# Study on the role of mineral elements in rice defense against herbivores

# 植食性昆虫に対するイネの防御機構におけるミネラルの役割の研究

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**DANDY AHAMEFULA OSIBE**

**77D21753**

Graduate School of Environmental and Life Sciences

(Doctor’s course)

OKAYAMA UNIVERSITY

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# Abstract

To defend against herbivore attack, plants have evolved a plethora of diverse and dynamic defense systems including several inherent morphological traits, such as trichomes, waxy cuticles, cell wall thickening or lignification, and also the production of numerous chemical compounds which collectively are either toxic or deter herbivores from feeding on plants. In addition to the production of these defensive structures, some plants can also hyperaccumulate certain minerals from the soil which may play some important defensive roles against insect attack. Apparently, as a defense strategy, plants under herbivore attack may recruit or re-direct mineral nutrients to the attacked tissues to aid in direct defense and/or the production of defensive metabolites. Mineral nutrients could also be reallocated away from the infested tissues to possibly starve the insect herbivores of its nutritional requirements. However, the underlying mechanisms on how plants recruit, reallocate and use mineral resources for defense when attacked by insect herbivores are not well understood. Consequently, focusing on rice plants (*Oryza sativa*), the primary objective of my thesis was to comprehensively analyze the mineral associated changes in rice anti-herbivore defense responses. Specifically, I aimed to grasp detailed understanding of which mineral elements are preferentially recruited by plants under herbivore attack and how these recruited minerals are used by plants to resist insect damage. In practical outcome, information about the dynamics of mineral use in herbivore stressed plants would be key in designing future herbivore-resilient crops through optimized mineral supply (smarter fertilizer applications).

Taking advantage of the available outstanding technical resources for minerals and rice-insect pest research (ICP-MS, EDX, LC-MS, GC-MS, etc.) available in IPSR Okayama University, I investigated the intrinsic role(s) of minerals in rice resistance to insect herbivory. For the first part of my PhD thesis, I attempted to construct a spatiotemporal map of mineral use during anti-herbivore defense in rice through detailed analysis of major mineral changes associated with herbivore attack. For this, rice plants grown hydroponically were subjected to herbivory treatment. Thereafter, rice tissues from herbivore-treated and untreated control plants were collected along spatial (different tissues) and temporal (different time) axes, in other words, proximal (locally-fed leaf) and

distal to feeding area (systemic leaves, stem, and roots); and at early (h) and late (day) time points after herbivore infestation and the mineral levels analyzed using ICP-MS. Mineral analyses revealed that the accumulation patterns of 20 elements in rice plants subjected to herbivory stress varied both spatially and temporally. For instance, relative to control, herbivory induced the differential accumulation of several mineral elements in the leaves while levels in the root tissues were not largely affected after 24 h exposure to herbivory stress. When the temporal accumulation of minerals at earlier time points after herbivore infestation was examined, the accumulation pattern of some macro elements (e.g., magnesium and phosphorus) in the locally attacked and old systemic leaves did not change relative to control even after 10 h of insect feeding. In contrast, significantly higher levels of micro elements (e.g., nickel and cobalt) accumulated in both locally attacked leaves and systemic old leaves just after 3 h of exposure to herbivore attack. Collectively, these results suggest that redistribution in the leaves rather than additional uptake of mineral elements in the roots may be regulated by herbivory stress in rice.

In the second part of my thesis, I leveraged on the well characterized silicon (Si) mineral transport and accumulation mechanisms in previous studies in rice to model the functional role of minerals in rice anti-herbivore defense outputs. To this end, I comprehensively evaluated the defensive and metabolic roles of exogenous Si in rice plants subjected to herbivory stress. To begin with, the distribution of Si in rice leaves, including local and systemic leaves when exposed to herbivore attack was explored using short-term Si uptake experiments in hydroponic system. Generally, Si-supplemented rice plants accumulated higher levels of Si when exposed to herbivory stress for 24 h compared to unexposed controls, although only the locally exposed leaf accumulated significantly higher levels of Si. Next, I asked whether the observed differences in Si accumulation in herbivore exposed rice plants relative to the unexposed control may be due to the transcriptional regulation of Si transporter genes by herbivory stress. Transcript levels of genes involved in Si uptake (*Lsi1* and *Lsi2*) and distribution (*Lsi6* and *SIET4*) in rice plants untreated or subjected to herbivory were analyzed. While *Lsi1* and *Lsi2* were only transiently induced in the roots, *Lsi6* and *SIET4* were clearly upregulated after herbivory treatments suggesting that distribution rather than additional uptake of Si may be regulated by herbivory stress in rice plant possibly to mount effective defense. To identify the possible metabolic sinks for Si during rice anti-herbivore defense, I analyzed the

accumulation of several primary and secondary metabolites including volatile organic compounds (VOCs) in Si-supplemented and Si-deprived rice plants exposed to herbivory stress. Intriguingly, herbivore feeding altered rice primary metabolism in an Si-dependent manner. However, leaf photosynthetic activity and chlorophyll contents could not explain the observed Si-associated primary metabolic changes during herbivory stress in rice. I next examined the accumulation of defense-related secondary metabolites in Si-supplied and Si-deprived rice plants exposed to herbivory stress. Overall, I detected more direct-defense related metabolites in Si-supplied relative to Si-deprived rice leaves after exposure to herbivore treatments. Taking into account these changes in direct defense-related metabolites levels in rice after Si supplementation, I decided to test whether Si can also modify the emission of VOCs which usually function in indirect defense in rice under herbivory stress. After herbivory treatment, Si-supplemented plants emitted higher quantities of several VOCs relative to Si-deprived plants, whereas the internal contents of the volatiles were largely unaffected by Si supply. These data suggest that volatiles in rice headspace can be promoted by Si either through facilitating higher release and/or the biosynthesis of these compounds. Indeed, the increased transcripts of key genes putatively associated with VOCs biosynthesis in Si-supplied compared to the Si-deprived rice plants after herbivory stress provided support for the observed higher levels of some VOCs in Si-supplemented rice headspace. Surprisingly, the accumulation of jasmonates, a key regulator of specialized metabolism and VOC release under herbivory stress, was not largely affected by Si supply in rice. Finally, I performed series of bioassays with either insect larvae or gravid female moths to assess the overall practical importance of Si in rice resistance to herbivores. In herbivore performance assays, larvae fed on rice plants grown without Si increased their mass, whereas larvae fed on Si-supplemented rice plants retained on average a lower mass relative to the initial conditions, corroborating a previously known defensive role of Si against chewing herbivores. In oviposition choice assays with gravid female moths, Si supply to rice plants alone did not affect the oviposition choice of the moths. In contrast, gravid female moths showed significant preference for herbivory-treated/Si-deprived compared to Si-supplemented plants. Preference for herbivory-treated/Si-deprived rice plants which generally released less volatiles may suggest that gravid moths may be selecting host plants based on the lower amounts of volatiles which probably indicates lower defense capacity of the host to herbivores.

Taken together, my thesis provided a comprehensive analysis of global mineral changes associated with rice anti-herbivore defense responses. Using Si as a model mineral element, I further deepened the understanding of defensive and metabolic roles, and ecological implications of minerals in rice challenged with insect herbivores. Collectively, my thesis provides exciting new opportunities from studying minerals as essential components for growth and development to including minerals as key players in plant defense systems against biotic stresses including herbivore and pathogen attacks.

# Chapter 1 General Introduction

## Overview of plant defense mechanisms against insect herbivores

Plants are often confronted with several (a)biotic stresses in their habitats, which can impose significant costs to their fitness. Because plants are sessile, they usually cannot escape from these stresses and must find a way to protect themselves to ensure survival (Mithöfer and Boland, 2012). Biotic stresses as a result of pathogen or insect attacks are a great threat to the productivity of many important crop species especially in certain compatible plant-organism combinations. For example, plant-herbivore interactions reportedly are essential determinants of crop productivity (Mitchell et al., 2016). Crop yield loss due to herbivore-associated damage is estimated to exceed 15% annually (Mitchell et al., 2016). Consequently, plants have evolved a plethora of strategies to either resist or tolerate herbivore attacks (Hanley et al., 2007).

To resist insect attack, plants have developed a diverse and dynamic defense systems which in principle can be broadly grouped into; (a) constitutive defense which are always present and (b) inducible defense which are usually activated when attacked by certain herbivores (Mithöfer and Boland, 2012). Each one of these defense strategies can either be direct or indirect.  In general, direct defense includes various structural traits such as trichomes, prickles, thorns, wax, cuticles, cell wall thickening or lignification, and chemical compounds including several classes of specialized metabolites produced by plants that collectively are either toxic to or deter insects from feeding on plants (War et al., 2012; Kaur and Kariyat, 2023). Indirect defense on the other hand is mediated by the release of certain volatile organic compounds (VOCs) by herbivore-attacked plants which can attract natural enemies of the feeding insect or may serve as host-location cues for ovipositing females of herbivores (Zhou and Jander, 2022; Achhami et al., 2021). Interestingly, many of these VOCs are only produced, when necessary, after plant damage, and their compositions may actually depend on the type of damage, for example mechanical injury, oviposition, or insect feeding (Hilker and Meiners, 2006; Mithöfer et al., 2005, Mithöfer and Boland, 2012).

In addition to the production of defensive structures, plants can also develop tolerance to herbivory through growth and certain compensatory physiological processes that help them withstand or recover from herbivory-associated damage (Koch et al., 2016; Mitchell et al., 2016). These plant tolerance traits to herbivory may include mobilizing and saving stored energy through carbon and nitrogen reallocation after insect attack, and the alteration in photosynthetic activity and growth (Strauss and Agrawal, 1999; Stowe et al., 2000; Schwachtje et al., 2006). Apparently, many of these plant tolerance mechanisms to herbivory involve significant changes in plant physiology usually requiring to some extent the synergistic interactions between primary metabolism, photosynthesis and defense responses (Koch et al., 2016). For instance, Wilson et al. (2011) demonstrated the differential regulation of nitrogen metabolism in plants exposed to aphid infestation which was due to the increased activities of nitrate reductase in aphid-infested plants relative to the un-infested plants. Seemingly, reallocation of resources is one of the key mechanisms with which plants tolerate attack by insects (Becker et al., 2022; Arias-Porras et al., 2023). For example, in *Nicotiana attenuata*, carbon and sugar were allocated to the less-vulnerable roots upon attack by the tobacco hornworm, *Manduca sexta* (Schwachtje et al., 2006). Similarly, rice infestation with the chewing herbivore *Cnaphalocis* *medinalis* Guenée induced the expression of sucrose transporter *OsSUT4* possibly to reallocate sucrose from the infested to unaffected tissues (Chang et al., 2019). Interestingly, belowground, Robert et al. (2014) demonstrated the induced reallocation of photoassimilates from source leaves to stems but not the roots, in maize plants infested by western corn rootworm *Daibrotica virgifera*. It was suggested that the allocation of photoassimilates to the shoots and not the roots, in root-attacked maize plants could be an important induced tolerance strategy to withstand belowground attack and also compensate for the loss of below ground tissues.

Insect herbivores performance and dynamics have been reported to depend on both the host plants defensive and nutritive traits (Wetzel et al., 2016; Kaspari et al., 2021). Wetzel et al. (2016), demonstrated how nutrient heterogeneity within plants contributes to the suppression of herbivore populations. Besides leveraging resource reallocation as tolerance strategy, plant can also allocate resources for the production of defense metabolites upon herbivore attack. Apparently, under herbivore attack, minerals must be re-directed for use in local defense to ensure plant survival (Figure 1). Previously, several N-containing defense compounds were shown to accumulate in herbivore-attacked rice (Alamgir et al., 2016; Aboshi et al., 2020). Several hypotheses including the carbon-nutrient balance, resource availability, optimal defense and mineral-balance had been proposed to describe the patterns of anti-herbivore defense compound production under different mineral nutrient regimes (Bryant et al., 1983; Coley et al., 1985; Rhoades, 1979; Phelan et al., 1996). For example, the mineral balance hypothesis predicts that optimal nutrient balance results to healthy plant growth, and better resistance to insect herbivores (Phelan et al., 1996). While the carbon-nutrient balance hypothesis posits that plant should allocate more to the production of N-containing defense metabolites when N is readily available and abundant (Mutikainen et al., 2000). However, these hypotheses offer limited explanations as to how plants recruit and remobilize all these necessary mineral resources for defense against herbivores and herbivore-triggered mechanisms for uptake of additional nutrients from the soil are not well known in rice.

