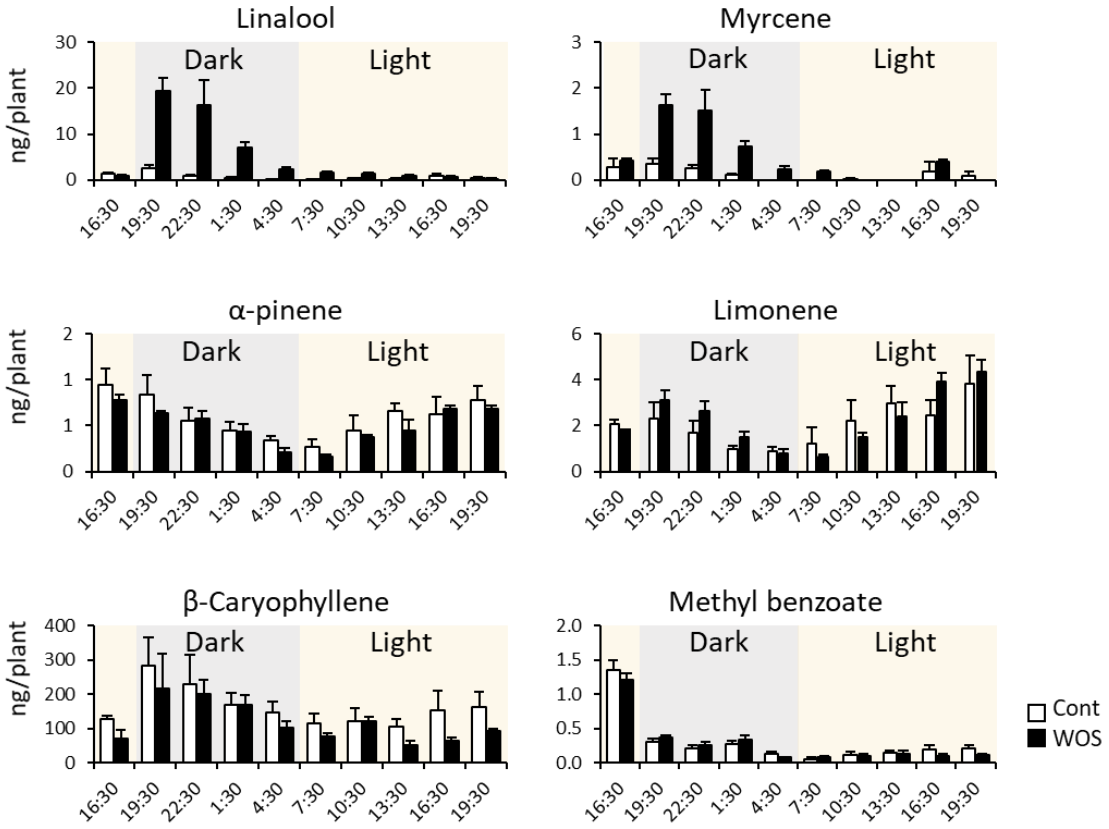

Fig. S1. Diurnal oscillation of VOCs in rice plants. Monoterpene linalool, myrcene, α-pinene, and limonene, sesquiterpene β-caryophyllene, and aromatic methyl benzoate compounds were determined by GC-MS, released volatiles from intact plants were collected by headspace (n=4). Plants were induced at 3 pm, then samples were taken consecutively every three hours. Cont, control; WOS, wounding followed by application of 20 μL diluted *M. loreyi* oral secretions.