## 1.2 Objectives of this study

The overall objective of this study was to comprehensively analyze the role of mineral elements in rice defense against herbivores. Using hydroponically grown rice plants, I aimed to evaluate the overall impact of mineral nutrition in rice anti-herbivore defense responses. Specifically, in Chapter 2, to identify major mineral changes associated with herbivore attack, I conducted a detailed analysis of the spatial and temporal dynamics of mineral ion accumulation in rice exposed to insect herbivory. Next in Chapter 3, I explored in detail the defensive and metabolic roles of Si, an important mineral in rice defense against herbivore attack in order to deepen our understanding on Si-mediated anti-herbivore defense response mechanisms in rice.

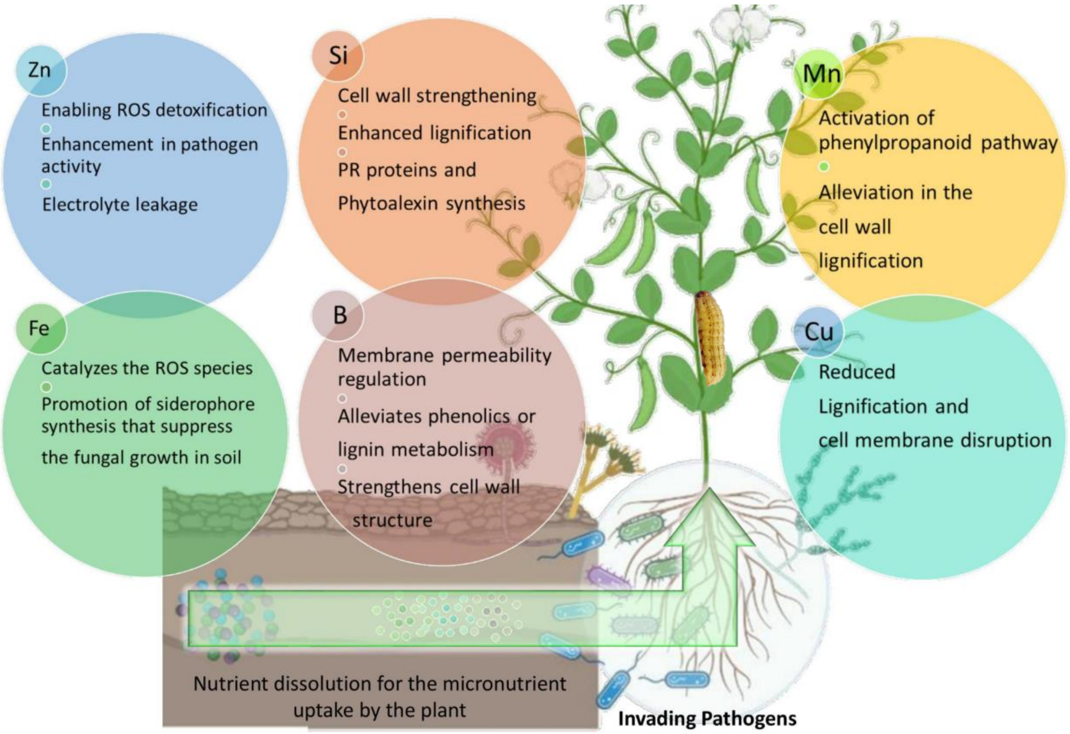


**Figure 1. Schematic overview of the biochemical and physiological roles of major micronutrients, adopted from Tripathi et al. (2022) with slight modification.** Some of the functions of these micronutrients in plants could have important implications in plant responses to biotic attack. Strengthening of the cell wall structures, enhancement of lignification and phytoalexins biosynthesis by boron and silicon, respectively may be beneficial in plant defense against chewing herbivores.

# Chapter 2: Mineral dynamics in rice exposed to herbivory stress

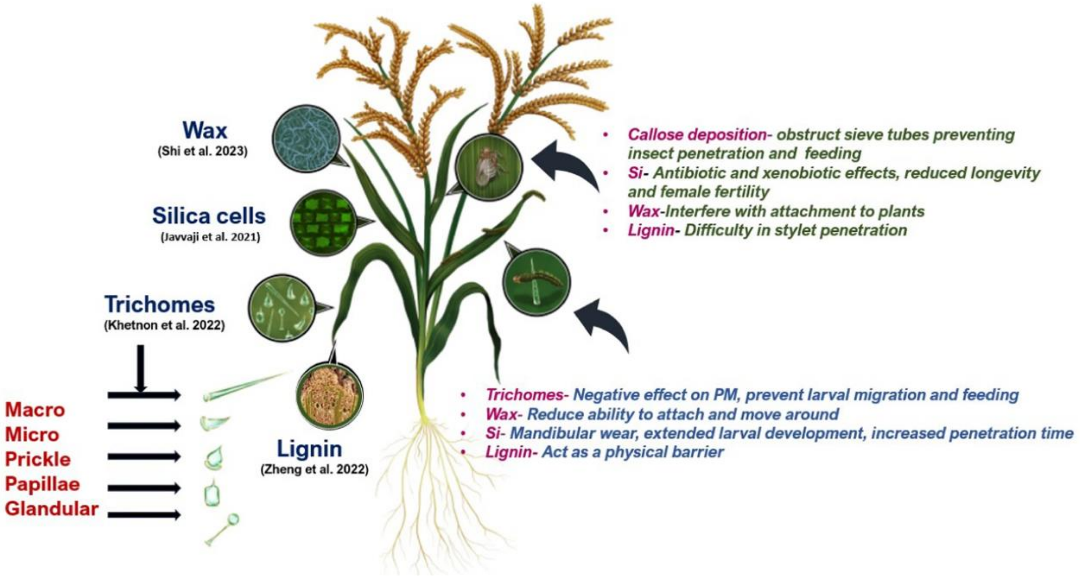
## Introduction

Rice is one of the most important staple crops in Asia and sub-Saharan Africa. Global annual milled rice production is estimated to be approximately 480 million metric tons with China and India accounting for up to 50% of rice grown and consumed (Muthayya et al., 2014). In Africa, rice production has also rapidly increased with Egypt, Nigeria and Madagascar leading the production chart (Bin Rahman and Zhang, 2023). Rice accounts for approximately 20% of the world's dietary energy supply with over 70% calorie supply in some Asian country's diets provided by rice (Bin Rahman and Zhang, 2023). Evidently, rice production is increasingly important in sustaining food security in a rapidly growing world's population. However, similar to other cultivated crops, rice is usually highly vulnerable to insect damage either from direct feeding or indirectly from viruses transmitted by insect vectors (Jiang et al., 2011; Wang et al., 2022). To cope with these challenges posed by insect herbivory, rice has evolved diverse array of defense strategies including direct physical (Balakrishnan et al., 2024) and chemical defenses (Zhao et al., 2023) as well as indirectly through the production of VOCs (Qi et al., 2018) to counteract insect attack.

Plant chemical defenses could be constitutive or induced upon insect attack and may include several chemical compounds such as protease inhibitors and wide variety of secondary metabolites which help plants resist herbivore attack. Many of these plant chemicals such as glucosinolates, alkaloids, terpenoids, phenolics and cyanogenic glycosides are toxic or antinutritive to non-adapted herbivores (Mithöfer and Boland, 2012). In rice, the accumulation of the phenolamides (PAs), *p*-coumaroylputrescine

(CoP) and feruloylputrescine (FP), have been reported to be induced in response to attack by both chewing and sucking insects and their defense functions clearly elucidated in BPH (Alamgir et al., 2016). Besides PAs, several other secondary metabolites such as isopentylamine (IPA) and momilactone diterpenes reportedly accumulate in rice upon attack by insect herbivores (Aboshi et al., 2021; Shinya et al., 2016). Furthermore, rice can also emit several VOCs after herbivore attack as a form of direct or indirect defense (Kuai and Lou, 2024; Aljbory and Chen, 2018).

Some morphological structures such as trichomes, cuticles, leaf surface wax and cell wall lignification function as physical barriers that act as first line of defense against herbivorous insects (Figure 2). In rice, trichome length, density, degree of hardness, form type and orientation may differ and even so among rice varieties (Xiao et al., 2017; Balakrishnan et al., 2024). Trichomes were reported to help rice resist the attack of rice leaf folder, *C. medinalis* by deterring the migration of the larvae and their ability to create feeding folds on the leaves (Punithavalli et al., 2013). More so, silicification of rice non-glandular trichomes was shown to be important in rice defense against chewing herbivores (Andama et al., 2020).  On the other hand, the density of prickle trichomes were not associated with insect injury level in rice infested by the brown plant hopper (BPH), *Nilaparvata lugens* (Khetnon et al., 2022). Furthermore, cuticular wax on plant leaf surfaces can also affect the feeding and foraging behaviors of insect pests (Eigenbrode 2004). For example, epicuticular wax has been demonstrated to be important in rice defense against herbivores. Using rice mutants with reduced epicuticular wax, Bernaola et al. (2021) showed the importance of rice cuticular wax in reducing the infestations of the *Spodoptera frugiperda* JE Smith and *Lissorhoptrus oryzophilus*, known to be important pests of rice. Another type of physical defense utilized by plants to resist insect attack is lignification or cell thickening. Recently, Zhang et al. (2022) demonstrated the importance of lignin in rice resistance to BPH. The authors found constitutively higher content of lignin in resistant rice accession compared to the susceptible accession. Furthermore, mineral supply such as nitrogen fertilization may also affect lignin accumulation in rice. Zheng et al. (2021) explored the impact of low and high nitrate levels in rice defense against the striped stem borer *Chilo suppressalis*. Interestingly, low nitrate supply resulted in high accumulation of lignin which enhanced the constitutive defense in the borer-infested rice plants suggesting that mechanical defenses may be dependent on soil and/or plant mineral status.



- *Callose deposition- obstruct sieve tubes preventing insect penetration and feeding*
- *Si- Antibiotic and xenobiotic effects, reduced longevity and female fertility*
- *Wax-Interfere with attachment to plants*
- *Lignin- Difficulty in stylet penetration*

- *Trichomes- Negative effect on PM, prevent larval migration and feeding*
- *Wax- Reduce ability to attach and move around*
- *Si- Mandibular wear, extended larval development, increased penetration time*
- *Lignin- Act as a physical barrier*

**Figure 2. Representative morphological traits in rice used as physical defense structures against insect herbivores, adopted from Balakrishnan et al. (2024).** Wax and trichomes are important in restricting movement of insect herbivores including reduction in weight gain, and increased mortality via penetration of the insect midgut especially by trichomes. Deposition of callose is specifically important in rice defense against sucking herbivores as they obstruct the penetration of the stylet into to the sieve tubes.

Mineral nutrients have been reported to be directly involved as structural components and metabolic regulators in plant defense (Huber, 1980). For instance, Si plays a crucial structural role in providing resistance to plants against insect herbivores by increasing the hardness and abrasiveness of plant tissues (Balakrishnan et al., 2024; Frew et al., 2018). Furthermore, several macro and micronutrients can reprogram plant defense through the activation of several enzymes involved in the production of defense metabolites or by indirectly altering soil microbial activity which in turn could modulate plant defense responses (Datnoff et al., 2007; Martinez-Medina et al., 2017). For example, in addition to the alteration of constitutive defense against herbivores, nitrogen availability was reported to influence herbivore-induced direct defense responses in *N. attenuata* attacked by *Manduca sexta* (Lou and Baldwin, 2004). Similarly, manganese is reportedly involved in the production of phenolic compounds usually used in plant defense against biotic attacks (Fernando et al., 2009). Some plants can also accumulate large quantities of certain elements such as nickel, selenium and zinc from the soil as a defensive tactic to protect themselves from herbivores or pathogen attacks (Boyd, 2012; Hanson et al., 2003; Ghaderian et al., 2000). Despite the widely accepted influence of mineral nutrients in plant structural and chemical defense mechanisms, surprisingly, how mineral elements are recruited or distributed in plants under herbivory stress is poorly understood.

In this chapter, ionome analysis of rice exposed to herbivore attack was examined to better understand the spatial and temporal dynamics of mineral use in rice defense against herbivores.

# Materials and methods

## Plant materials and growth conditions

Hydroponically-grown rice plants (*Oryza sativa* L. cv. Nipponbare) were used in all experiments. Seeds were soaked in water, covered with aluminum foil, and kept in the dark for 2 d at 30 °C. The seeds were then germinated in 1.2 L plastic pots containing 0.5 mM $CaCl_2$ for 4 d on a floating net (30 x 20 cm). Thereafter the seedlings on net were then transferred to a half-strength Kimura B solution containing 0.5 mM Si as silicic acid (Ma et al., 2002). The composition of the Kimura B solution is described in Ma et al. (2001). Silicic acid was prepared using potassium silicate passed through a cation-exchange resin (Amberlite IR-120B, H + form, Organo, Tokyo). The final pH of the Kimura B solution was adjusted to 5.6 with NaOH. Ferrous sulfate was freshly prepared and added to Kimura B solution just before use at a final concentration of 5 µM. After 2 weeks, 50 seedlings growing on the net were transferred to a 3.5 L pot containing half-strength Kimura B solution. Pairs of seedlings were supported by soft sponge inserted in pot cover with 2 cm round holes. Nutrient solution was replaced once every 2 d. Four weeks after germination, seedlings were individually transferred to a 300-mL plastic black bottle containing half-strength Kimura B solution. The Kimura B solution was changed twice every week. Plants were grown in a cultivation room with a 14 h photoperiod and temperature of 28 ±3 °C. Rice plants were used for most experiments 7-8 weeks after germination.

## Plant treatments

### *Insects*

Loreyi armyworm, *Mythimna loreyi* Duponchel (Lepidoptera: Noctuidae) or MYL originally collected from rice in the paddy field (Kurashiki, Okayama Prefecture, Japan) were maintained in the laboratory on artificial pinto bean-based diet and rice leaf. Insects were reared at 30 ±1 °C. Neonates hatched from eggs on the rice leaves were transferred to artificial pinto bean-based diet (Gulzar and Wright, 2015) and maintained until pupation. Thereafter, pupae were kept in paper tissue for hatching in flight cages supplied with 10% diluted honey solution cups and rice seedlings serving as moth diet during mating period and oviposition substrate, respectively. Prior to use for bioassays, larvae at the 2nd-3rd instar stage were pre-fed with rice seedlings overnight and subsequently starved for at least 3 h before placement on mature leaves of rice plants (defined as second fully expanded leaf after the youngest fully expanded leaf).

### *Herbivory treatments*

Prior to herbivory treatment, 7-8-weeks-old hydroponically grown plants were transferred to freshly prepared half-strength Kimura B solution and then randomly arranged on tables in the glasshouse. The mature leaves were individually infested with starved 3rd instar larva as described above in clip cages (7.5 cm x7.5 cm). Local leaves (second mature leaves), systemic leaves (youngest fully expanded and third old mature leaves) were harvested from MYL-treated and untreated plants after 0, 1, 3, 6, 10, and 24 h. Root samples were rinsed thrice with water. All samples were collected, frozen immediately in liquid nitrogen, and stored at -80 °C until use for mineral element content analysis.

### *Nickel treatments*

Rice plants were grown in half-strength Kimura B solution for about 7 weeks. Thereafter, plants were transferred to freshly prepared Kimura B solution containing 0, 20, 100, or 500 ppb Ni and kept overnight before herbivory treatments on second mature leaves (local leaves). Herbivory exposed leaves were sampled at designated time intervals (0, 1, 3, and 10 h) for Ni analysis.

### Ionome analysis by inductively coupled plasma mass spectrometry (ICP-MS)

After grinding frozen samples into fine powder over liquid nitrogen, 100 and 200 mg aliquots of the leaves and root samples, respectively were transferred into 1.5 mL tubes and oven dried overnight (24 h) at 75 °C. Samples dry weights were determined after oven drying and then transferred individually into labelled 15 mL glass or plastic tubes for nitric acid digestion. Acid digestion of samples were performed by Dr. Namiki Mitani-Ueno. After digestion, metal concentrations in samples were determined with ICP-MS (7700X; Agilent Technologies, Santa Clara, CA, USA) by Ms. Sanae Rikiishi of the Plant stress physiology group, IPSR, Okayama University, Japan.

### Statistical analyses

Statistical analyses were performed by Student's t-test (Microsoft Excel) and significant differences were defined as $^{*}P<0.05$; $^{***}P<0.001$.

# Results

## Variation in the ionome profile of rice exposed to herbivory stress

To explore the dynamics of changes in mineral elements accumulation and distribution in rice plants upon herbivore attack, I performed a detailed spatial analysis of the accumulation levels of 20 elements in hydroponically grown rice plants exposed to chewing herbivore feeding for 24 h. After 24 h, rice tissues including the locally treated leaves and the systemic parts; the roots, basal stem, old, and youngest fully developed were collected from herbivore-treated and untreated plants and analyzed for elemental composition. Changes in the mineral concentration in rice tissues after herbivore exposure were expressed relative to the unexposed control plants and presented with a heatmap (Figure 3). Generally, herbivory induced higher mineral element accumulation in old systemic leaves compared to the local and other systemic tissues (Figure 3). Interestingly, the accumulation of Nickel (Ni), Cobalt (Co), Copper (Cu), and Arsenic (As) increased by approximately 2-fold in the old systemic rice leaves of herbivore-exposed plants after 24 h of feeding relative to the unexposed control plants (Figure 3). In the other tissues, higher of levels of Na (sodium) accumulated in the basal stem and the youngest fully developed leaves in herbivore exposed rice plants, whereas Boron (B) and Selenium (Se) levels tended to be higher in herbivore-fed local leaves relative to the control and other systemic tissues (Figure 3). Overall, mineral levels in the root tissues were not largely affected by herbivory suggesting that mineral elements redistribution rather than addition uptake could be affected when rice plants are exposed to herbivore attack.

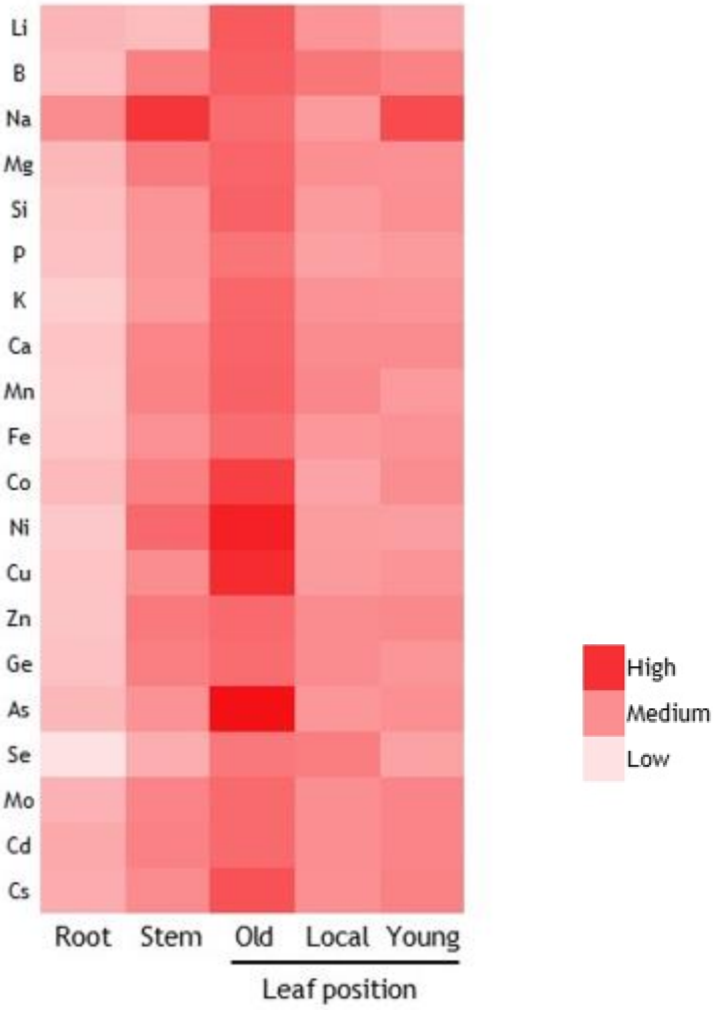


**Figure 3. Distribution of mineral elements in rice exposed to herbivory stress.** Heat map of the global changes in the accumulation of minerals in WT rice plants grown in hydroponic solution containing 0.5 mM Si and exposed to *M. loreyi* feeding for 24 h. After 24 h exposure to herbivore feeding, both local (mature leaves) and systemic tissues (roots, basal stem, old leaves, and youngest fully expanded leaves) were harvested and the elements analyzed using ICP-MS. Colour gradients represent values for mineral element contents in rice tissues exposed to herbivory expressed relative to the unexposed controls.

Since many macro and micro elements with potential defensive functions accumulated in the old systemic leaves of rice after exposure to herbivore attack, we next examined the temporal accumulation of these minerals in the local herbivore-fed, and systemic old leaves at earlier time points after herbivore infestation. Accumulation of Magnesium (Mg) and Potassium (K) was not affected by herbivore attack in both the locally attacked leaves and systemic old leaves (Figure 4), however, the levels of Calcium (Ca) and Phosphorus (P) decreased significantly in locally attacked leaves after 10 h of feeding relative to the control (Figure 4). Interestingly, both locally attacked leaves and systemic old leaves accumulated significantly higher levels of Ni and Co after 3 h of exposure to herbivore attack (Figure 5A). The accumulation of Zinc (Zn) was only transiently induced in both locally attacked and systemic old leaves 1 h after herbivore exposure (Figure 5). Cu showed similar accumulation patterns in both the locally attacked and systemic old leaves, with higher but not significant accumulation relative to control detected only after 10 h of herbivore feeding. To further confirm that the observed higher accumulation of Ni in rice when exposed to herbivory stress reflects an intrinsic anti-herbivore defense response, the pattern of accumulation of Ni in the local leaves of herbivore-treated and untreated control plants when exposed to exogenous Ni supply was analyzed. Again, in all concentrations of Ni including rice plants not supplied with Ni, insect feeding resulted in higher accumulation of Ni in the locally-fed rice leaves compared to the unfed controls (Figure 5B). Collectively, these results suggest that rice plants under herbivore attack may recruit additional amounts of certain micro-elements which could potentially perform direct defensive function against insect herbivores or may be involved in the biosynthesis of other specialized defensive metabolites in rice.