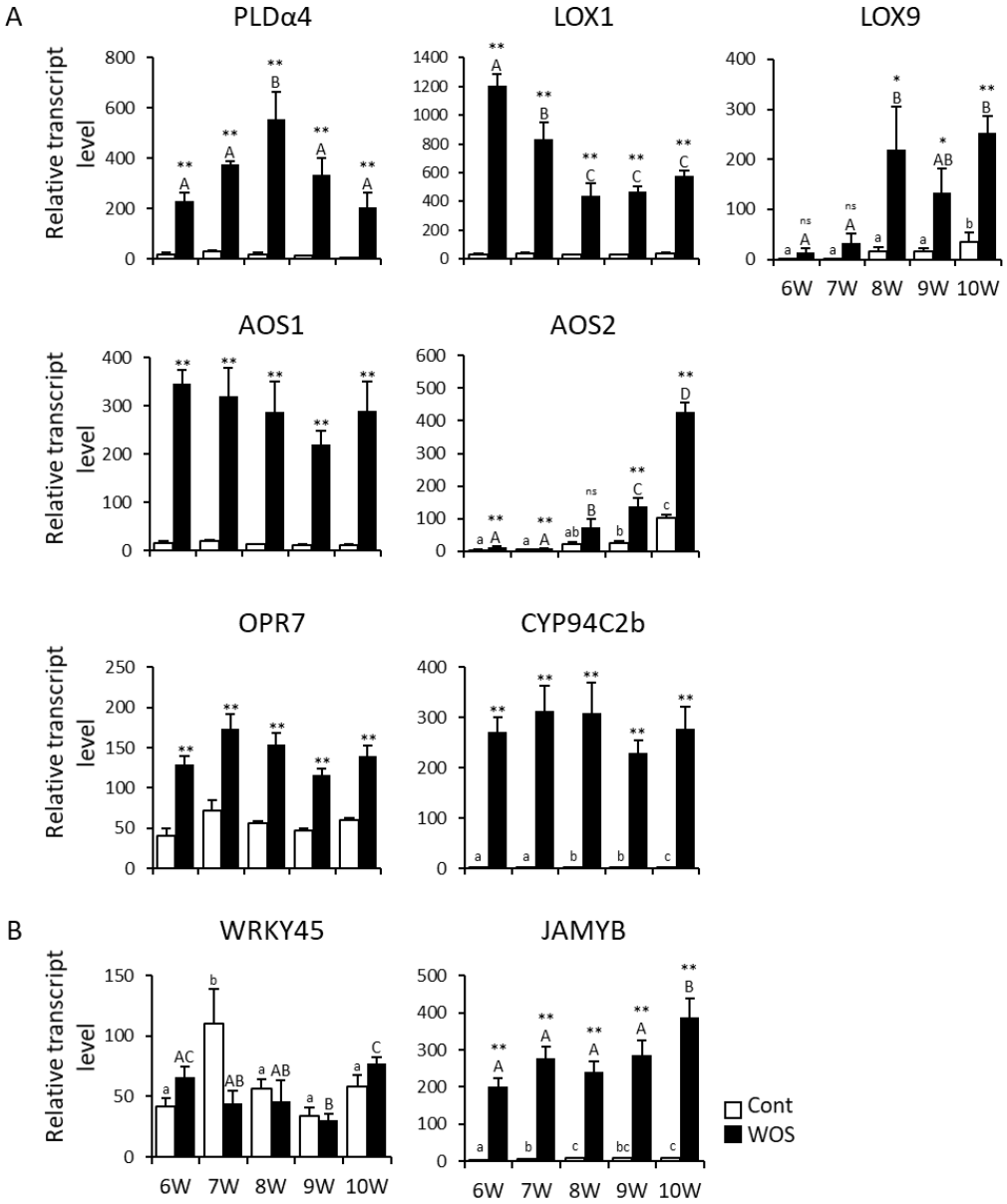

Fig. S2. Relative constitutive and induced transcript levels of additional jasmonate biosynthesis and signaling-related genes in rice plants at different developmental stages. Gene expression was determined by qRT-PCR in rice leaves of 6-10-week-old plants before, and 1 h after WOS elicitation. Induction of (A) JA metabolism-related genes (*OsPLDα4*, *OsLOX1*, *OsLOX9*, *OsAOS1*, *OsAOS2*, *OsOPR7*, *OsCYP94C2b*), and (B) rice signaling components (*OsWRKY45*, *OsJAMYB*). *OsAOC* is shown in main Figure 3. Data (n = 5) are shown as means ± SE; Cont, control; WOS, Wounding followed by application of 20 µL diluted *M. loreyi* oral secretions. Statistical differences between pairs of control (Cont) and WOS-treated plants at each age were analyzed by Student's t-test (*P < 0.05; ** P < 0.01; ns, not significant). Different letters (a-c, or A-D) indicate significant differences (p ≤ 0.05) between different plant age determined separately for each group (Cont or WOS) by one-way ANOVA followed by Fisher's LSD test. W, weeks old.