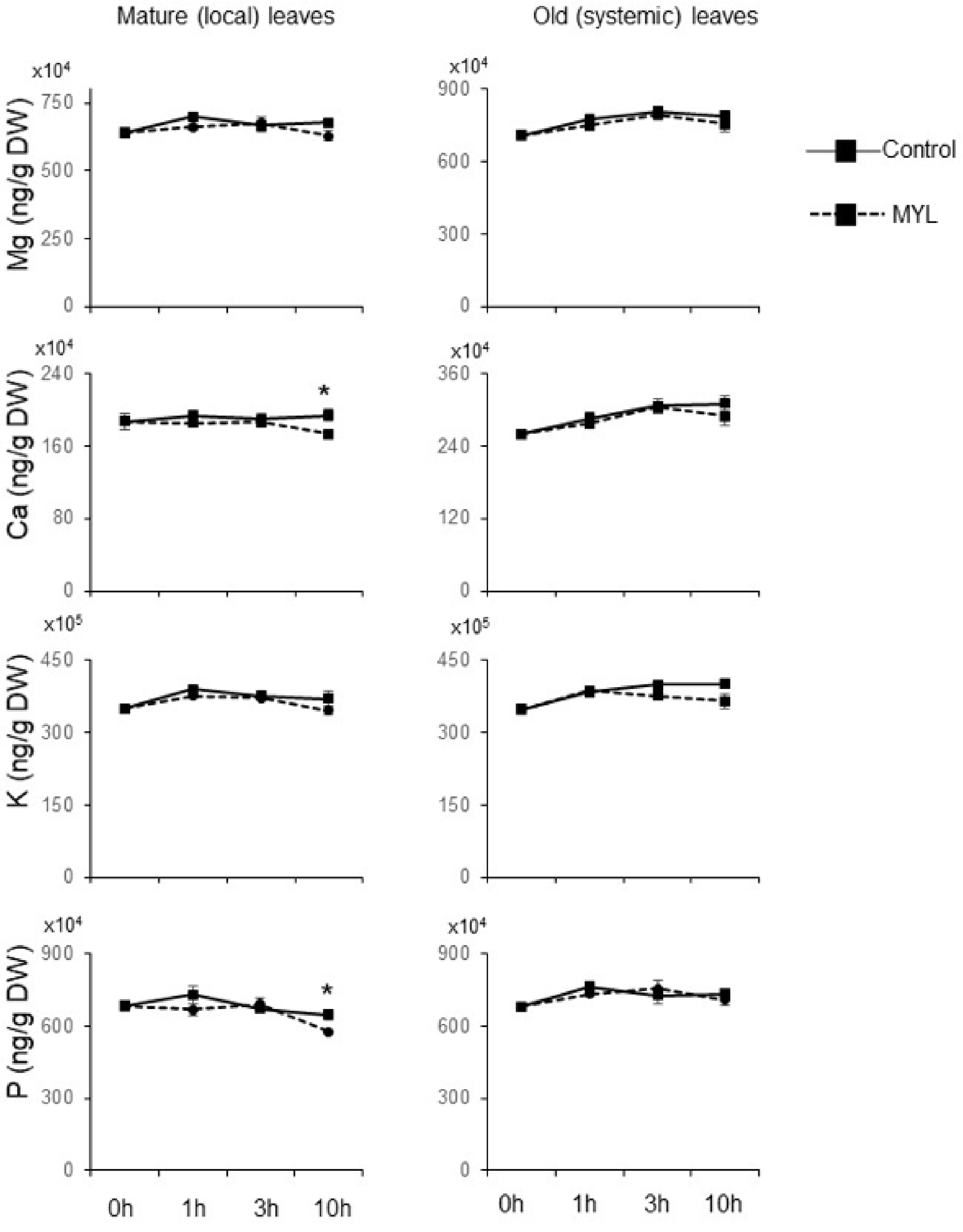


**Figure 4. Impact of herbivory stress on the spatiotemporal accumulation of macronutrients in rice.** Elemental concentrations in local (mature) and systemic (old) leaves of hydroponically grown rice plants after exposure to *M. loreyi* feeding. Data are mean ± SE (n=4). Statistical differences between treatments (Control and herbivory) were analyzed by Student's t-test (*P<0.05; no symbol, not significant).

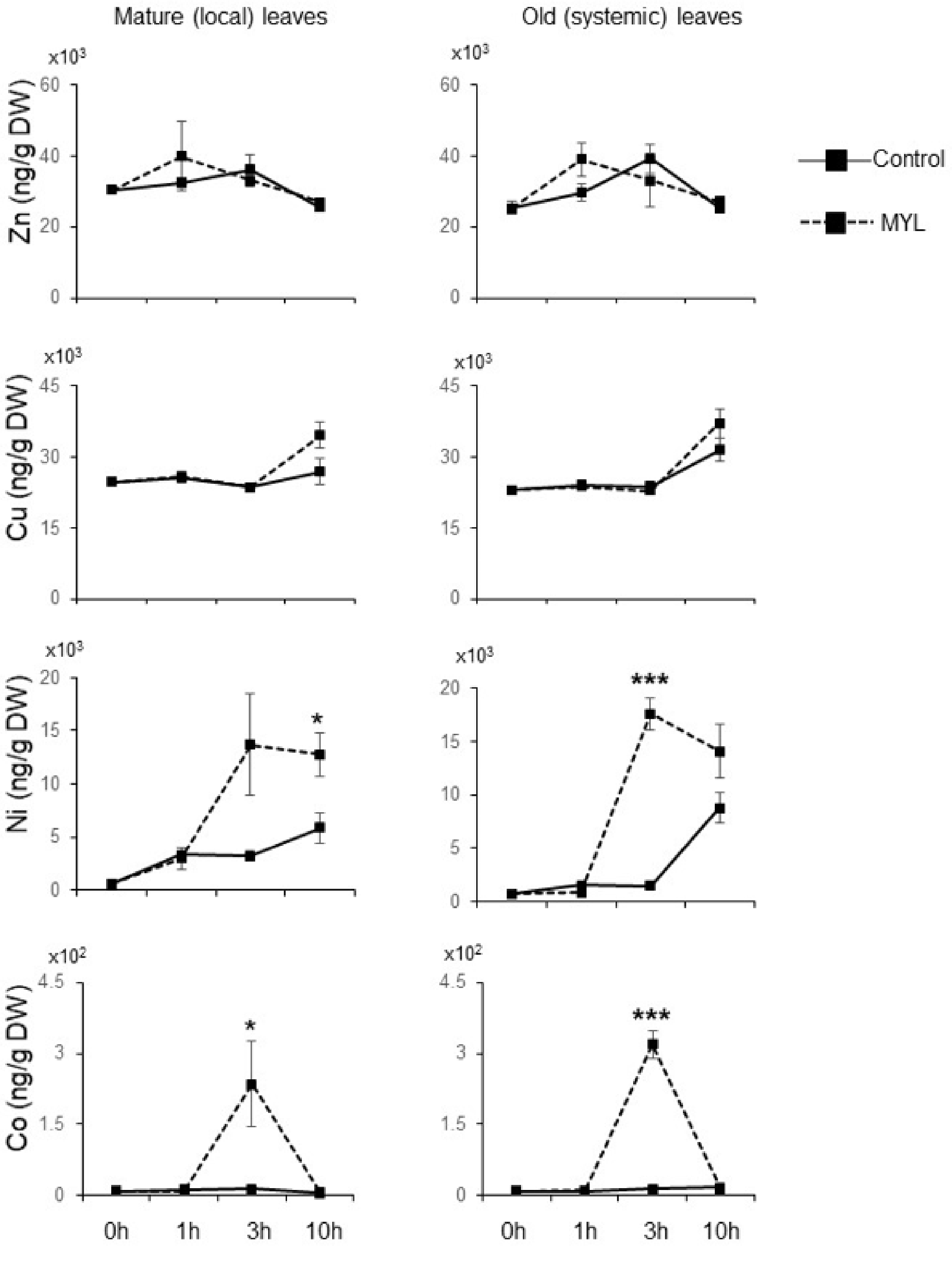


**Figure 5A. Impact of herbivory stress on the spatiotemporal accumulation of micronutrients in rice.** Elemental concentrations in local (mature) and systemic (old) leaves of hydroponically grown rice plants after exposure to *M. loreyi* feeding. Data are mean ± SE (n=4). Statistical differences between treatments (Control and herbivory) were analyzed by Student's t-test (*P<0.05; ***P<0.001; no symbol, not significant).

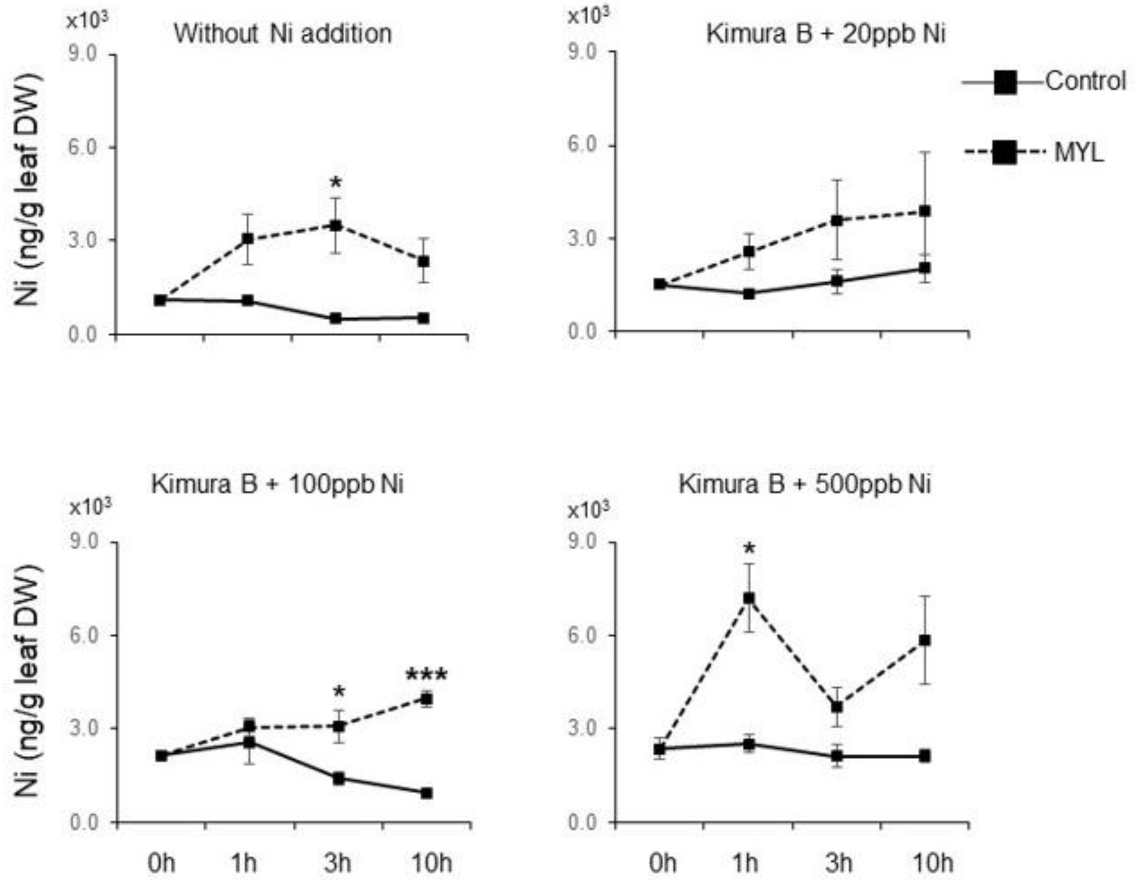


**Figure 5B. Rice Ni accumulation patterns when exposed to herbivory stress and different concentrations of exogenous Ni.** WT rice plants were grown in hydroponic solution containing 0.5 mM Si for 7 weeks and then supplied with different concentrations of Ni prior to exposure to *M. loreyi* feeding. Herbivore-fed mature leaves at designated time intervals were harvested and Ni concentration in the leaves analyzed using ICP-MS. Data are mean ± SE (n=4). Statistical differences between treatments (Control and herbivory) were analyzed by Student's t-test (*P<0.05; ***P<0.001; no symbol, not significant).

# Discussion

In general, plant mineral compositions are reportedly recognized as important regulators of plant defense responses (Hu et al. 2021; Wetzel et al. 2016). Furthermore, plant nutritional quality may directly affect the performance of herbivores that feed on them (Awmack and Leather, 2002; Yu et al., 2024). On the other hand, upon herbivore infestation, plants may re-direct mineral nutrients either for the production of defensive metabolites or away from the attacking insects to starve them of their nutritional requirements. While many reports had focused on the role of plant mineral compositions as indicators of host plant quality in plant-herbivore interactions (Beanland et al., 2003), little is known about whether herbivory may cause variation in the accumulation of plant mineral nutrients.

Several earlier studies had demonstrated that plant foliar elemental compositions can vary considerably in response to local environmental conditions (Joern et al., 2012; Chapin 1991; Aerts and Chapin, 2000). In this study, the accumulation patterns of several elements in hydroponically grown rice plants subjected to herbivore attack was shown to vary both spatially and temporally. Mineral analyses revealed on the average about 2-fold increase in the accumulation of Ni, Cu, As, and Co in the systemic old leaves of herbivore-exposed relative to unexposed plants (Figure 3). Previously, herbivore infestation of *Arabidopsis helleri* leaves was reported to result in higher accumulation of heavy metals in the phloem (Stolpe et al., 2017). Upon infection by rice stripe virus, rice accumulated higher amounts of Cu in the shoots to counteract viral infection (Yao et al., 2022). Plants can be subjectively classified into normal, accumulator, and hyperaccumulator based on the concentrations of metals in their tissues (Reeves and Baker, 2000).

Hyperaccumulating plants take up very high concentrations of metals from the soil and sequester these metals in the above ground tissues (Davis and Boyd, 2000). While considerable experimental evidence suggests that hyperaccumulation of trace metals such as Ni, Se, Zn, and cadmium (Cd) could have a defensive function against herbivory (Boyd, 2012; Hanson et al., 2003; Jhee et al., 1999), it is unclear whether other non-hyperaccumulator plants can utilize this hyperaccumulation strategy to recruit trace metals in response to herbivore attacks to protect themselves. In the Ni hyperaccumulator, *Streptanthus polygaloides*, exposure to simulated herbivory or live folivores did not induce the accumulation of additional Ni suggesting that Ni-based defenses may be constitutive in hyperaccumulators (Davis and Boyd, 2000). Interestingly, our results revealed that rice subjected to chewing herbivore feeding accumulated significantly higher levels of Ni and Co after 3 h of feeding relative to untreated control plants (Figure 5). These results suggest that in non-hyperaccumulating plants such as rice, higher Ni accumulation may be used as a form of induced defense strategy to defend against herbivore damage. It is also noteworthy to state that B and Se levels in this study tended to be higher in herbivore-fed local leaves relative to the control and other systemic tissues (Figure 3). Given the reported important roles of B in phenolic-based biosynthetic pathway and synthesis of lignin (Beanland et al., 2003) and their effect against herbivore attack (Ruuhola et al., 2011), it plausible to assume that rice under herbivore attack could be recruiting B in the attacked tissues to directly or indirectly perform defensive functions. Similarly, Hanson et al. (2003) demonstrated that hyperaccumulation of Se in *Brassica juncea* offer protection against invertebrate herbivory and fungal infection. Overall, this study provides evidence that herbivory may stimulate rice plants to take up additional amounts of some of the metals widely reported to be essential for plant defense against herbivory in other naturally hyperaccumulating plants species. However, it is still unclear how rice plants use the accumulated micro elements (Ni, Co) in defense against herbivory. Presumably, the higher accumulation of Ni in rice under herbivore attack may be directly used to poison the herbivore or could be integrated for the production of defensive secondary metabolites. Although our preliminary results (data not shown) revealed that in artificial diet assay, concentrations of nickel higher than the amounts accumulating in rice leaves after herbivore attack did not directly inhibit the growth of MYL larvae, nonetheless, these need to be experimentally evaluated *in planta* in future studies.

# Chapter 3: Comprehensive analysis of silicon impact on defense and metabolic responses in rice exposed to herbivory stress

## Overview

Silicon (Si) uptake is generally beneficial for plants that need protection from insect herbivores. In pursue of mechanisms involved in Si-mediated defense, in this chapter, I comprehensively explored the impact of Si on several defensive and metabolic traits in rice exposed to simulated and real herbivory of *M. loreyi* larvae. Hydroponic experiments showed that Si-deprived rice supplemented with Si 72 h prior to insect infestation were similarly resistant to larvae as plants continuously grown in Si-containing media. Both Si and herbivory altered primary metabolism in rice, including the levels of several sugars, amino acids, and organic acids. While the accumulation of sugars was generally positively correlated with Si presence, multiple amino acids showed a negative correlation trend with Si supplementation. The levels of secondary metabolites, including IPA, CoP and FP, were typically higher in the leaves of Si-supplemented plants exposed to herbivory stress compared to Si-deprived plants. In addition, simulated herbivory treatment in Si-supplemented plants induced more volatile emissions relative to Si-deprived plants, which was consistent with the increased transcripts of key genes involved in volatile biosynthesis. In ecological interactions, Si alone did not affect the oviposition choice of *M. loreyi* but gravid females showed a significant preference for simulated herbivory-treated/ Si-deprived compared to Si-supplemented plants. These data collectively suggest that apart from mechanical defense, Si may affect rice metabolism in multiple ways that might enhance/modulate defense responses of rice under herbivory stress.

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## Introduction

In their habitats, plants are often confronted with the attacks of insect herbivores, which requires proper balancing of growth and defense responses (McMillan, 2023). Consequently, plants have evolved several mechanical and biochemical defense strategies; some of which are constitutive, and others inducible to counter herbivores and preserve their own fitness (Schuman and Baldwin, 2016). Mechanical defense includes various inherent structural traits, such as waxy cuticles, trichomes, hairs, and spines which collectively deter insects from feeding on plants (Mitchell et al., 2016; Kaur and Kariyat, 2023; Balakrishnan et al., 2024). Some plants are known to hyperaccumulate minerals from the soil that acts as a physical barrier to insect feeding (Hanley et al. 2007; Debona et al., 2017; Korth et al., 2006). For instance, *Medicago truncata* Gaertn., mutants with reduced levels of calcium oxalate were more susceptible to damage by beet armyworms (*S. exigua*) compared to the WT lines (Korth et al., 2006). Needle-like calcium oxalate crystals (raphides) and cysteine protease exerted synergistic defensive function against insect Eri silkmoth (*Samia ricini* Donovan) (Konno et al., 2014). Similarly, impregnation of non-glandular trichomes with silicon was demonstrated as one of the essential traits in defense of rice (*Oryza sativa* L.) cv. Nipponbare against chewing insect herbivores (Andama et al., 2020).

Besides increasing the physical strength to insects *via* deposition on trichomes and beneath the leaf cuticles, several studies have also suggested that Si accumulation could be affecting the insect damage by modulating the levels of inducible chemical defenses in herbivore-exposed plants (Alhousari and Greger, 2018; Reynold et al., 2016). For example, Si pretreatment enhanced the activity of several plant defense-related enzymes after insect attack (Ye et al., 2013; Han et al., 2016). Such enzymes are often associated with the biosynthetic pathways for various types of defensive secondary metabolites, including various types of phenolics and momilactones (Fawe et al., 1998; Ahanger et al., 2020). Previously, we characterized several novel herbivore-induced metabolites in rice, which is a typical hyperaccumulator of Si (Alamgir et al., 2016; Aboshi et al., 2021); however, whether Si could be mediating the content of these metabolites in rice defense remains unknown.

In addition to specialized metabolites, Si application altered primary metabolism in unstressed rice by inducing the amino acid remobilization (Detmann et al., 2012). Under abiotic stress conditions, such as iron toxicity and drought, Si application enhanced photosynthesis (dos Santos et al., 2019), carbohydrates and amino acid accumulations (Hajiboland et al., 2017). The impact of Si on primary metabolism in plants under biotic stress was mainly explored in plant-pathogen systems, especially with the help of transcriptomics. For instance, Si treatment alleviated the down-regulation of genes involved in primary metabolism in *Arabidopsis* inoculated with powdery mildew fungus (Fautex et al., 2006). In addition, Si pretreatment increased the photorespiration rates in brown spot-infected rice plants, a mechanism thought to protect the photosynthetic machinery of plants under the stress conditions (Van Bockhaven et al., 2015). Although significant alterations in primary metabolism of plants also occur during herbivory stress

(Steinbrenner et al., 2011; Zhou et al., 2015), possible effect(s) of Si on such metabolism in plants exposed to insect herbivores remain to be explored in detail (Frew et al., 2018).

After herbivore attack, in addition to direct defense metabolites, plants also produce VOCs, such as terpenoids, green-leaf volatiles and aromatic compounds, which can either be directly toxic or indirectly protect plants by recruitment of natural enemies of herbivores, when retained or released from plants, respectively (Aljbory and Chen, 2018; Zhou and Jander, 2022). Previously, Si treatment was shown to affect the release of herbivory-induced volatiles (HIPVs) in herbivore-infested rice, and these alterations were ecologically important in attracting the natural enemies of herbivores to attacked plants (Liu et al., 2017). While the control of HIPV emissions in rice involves both biosynthesis and/or release mechanisms (Mujiono et al., 2021), which of these are modulated by Si application is not well understood. In addition, HIPVs are well known to serve as danger and/or host-location cues for ovipositing females of herbivorous insects (Zakir et al., 2013; Achhami et al.,2021). So far in maize, Si supplementation did not alter the oviposition choice of *S. exigua* (Leroy et al., 2022) but suppressed the *S. frugiperda* oviposition on Si-enriched plants (Pereira et al., 2021).

In this chapter, I aimed for a more comprehensive approach to evaluate both defensive and metabolic roles of Si in rice plants under herbivory stress, represented by real herbivory of generalist chewing herbivore *M. loreyi* (MYL), and simulated herbivory treatment (WOS), when mechanical wounds were treated with the oral secretions from MYL. Short-term Si uptake experiments in hydroponic system were used to monitor the distribution of Si in rice leaves, including local and systemic leaves after herbivore attack. The impact of Si on rice metabolism was analyzed by monitoring the accumulation of several primary and secondary metabolites, including HIPVs, in Si-supplemented and Si-deprived rice plants exposed to insect herbivory. By measuring the transcripts levels of genes putatively associated with HIPV biosynthesis in rice, we further attempted to elucidate the possible mechanisms involved in Si-modulated HIPV emissions in rice. In summary, these results further deepen the understanding of defensive and metabolic roles, and ecological implications, of Si in rice challenged with insect herbivores.

## Materials and methods

### Plant materials and growth conditions

Wild type (WT) rice plants (*Oryza sativa* L. cv. Nipponbare) and the corresponding Si-transporter-deficient mutants, *lsi1* grown hydroponically in half-strength Kimura B solution as described in Chapter 2 were used in all experiments, except in herbivore performance assay where *lsi1* and the corresponding WT plants were grown in pots with paddy field soil. Nipponbare was selected for this study because it is a reference rice cultivar with well characterized Si accumulation mechanisms (Ma et al. 2007a). Seeds of *lsi1* mutant were obtained from Dr. Jian Feng Ma, Okayama University, Japan. Seedlings of WT (Nipponbare) and corresponding *lsi1* mutant were grown in pots with paddy field soil as described in Mujiono et al. (2020). Plants were kept in a cultivation room with a 14 h photoperiod and temperature of 28 ±3 °C. Rice plants were used for most experiments 7-9 weeks after germination.

### Plant treatments

*Real herbivory/MYL treatment*

Larvae of MYL used for all experiments were reared and maintained in the laboratory as described in Chapter 2. Before use for experiments, larvae at the 2nd-3rd instar stage were pre-fed with rice seedlings overnight and subsequently starved for at least 3 h before placement on mature leaves of rice plants (defined as second fully expanded leaf after the youngest fully expanded leaf).