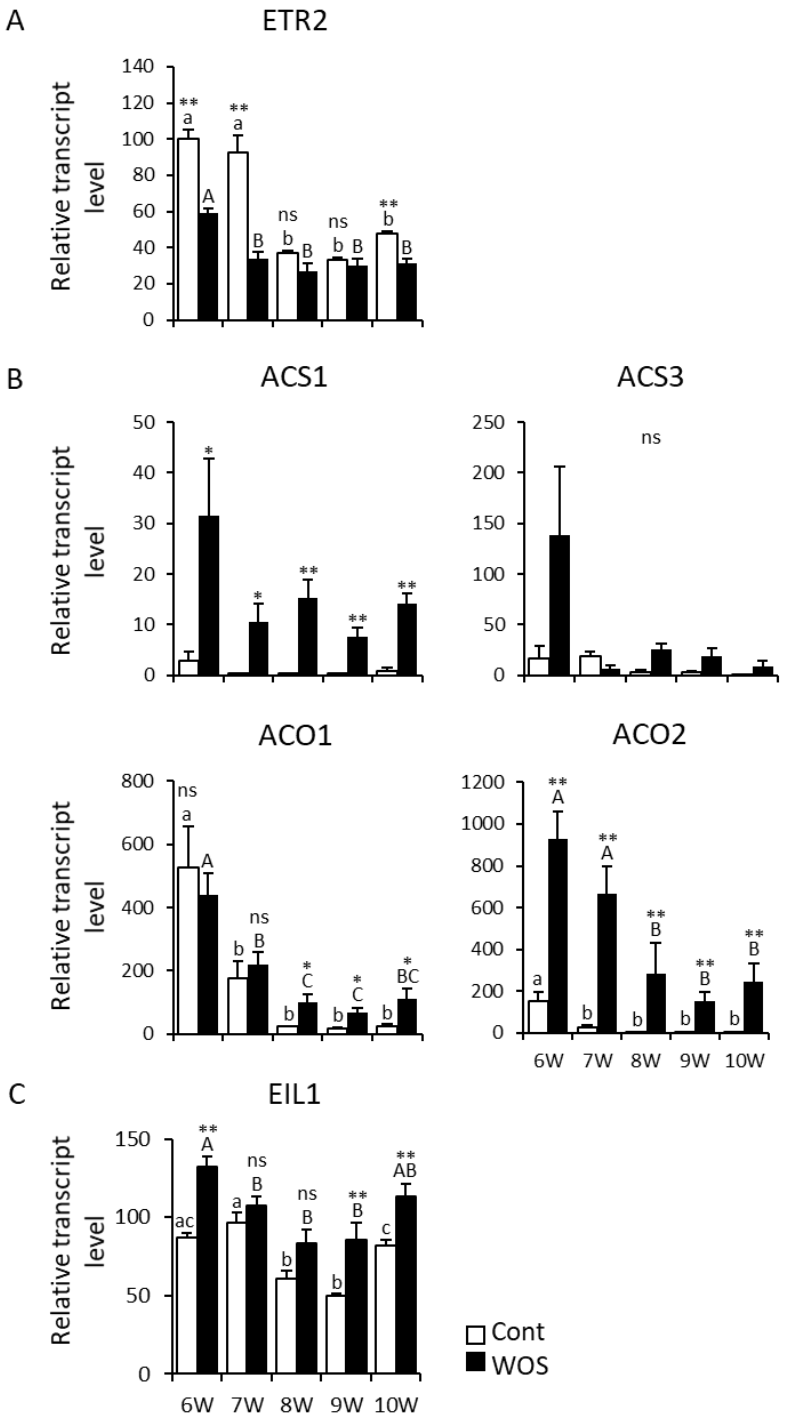

Fig. S3. Relative constitutive and induced transcript levels of ethylene biosynthesis and signaling-related genes in rice plants at different developmental stages. Gene expression was determined by qRT-PCR in rice leaves of 6-10-week-old plants before, and 1 h after WOS elicitation. Induction of (A) ethylene receptor (*OsETR2*), (B) ethylene biosynthesis genes (*OsACS1*, *OsACS3*, *OsACO1*, *OsACO2*), and (C) ethylene signaling gene (*OsEIL1*). Data (n = 5) are shown as means ± SE; Cont, control; WOS, Wounding followed by application of 20 µL diluted *M. loreyi* oral secretions. Statistical differences between pairs of control (C) and WOS-treated plants at each age were analyzed by Student's t-test (*P < 0.05; ** P < 0.01; ns, not significant). Different letters (a-c, or A-C) indicate significant differences ($p \leq 0.05$) between different plant age determined separately for each group (Cont or WOS) by one-way ANOVA followed by Fisher's LSD test. W, weeks old.

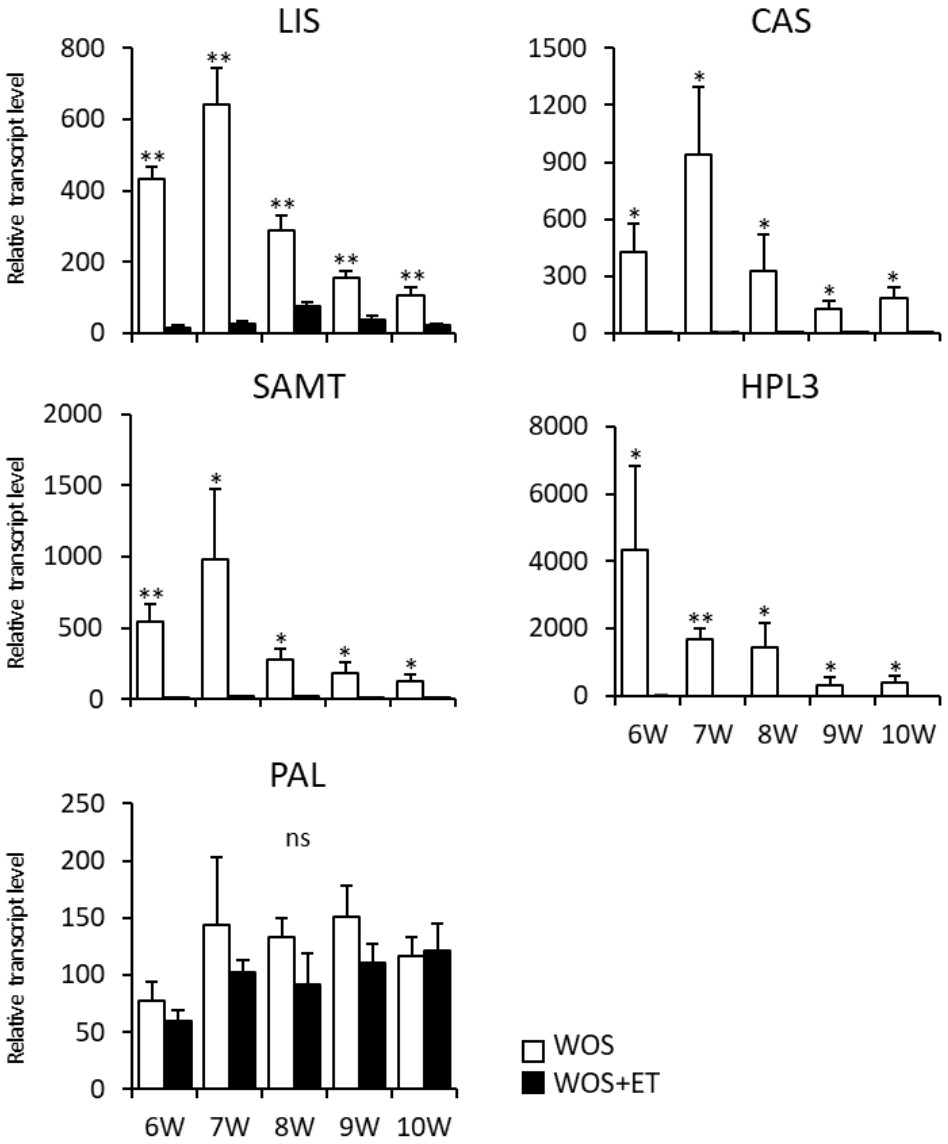

Fig. S4. Effect of ethylene on selected set of VOC biosynthesis-related transcription in rice at 4 h post treatment in differently aged rice. Gene expression was determined by qRT-PCR in rice leaves of 6-10-week-old plants after WOS elicitation complemented with ethylene (WOS+ET), or without ethylene (WOS). Induction of VOC biosynthesis (*OsLIS*, *OsCAS*, *OsSAMT*, *OsHPL3*, and *OsPAL*) genes. Data (n = 5) is shown as means ± SE; WOS, Wounding followed by application of 20 µL diluted *M. loreyi* oral secretions. Statistical differences between pairs of WOS and WOS+ET-treated plants at each age were analyzed by Student's t-test (*P < 0.05; ** P < 0.01; ns, not significant). W, weeks old.