*Mechanical wounding and oral secretions treatment*

Mechanical wounds were made using a fabric pattern wheel on both halves of the treated leaf along the midvein, followed by treatment with 20 µL of water-diluted (3:1) oral secretions from MYL (WOS) evenly spread on the leaf surface by gentle rubbing. Oral secretions used in all experiments were prepared essentially as described in Shinya et al. (2016).

### MYL Performance Bioassays

Prior to performance assay, 7-week-old rice plants grown hydroponically with or without Si supply were respectively transferred to freshly prepared half-strength Kimura B solution with or without Si addition. Youngest fully expanded leaves of WT plants with or without Si amendments and *lsi1* mutant were individually infested with one pre-weighed 3rd instar larva in clip cages (7.5 cm x 7.5 cm). All larvae were weighed at 2 d intervals until 6 d and the percentage mass gain on each plant was calculated. Thirty plants on each Si amendment regime were used for the assays except for the short-term Si supply performance assay where fifteen plants were used for each treatment.

### Determination of Si uptake and accumulation

To observe the tissue-specific accumulation of Si upon exposure to herbivory, mature leaves of 7-weeks-old plants grown with or without Si in hydroponic solution and the *lsi1* mutant with the corresponding WT grown in pots with paddy field soil were infested with 3rd instar larva in clip cages. After 48 h, the local leaves (second mature leaves) and the systemic leaves (youngest fully expanded and third old mature leaves) were harvested, frozen in liquid nitrogen and stored at -80 °C until use for determination of Si concentration. To examine the dynamics of Si uptake in rice seedlings exposed to either Si alone or in combination with insect infestation, we performed a short-term Si uptake experiment. Plants grown without Si for 7 weeks in hydroponic solution were either supplied with Si alone or in combination with herbivore treatments. Local and systemic leaves were sampled at designated time intervals (0, 3, 6, and 24 h) for Si analysis. Determination of Si content in rice leaves was performed as described by Wei-min et al. (2005) with some modifications. Frozen leaf samples were pulverized into fine powder over liquid nitrogen and 100-150 mg aliquots were transferred into a 5-mL Eppendorf tubes, and oven dried at 60 °C for 48 h. After oven drying, each tube was added 750 µL of 50% NaOH and gently vortexed. The tubes were then covered with screw caps and autoclaved at 121 °C and 138 kPa for 1 h. Ten µL aliquots from the digested sample were diluted with 157 µL of water, thereafter 20 µL of the diluted solution was transferred to 1.5 mL tube and combined stepwise with 600 µL of 20% (vol/vol) acetic acid and 200 µL ammonium molybdate (54 g/L, pH 7.0). Samples were vortexed and kept at room temperature for 5 min before the addition of 100 µL of 20% (wt/vol) tartaric acid and 20 µL of freshly prepared reducing agent (40 mg $Na_2SO_3$, 8 mg 1-amino-2-naphtol-4-sulfonic acid, 500 mg $NaHSO_3$ in 5 mL of milli-Q water). Final reaction volume was adjusted to 1 mL with acetic acid before incubating at room temperature for 30 min. Absorbance was measured at 650 nm after 30 min incubation at room temperature using a spectrophotometer.

## Visualization of Si deposition in rice leaves by SEM-EDX

Scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectrometry (EDX) was used to observe the pattern of deposition of Si in rice leaves exposed to either Si alone or in combination with insect feeding. Rice seedlings (7-weeks-old) grown in the absence of Si were transferred to a Kimura B solution containing 0.5 mM Si alone or in combination with herbivore exposure for 24 h. Leaf discs (4 mm) collected from mature leaf of herbivore-fed and control plants were placed on double-sided adhesive tapes and oven-dried at 40 °C for at least 24 h before SEM imaging. The abaxial surface of the leaf discs was used for SEM observation on Miniscope TM 3000 (Hitachi High-Technologies, Tokyo, Japan) at 15 kV. Scanning electron micrographs obtained at 500-fold magnification were used to map Si deposition pattern on leaf surface using Swift ED3000 X-ray microanalyzer coupled to the microscope.

## Leaf photosynthetic parameters and chlorophyll contents

The impact of Si amendment on rice leaf photosynthetic parameters and chlorophyll content was estimated with rice plants grown hydroponically with or without Si amendment. Leaf gas exchange rates ($\mathrm{G_s}$ and $\mathrm{T_r}$) and photosynthetic activity ($\mathrm{P_n}$) were measured using a portable gas exchange analysis system LI-6400XT coupled with a leaf chamber Fluorometer 6400-40 (LI-COR Biosciences, Lincoln, NE, USA). The ratio of $P_n$ and $G_s$ was calculated as the intrinsic water use efficiency (iWUE). The temperature of the leaf chamber was set at 28 °C to match with the temperature in the plant growth room at the start of measurement. During measurement, light intensity and $CO_2$ concentration in the leaf chamber were maintained at 1000 μ mol $m^{-2}$ $s^{-1}$ and 400 ppm, respectively. The leaf was allowed to acclimate for 30 min after enabling the light source of LI-6400XT, before each measurement. Mature leaves (second fully expanded leaf) were used for the measurements. WOS treatments were performed as described above on mature leaves. After 24 h, leaf photosynthetic activity was measured on the WOS-treated leaf. The Soil Plant Analysis Development (SPAD) chlorophyll meter was used to measure the chlorophyll contents in leaves of rice plants grown with or without Si and treated with or without WOS. Measurements were performed on the local leaves (second mature leaves), and systemic leaves (youngest fully expanded and third old mature leaves) of untreated plants and WOS-treated plants after 24, 48, and 72 h. Four locations on each leaf were selected for SPAD measurements, with two of the locations near either the leaf tip or base, while the remaining two locations were measured around the middle of the leaf. The means of the four measured SPAD values were recorded for each leaf.

### Primary and secondary metabolites analyses

Sampled leaf tissues (70-150 mg fresh mass) were ground to fine powder in liquid nitrogen before adding 4-5 ceramic beads for sample homogenization by reciprocal shaking using a FastPrep 24 instrument (MP Biomedicals, Santa Ana, CA, USA). Sample primary metabolites extraction, quantification, and analysis with an Agilent 7890A-GC/240-MS instrument (Agilent Technologies, Santa Clara, CA, USA) were performed as described in Raviv et al. (2020). For the identification of primary metabolites after GC-MS analysis, commercially available authentic standards for each compound were used for comparison of retention times and fragmentation patterns, together with the information from the NIST Mass Spectral Library version 2.0g build 2011 (National Institute of Standards and Technology, Gaithersburg, MD, USA). Secondary metabolite content in leaf samples was measured with 6410 Triple Quadrupole LC/MS (Agilent

Technologies) after extraction and purification as described previously with slight modifications (Tanabe et al., 2016). For secondary metabolite sample purification with C18 column, supernatants from sample extractions were combined and diluted with 84 mM ammonium acetate buffer (pH 4.8) to a final methanol concentration of 20% (v/v) before purification with a C18 solid-phase extraction column (Bond Elut C18: Agilent Technologies). Secondary metabolites contents in the samples were determined after LC-MS analysis using synthetic standards of phenolamides prepared in Alamgir et al. (2016) and authentic isopentylamine (Tokyo Chemical Industry Co., Ltd., Japan).

### Phytohormone measurements

Mature leaves of rice plants grown hydroponically with or without Si amendment were individually infested by MYL larvae or were elicited with WOS. Mature leaves were harvested from MYL- and WOS-treated and untreated after 0, 1, 3, 6, 10, and 24 h. Harvested samples were immediately put in liquid nitrogen, and were then stored at -80 °C until use for phytohormone analysis. Phytohormone contents in leaf samples were measured as essentially described in a previously published method (Fukumoto et al., 2013) using 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). Endogenous phytohormone amounts were quantified using deuterium-labeled internal standards from commercial sources (d6-ABA, Funakoshi Co. Ltd. Japan; d4-SA, Toronto Research Chemicals Inc., Canada) or d3-JA, d3-JA-Ile donated by Dr. H. Matsuura (Hokkaido University).

## Quantification of headspace volatile organic compounds (VOCs)

A headspace VOC collection system previously described in Sobhy et al. (2017) was used to collect and quantify the VOCs released from rice plants grown with or without Si amendment and treated with or without WOS. For the WOS-treated sets, the youngest fully expanded leaf and the mature leaf (second fully expanded leaf) were subjected to WOS treatment as described earlier, while the control set remained unwounded. Briefly, for VOC collection, treated or control plants grown hydroponically in 300-mL black bottles were individually inserted in an acrylic cylinder (67 cm high x 10 cm internal diameter) with an inlet port that allowed purified air into the cylinder, and an outlet port at the top fitted with volatile traps containing two MonoTrap devices (cylindrical monolithic adsorbents; GL Sciences Inc., Tokyo, Japan). After 24 h of trapping, Mono Traps were transferred to GC vials and eluted by sonication with 300 µL dichloromethane (DCM; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) spiked with 400 ng tetralin (1,2,3,4-tetrahydronaphthalene; FUJIFILM Wako Pure Chemical Corporation) as an internal standard. Eluted samples were analyzed on an Agilent 7890A-GC/240-MS instrument (Agilent Technologies) using previously optimized instrument parameters (Sobhy et al., 2017). For the identification of VOCs after GC-MS analysis, commercially available authentic standards for each compound were used for comparison of retention times and fragmentation patterns, together with the information from the NIST Mass Spectral Library version 2.0g build 2011 (National Institute of Standards and Technology, Gaithersburg, MD, USA).

### Extraction and quantification of internal leaf volatiles

A solid phase micro-extraction method previously described in Mujiono et al. (2020) was used for the extraction of volatiles from leaves of rice plants grown hydroponically with or without Si supply and treated with or without WOS. Both the youngest fully expanded leaf and the mature leaf (second fully expanded leaf) were collected from WOS-treated and untreated plants after 4 h, weighed and stored at -80 °C in 16 mL glass tubes until use. WOS-treatments were performed as described earlier on the youngest fully expanded and mature leaves. Briefly, for the extraction of internal volatiles from the leaves, frozen leaf samples in glass tubes were spiked with 5 µL tetralin (400 ng) in DCM as internal standard. Thereafter, tubes were tightly covered with polytetrafluoroethylene lids with MonoTraps suspended on a stainless pin pushed into the nitrile packing of the lid. The tubes were then inserted into a programmable heat block with the following volatile heat elution program: increase from room temperature to 150 °C and hold for 30 mins, natural cooling to 60 °C and hold for another 30 mins. MonoTraps were then detached from the lids and gently dried with stream of nitrogen gas before elution with 200 µL of DCM by sonication. After elution, MonoTraps and pins were removed and the volatiles in DCM-eluted samples were analysed on GC-MS.

### Gene expression, quantitative RT-PCR

Expression of selected genes of rice plants in response to Si amendments and WOS treatments was determined by quantitative RT-PCR essentially as described previously in Fukumoto et al. (2013). Total RNA was extracted from leaf tissues (100 mg) using the Trizol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Total RNA (1 µg) was used for the first-strand cDNA synthesis using PrimeScript reverse transcriptase (Takara Bio Inc., Japan) following the manufacturer's protocol, and qRT-PCR was performed using the qTOWER3 G touch Real-Time PCR thermal cycler (analytikjena, Jena, Germany). The rice housekeeping gene, *Actin* was used as endogenous control for transcript levels normalization. Gene-specific oligonucleotides primer sequences used for qRT-PCR are outlined in Supplementary Table 1.