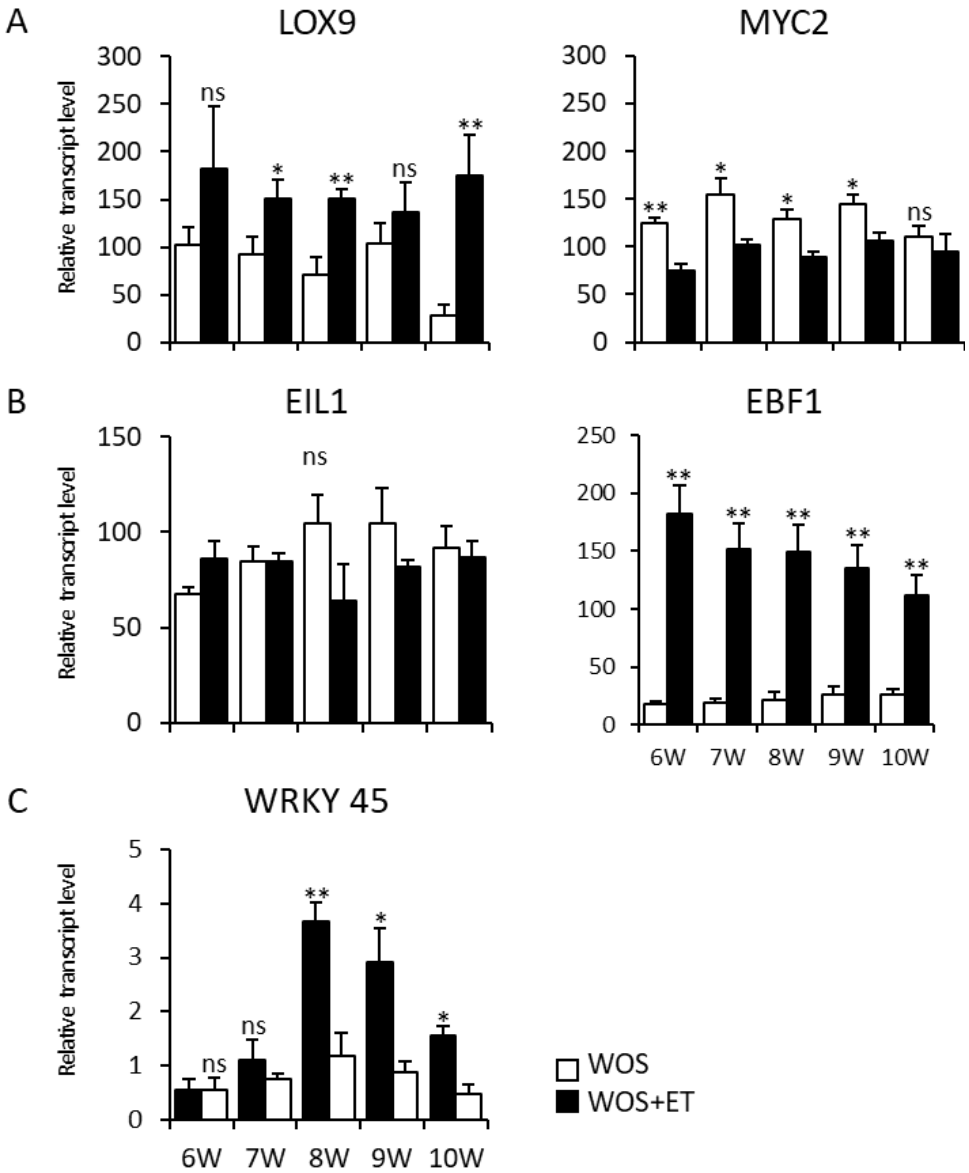

Fig. S5. Effect of ethylene on selected set of jasmonate/ethylene biosynthesis and signaling-related transcripts in rice at 4 h post treatment in differently aged rice. Gene expression was determined by qRT-PCR in rice leaves of 6-10-week-old plants after WOS elicitation complemented with 1 ppm ethylene (WOS+ET), or without ethylene (WOS). (A) Induction of JA biosynthesis (*OsLOX9*) and signaling–related (*OsMYC2*) genes, (B) ethylene signaling (*OsEIL1*) and response (*OsEBF1*) genes, and (C) rice defense signaling-related gene (*OsWRKY45*). Data (n = 5) are shown as means ± SE; WOS, Wounding followed by application of 20 µL diluted *M. loreyi* oral secretions. Statistical differences between pairs of WOS and WOS+ET-treated plants at each age were analyzed by Student's t-test (*P < 0.05; ** P < 0.01; ns, not significant). W, weeks old.

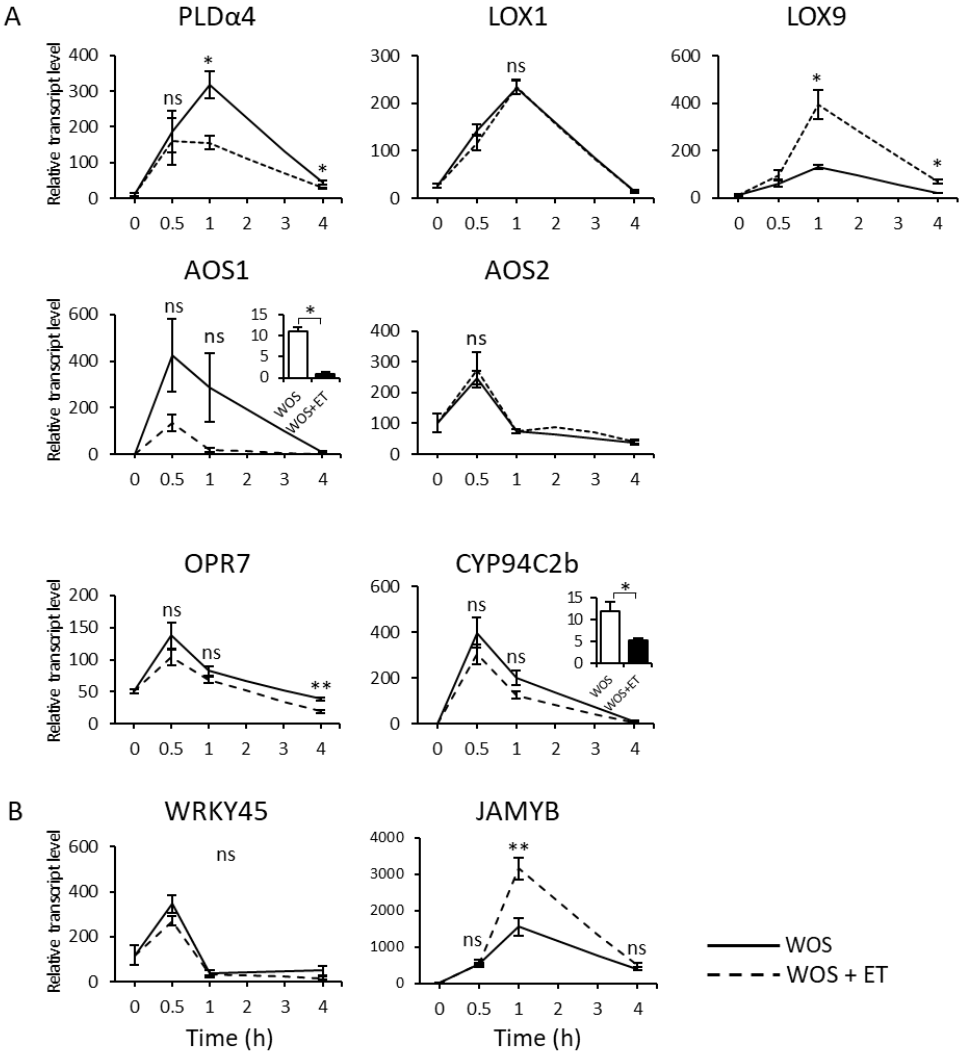

Fig. S6. Effect of ethylene on jasmonate biosynthesis and signaling-related transcripts in rice. Leaf samples were collected before and 0.5, 1, and 4 h after WOS treatment from plants kept in the presence (WOS+ET, dashed line) and absence (WOS, solid line) of ethylene (1 ppm). (A) relative transcript levels of JA metabolic genes (*OsPLDα4*, *OsLOX1*, *OsLOX9*, *OsAOS1*, *OsAOS2*, *OsOPR7*, *OsCYP94C2b*), and (B) rice signaling components (*OsWRKY45*, *OsJAMYB*). *OsAOC* is shown in main Figure 5. Data (n = 4) are shown as means ± SE. Statistical differences between treatments were analyzed at each time point by Student's t-test (*$P < 0.05$; ** $P < 0.01$, ns, not significant).

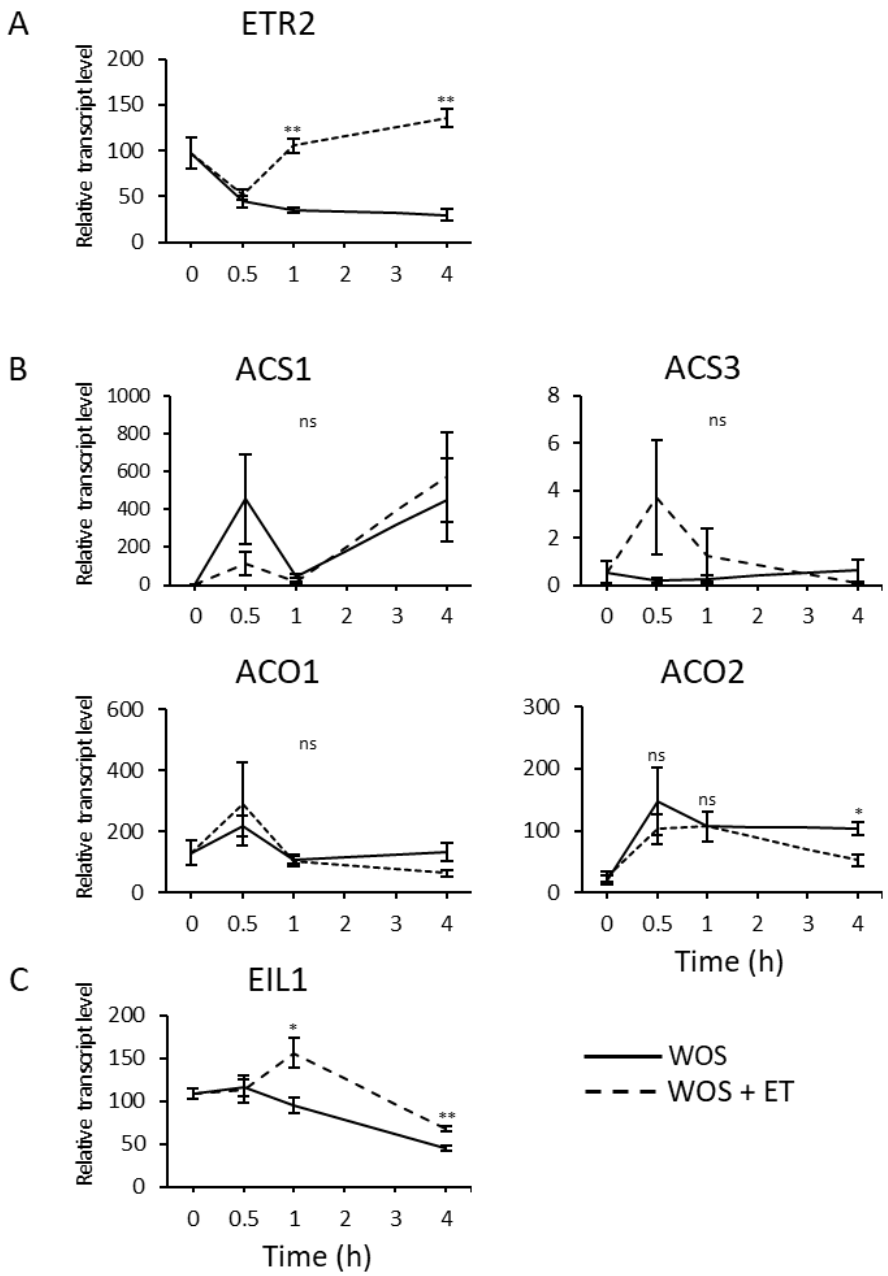

Fig. S7. Effect of ethylene on ethylene biosynthesis and signaling-related transcripts in rice. Leaf samples were collected before and 0.5, 1, and 4 h after WOS treatment from plants kept in the presence (WOS+ET, dashed line) and absence (WOS, solid line) of ethylene (1 ppm). (A) ethylene receptor (*OsETR2*), (B) ethylene biosynthesis genes (*OsACS1*, *OsACS3*, *OsACO1*, *OsACO2*), and (C) ethylene signaling gene (*OsEIL1*). Transcripts of ethylene response gene *OsEBF1* are shown in Figure 5. Data (n = 4) are shown as means ± SE. Statistical differences between treatments were analyzed at each time point by Student's t-test (**P* < 0.05; ** *P* < 0.01; ns, not significant).