### Oviposition preference bioassays

All oviposition preference assays were performed under outdoor conditions in a wire-mesh screen house. The experimental setup consisted of rice plants randomly arranged on tables in the screen house such that each plant with Si amendment was placed at distance of 50 cm from those without Si amendment. For each test, 20 adult females and 5 adult males of MYL moths were released in screen house containing 24 plants (12 plants each for each treatment either Si-treated or non-treated plant or Si regimes with WOS). Diluted honey solution was provided *ad libitum* as food source. To determine oviposition preference, the number of plants with egg masses for each treatment was counted after 1 and 2 d, and an oviposition preference percent: {(no. of plants with egg masses/total no. of plants) x 100} was calculated. The bioassay was independently performed with rice plants grown hydroponically with or without Si, and then plants grown with or without Si treated with WOS to simulate herbivory stress. For the simulated herbivory stress sets, the youngest and mature fully expanded leaves were subjected to WOS treatment in the screen house after which the MYL moths were released and oviposition preference was determined after 1 and 2 d. Each set was repeated at least 5 times with new sets of plants and insects used for each repeat.

## Statistical analyses

Data were first examined for normality using the online version of Shapiro-Wilk test (www.statskingdom.com/shapiro-wilk-test-calculator.html), and when not normally distributed, logarithmic transformations of data were used before statistical analysis. Statistical differences between pairs of datasets were determined using Student’s t-test (Microsoft Excel), and multiple samples were compared by analysis of variance (ANOVA) in *multcomp* package in R version 4.3.2 (R Core Team, 2023). The impact of Si and herbivory on the identified primary metabolites were visualized with Pearson’s correlation, and principal component analysis (PCA) packages in MetaboAnalyst 6.0 (http://www.metaboanalyst.ca).

# Results

## Supply of silicon (Si) enhances rice resistance to MYL

To assess the overall importance of Si in rice resistance to chewing herbivores, we first performed a bioassay using larvae of MYL that fed on hydroponically-grown Nipponbare (WT) rice plants. The plants in Kimura B culture media were supplied with or without 0.5 mM silicic acid, and replenished with a fresh media in twice a week time interval. MYL larvae fed on 7-week-old WT plants without Si increased their mass to 152.2% at 2 d of infestation, whereas larvae fed on Si-supplemented WT plants retained on average only mass of 65.3% relative to initial conditions (Figure 6A). To further confirm that the observed herbivore growth patterns reflect the accumulation of Si in the rice leaves, we compared MYL performance on Si-transporter-deficient Nipponbare *lsi1* mutants, and their corresponding WT plants grown in pots with paddy field soil. Again, MYL larvae grew better on the *lsi1* mutant plants, which was significant after 2 d of insect infestation when compared to similarly grown WTs (Figure 6A). These results corroborated a previously known defensive role of Si in rice against chewing herbivores that requires impregnation of rice tissues with silicon. We then examined the time interval required for Si to establish resistance against MYL larvae in rice leaves. WT plants were kept in Si-free hydroponic solution for 7 weeks, and then treated with different Si regimes before starting herbivore performance assays (Figure 6B): (1) grown without Si, (2) supplemented with Si 1 h before setting larvae on plants (-Si/+Si 1h) or (3) supplemented with Si 72 h prior to herbivory (-Si/+Si 72h). As before, plants continuously supplied with Si (+Si), used as a positive control, strongly inhibited MYL performance; interestingly, when the plants were supplied with Si 72 h prior to MYL exposure, we observed a similar

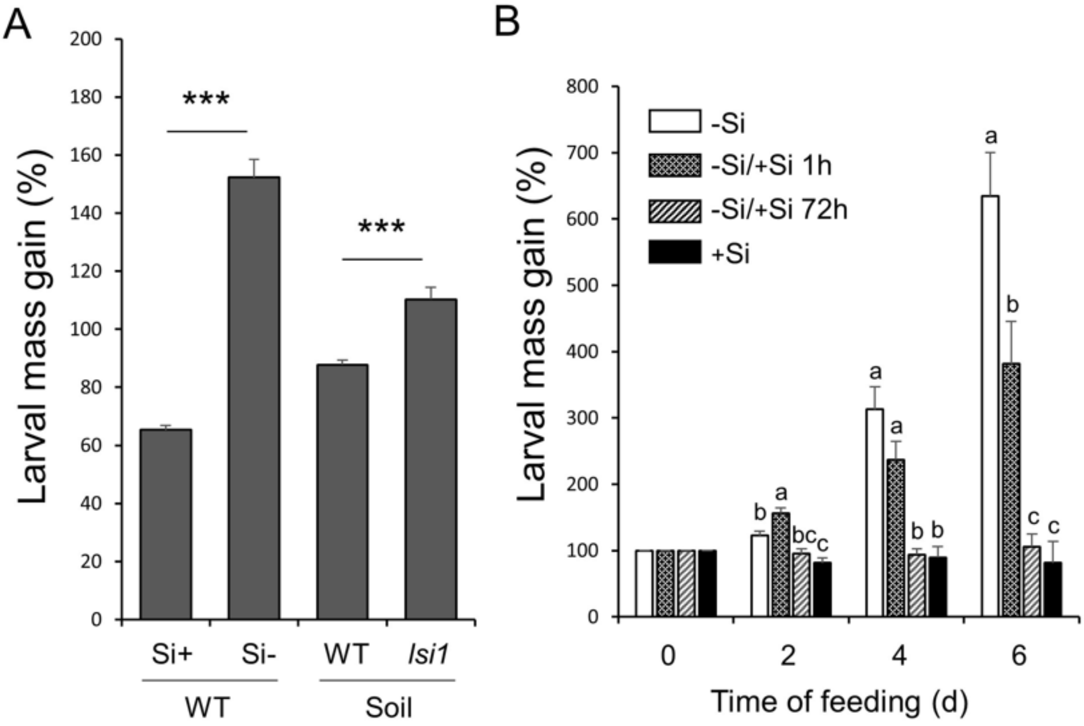

**Figure 6. Gain in mass of *M. loreyi* fed on rice plants.** (A) Nipponbare (WT) rice plants grown in hydroponic solution supplied with or without 0.5 mM Si and Si transporter deficient Nipponbare rice mutant (*lsi1*) grown in pots with paddy soil. (B) WT rice plants grown in hydroponic solution under different Si regimes:(**1**) grown without Si (-Si), (**2**) supplemented with Si 1 h before setting larvae on plants (-Si/+Si 1h), (**3**) supplemented with Si 72 h prior to herbivory (-Si/+Si 72h) or (**4**) continuously supplied with Si (+Si). Youngest fully expanded leaves of all plants were individually infested with third instar larvae. All larvae were weighed after 2 d for (A) or at intervals of 2 d for (B) and the percentage mass gain was calculated. Data are mean ± SE (n=30 for A, n=15 for B). Statistical differences between Si treatments were analyzed by Student's t-test (***P<0.001). Different letters (a-c) indicate significant differences (P≤0.05) between different Si regimes at each time point according to ANOVA followed by Tukey's HSD test.

level of resistance to that of the positive control rice plants (+Si) (Figure 6B). However, plants treated with Si 1 h prior to larvae exposure did not show any significant reduction in the larval growth until after 4 d of infestation. These results indicated a 3-4 d period as a minimum time necessary for Si to establish its defensive role in rice, at least under the model conditions when the plants were first deprived of Si in the culture media.

### Si accumulation and deposition patterns in rice leaves exposed to herbivory stress

Taking into account a gradual build-up of anti-herbivore Si-dependent defense, herbivores may be affecting the distribution of Si in the insect exposed plants, although most of the mineral is likely to remain immobile due to a fixation in specialized rice cells, such as silica bodies and impregnated trichomes (spikes). We first analyzed the leaf Si distribution in both hydroponically grown rice plants in the continuous presence and absence of Si, and *lsi1* mutants with the corresponding WT plants grown in pots with paddy field soil, which were exposed to feeding of MYL larvae for 48 h. Notably, regardless of herbivory, hydroponically grown plants without silica showed a reduced fresh mass of the aboveground parts (Figures 7A and B). As expected, plants in Si-containing culture media accumulated large amounts of Si (5-10% dry weight) in all tested leaves, compared to the non-Si treated plants (Figure 7C). Similarly, WT plants grown in paddy filed soil with normal Si levels in the laboratory accumulated higher amounts of Si in all samples leaf tissues relative to the *lsi1* mutant impaired in Si uptake (Figure 7D). Notably, Si accumulation differed with leaf age. Exposure to insect feeding for 48 h resulted in the significantly higher accumulation of Si, but only in the locally-fed leaves in the hydroponically grown plant sets (Figure 7C).

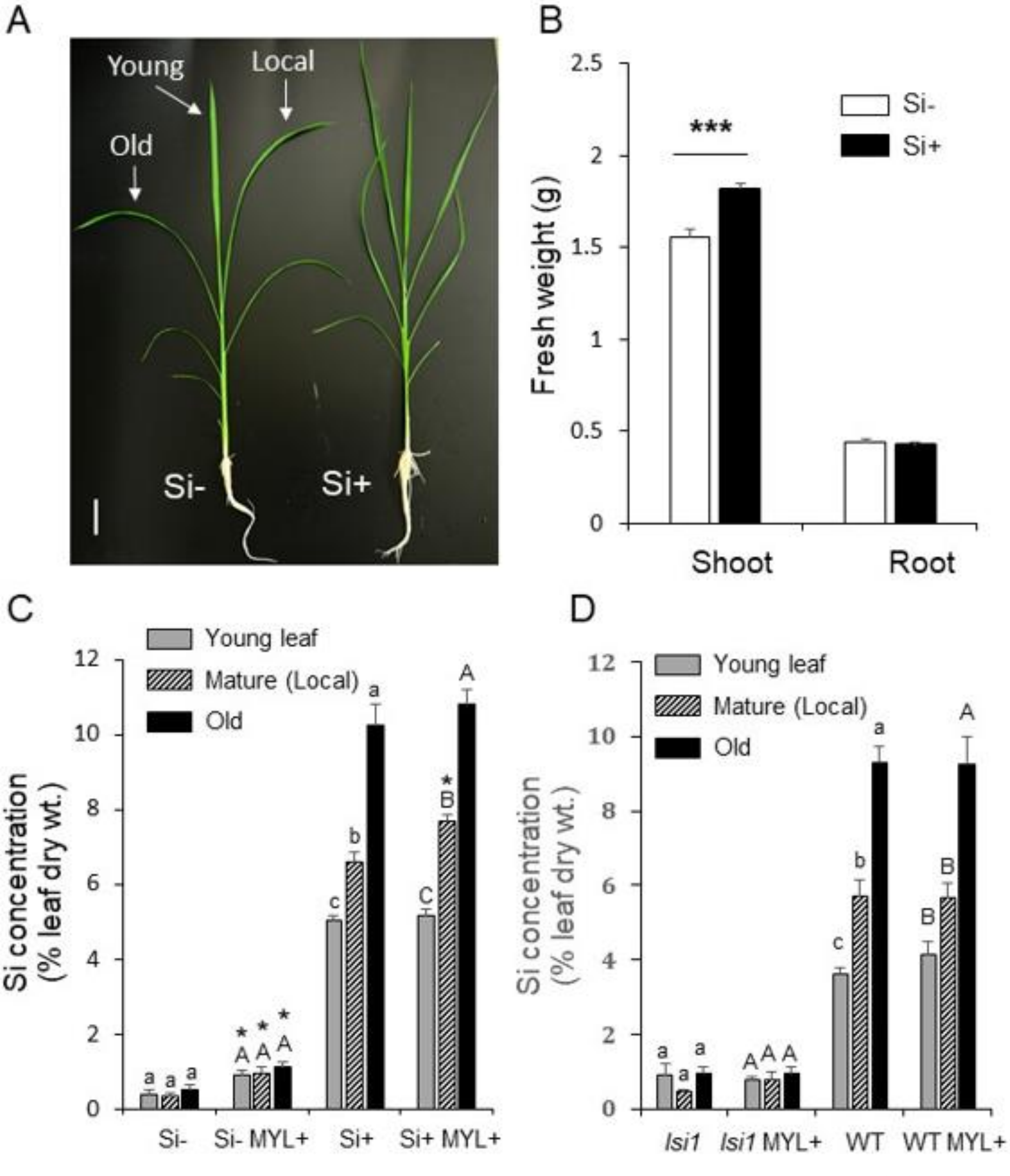

**Figure 7. Silicon accumulation and impact on growth of rice plants.** (A) Growth phenotype of WT rice plants; (B) Shoot and root fresh weight of WT (n=15); (C) Accumulation of Si in leaves of WT plants grown in hydroponic solution, and (D) Si transporter deficient mutant (*lsi1*) and the corresponding WT plants grown in pots with paddy field soil after 48 h exposure to *M. loreyi* feeding (MYL). WT and core *lsi1* mutant rice plants were grown for 7 weeks before herbivore exposure. Statistical differences between treatments (with or without herbivory) were analyzed by Student’s t-test (*P<0.05; ***P<0.001). Different letters (a-c or A-C) indicate significant differences (P≤0.05) between different leaf ages determined separately for each group (Si or herbivory treatments) according to ANOVA followed by Tukey’s HSD test. Scale bar indicates 5 cm.