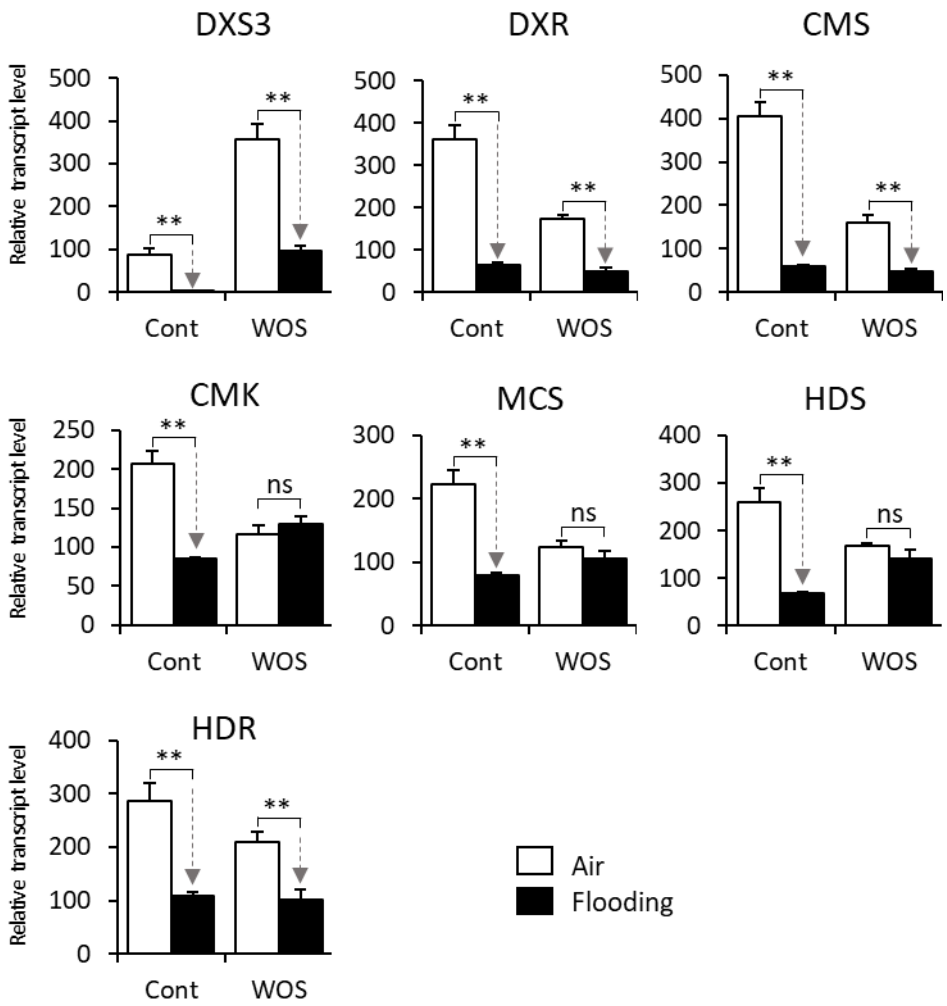

Fig. S8. Effect of water submergence on MEP pathway genes in rice. Rice plants at 7 weeks were submerged in water for 24 h (Flooding), or kept without water (Air), and subsequently treated with WOS, or left untreated (Cont). Samples were collected 1 h after last treatment, and analyzed by qRT-PCR. Methylerythritol 4-phosphate (MEP) pathway-related genes: *OsDXS3* (also shown in Fig. 7A), *1-DEOXY-D-XYLULOSE 5-PHOSPHATE REDUCTOISOMERASE* (*OsDXR*), *4-(CYTIDINE 5'-DIPHOSPHO)-2-C-METHYL-D-ERYTHRITOL SYNTHASE* (*OsCMS*), *4-DIPHOSPHOCYTIDYL-2-C-METHYL-D-ERYTHRITOL KINASE* (*OsCMK*), *2-C-METHYL-D-ERYTHRITOL 2,4-CYCLODIPHOSPHATE SYNTHASE* (*OsMCS*), *1-HYDROXY-2-METHYL-2-(E)-BUTENYL 4-DIPHOSPHATE SYNTHASE* (*OsHDS*), and *1-HYDROXY-2-METHYL-2-(E)-BUTENYL 4-DIPHOSPHATE REDUCTASE* (*OsHDR*). Data (n = 6) are shown as means ± SE. Statistical differences between pairs of flooded and air samples were analyzed by Student's t-test (** $P < 0.01$; ns, not significant).

Supplementary Table 1

Oligonucleotide primers used in the study

Oligonucleotide primers	RAP-DB	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
1-DEOXY-D-XYLULOSE 5-PHOSPHATE REDUCTOISOMERASE (*OsDXR*)	*Os01g0106900*	GCTCCATGCATAGTCAGCAG	GCACGGACGAACGATTTATT
4-DIPHOSPHOCYTIDYL-2-C-METHYL-D-ERYTHRITOL KINASE (*OsCMK*)	*Os01g0802100*	GGAAAAGAACCATTCCACCTT	TGTCACTTGGTATGCCCGTA
JA-REGULATED MYB TRANSCRIPTION FACTOR (*OsJAMYB*)	*Os01g0853400*	CTGGTGTAACAATGGCCACTG	TCAAATCTTGTGACTCCGACG
4-(CYTIDINE 5'-DIPHOSPHO)-2-C-METHYL-D-ERYTHRITOL SYNTHASE (*OsCMS*)	*Os01g0887100*	ACGGGATGGACTTGAGGTCA	TTATTTCTCATTCATCAGGCG
HYDOPEROXIDE LYASE 3 (*OsHPL3*)	*Os02g0110200*	AAACAGTGTGCCGCCAAG	TGTGCATGGAGATCGAGTGA
LINALOOL SYNTHASE (*OsLIS*)	*Os02g0121700*	CCAGGATGGTCGGCGTCATG	CACGCCATTATGCATGGACGATG
1-HYDROXY-2-METHYL-2-(E)-BUTENYL 4-DIPHOSPHATE SYNTHASE (*OsHDS*)	*Os02g0603800*	TCGACCTTTATGTTGGCAAG	CATGCTACGGCCTACTCCTC
PHENYLALANINE AMMONIA-LYASE (*OsPAL*)	*Os02g0626600*	CTACCCGCTGATGAAGAAGC	GCACCTTGTTCAGCTCCTCG
2-C-METHYL-D-ERYTHRITOL 2,4-CYCLODIPHOSPHATE SYNTHASE (*OsMCS*)	*Os02g0680600*	CCCAAAATTAGCCCATTCAA	GTCGACTTTCTCGTGCGTCT
S-ADENOSYL-L-METHIONINE:SALICYLIC ACID CARBOXYL METHYLTRANSFERASE (*OsSAMT*)	*Os02g0719600*	CTCATCGCTCGTCATTTCGG	ACACACTGGCACGCTACTTA
ELONGATION FACTOR 1a	*Os03g0177900*	CTGCCACACCTCCCACATTGC	CCGCACGGCAAAACGACCA
JASMONATE ZIM-DOMAIN 9 (*OsJAZ9*)	*Os03g0180800*	GCCGGCGAAGAAAGCGAAGG	GCCGTTAATGCGACGAGAACCA
JASMONATE ZIM-DOMAIN 11 (*OsJAZ11*)	*Os03g0180900*	ACTGCCGGAGTCGGAGAAGAAG	ACGGTTCCACACTTTCCACAGAC
ALLENE OXIDE SYNTHASE 2 (*OsAOS2*)	*Os03g0225900*	CCTCCGCCGTCAATCGTA	GACGACCCACGCCTGAAGT
ETHYLENE INSENSITIVE3-LIKE1 (*OsEIL1*)	*Os03g0324300*	ACAATGCCACGATCATGGAG	TCAGTAGTACCAATTCGAGC
LIPOXYGENASE 1 (*OsLOX1*)	*Os03g0700700*	CCACATGTTATCAAAGAGGACAAATT	CGAGCATTTCTCGTGCAAAC
1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 1 (*OsACS1*)	*Os03g0727600*	TCGGCCAAGACCCTCGACG	CGAAAGGAATCTGCTACTGCTGC
1-HYDROXY-2-METHYL-2-(E)-BUTENYL 4-DIPHOSPHATE REDUCTASE (*OsHDR*)	*Os03g0731900*	CTGATGGCTTGGTGAAGGTT	CAGCACATGCCGTAGTATGC
ALLENE OXIDE SYNTHASE 1 (*OsAOS1*)	*Os03g0767000*	GCCCGGTCATCTTATTTTCC	ACCAGTGCAACTCCGTATCC
ETHYLENE RESPONSE 2 (*OsETR2*)	*Os04g0169100*	TGCTTGCCCTTCAGTTCCA	GTCCGAGGATGCTCCAGAGA
1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 3 (*OsACS3*)	*Os05g0196600*	GCTGCTTCATCAAGAAATGGGAGC	GATGGAGCCATAGAGGGAGC
TRANSCRIPTION FACTOR WRKY45 (*OsWRKY45*)	*Os05g0322900*	GCCGACGACCAGCACGATCACC	ACGAGCCGACGCCGCCCTC
JASMONATE RESISTANT 1 (*OsJAR1*)	*Os05g0586200*	CTTCATTGACGCAGGCTACA	CACCACCAAGGCTTAGGAAA
PHOSPHOLIPASE Dα4 (*OsPLDα4*)	*Os06g0604200*	GCTTGCAAGAGGTCAAATCCA	GCGGTTCGCCTCCTAGGT
EIN3-BINDING F-BOX PROTEIN 1 gene (*OsEBF1*)	*Os06g0605900*	CGCGAAGCTGCAGTCGGTGA	GACTCCCGGGCAGGAGCTGA
1-DEOXY-D-XYLULOSE 5-PHOSPHATE SYNTHASE 3 (*OsDXS3*)	*Os07g0190000*	GGGGGAGGTTCCAGTAAGAA	TCATTTTGCATTTGGAAGCA
CARYOPHYLLENE SYNTHASE (*OsCAS*)	*Os08g0139700*	ATCGCCGGGGAGTGTCTC	GGAGTGTATTGTATCCTTGAGCG