A short-term Si uptake experiment, wherein the non-Si treated plants were either exposed to Si alone or in combination with insect infestation, showed somewhat different pattern. Firstly, higher Si distribution to young, yet expanding leaves was observed, compared to second (mature) and third (older mature) leaves (Figure 8A), suggesting a preferential flow of Si into newly developing tissues. Furthermore, Si accumulation tended to be higher in all leaves of MYL-exposed plants, whilst the difference was only significant in the locally exposed, i.e. second (mature) leaf 24 h after exposure to herbivory (Figure 8A). In addition, spatial distribution of Si in rice leaves after short term supply of Si was explored by SEM coupled to energy dispersive X-ray microanalysis (EDX) (Figure 8B). Si was similarly distributed in the control rice leaves and MYL-fed leaves after 24 h of Si uptake, namely, Si was deposited in silica bodies and hardened spikes. Notably, EDX was not able to capture the subtle quantitative differences observed in chemically-analyzed rice leaves with and without exposure to herbivory (Figures 7C, 8A).

## Transcriptional responses of Si transporter genes to herbivory stress

While Lsi1 and Lsi2 are required for Si uptake by roots (Ma et al., 2006; Ma et al., 2007b), Lsi6 and SIET4 have important roles in the distribution and deposition of Si in rice leaves (Yamaji et al., 2008; Mitani-Ueno et al., 2023). Because real herbivory experiments are difficult when precise timing is required, such as determination of early gene expression patterns, we used a simulated herbivory approach. In this method, mechanical wounds introduced by a fabric pattern wheel are treated with oral secretions (WOS) isolated from MYL larvae, after which the transcript levels of *Lsi1* and *Lsi2* in the roots, and *Lsi6* and *SIET4* in the leaves of Si-supplemented rice, untreated or exposed to herbivory, were analyzed.

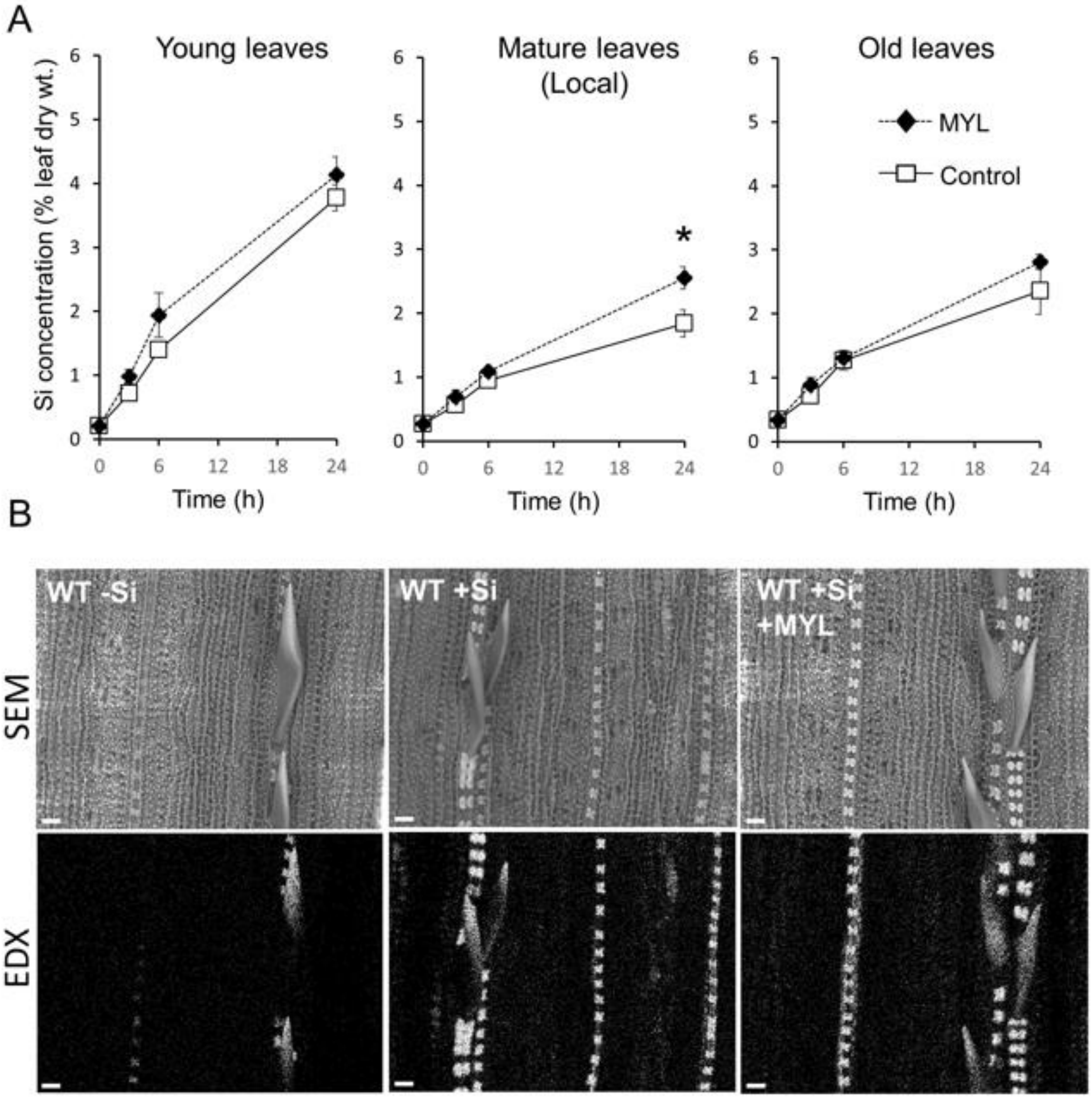

**Figure 8. Distribution and deposition patterns of Si in rice exposed to herbivory stress.** (A) Si concentration in different rice leaves after short-term exposure to Si and *M. loreyi* feeding (MYL), data are mean ± SE (n=5). (B) Scanning electron micrographs with the corresponding Si EDX map images of mature leaf surface of rice plants. WT rice plants (7 weeks) grown in Si-free hydroponic solution were transferred to a hydroponic solution containing 0.5 mM Si and exposed to *M. loreyi* feeding for 24 h. At indicated time intervals, mature herbivore fed (local), and systemic (young, old) untreated leaves were collected for Si determination. After 24 h exposure to either Si alone or in combination with herbivore feeding, 4 mm leaf discs were excised from mature leaf of herbivore fed and control leaves and subjected to SEM-EDX imaging. Scale bars indicate 20 µm. Statistical differences between treatments (Control and herbivory) were analyzed by Student's t-test (*P<0.05; no symbol, not significant).

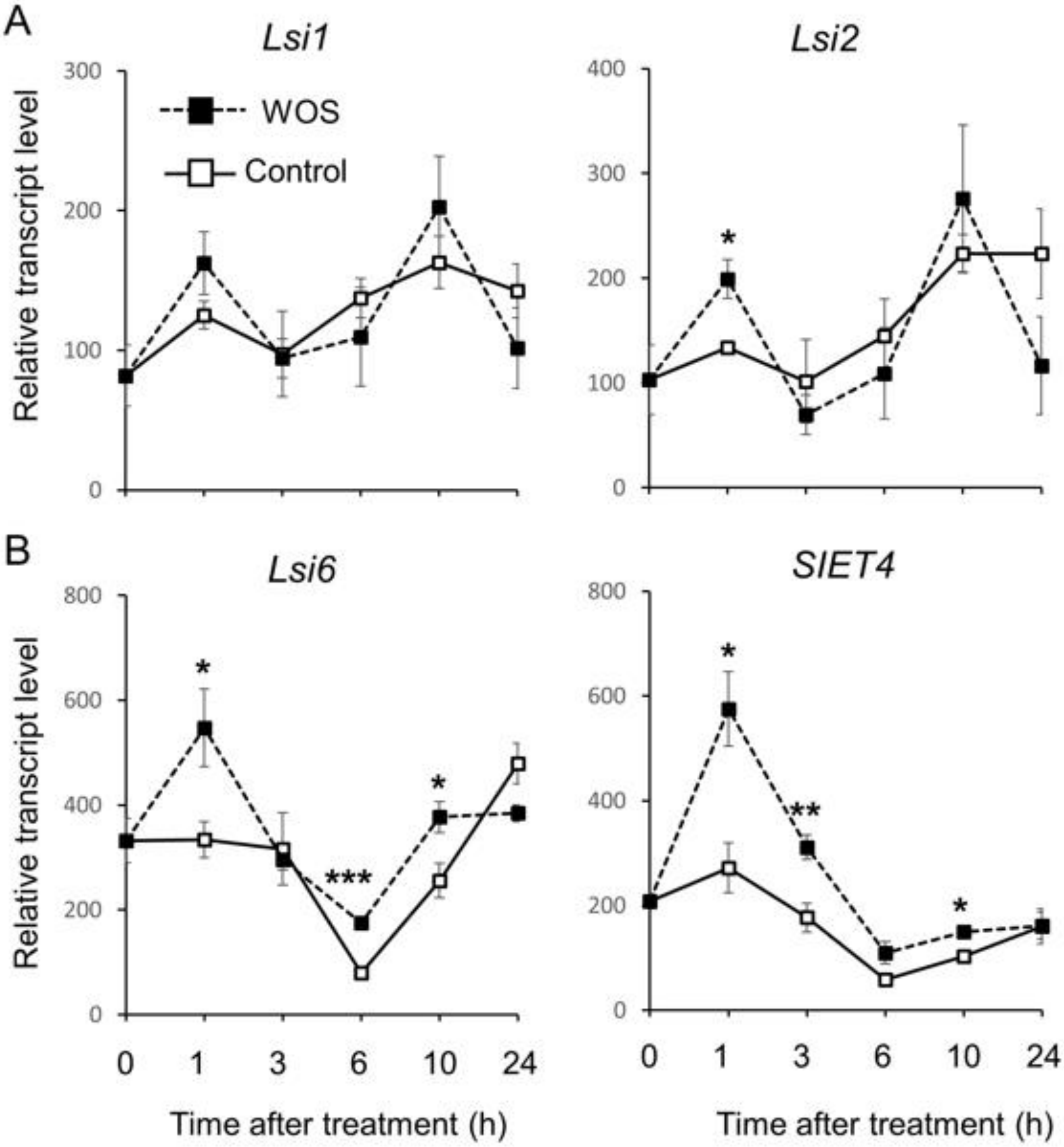

**Figure 9. Relative transcript levels of Si transporter genes in WT rice plants after exposure to herbivory stress.** Transcript levels of *Lsi1* and *Lsi2* in roots (A), and of *Lsi6* and *SIET4* in fully expanded rice leaves (young and mature leaves; (B). Gene expression was determined at indicated time intervals by qRT-PCR in 7-week-old WT rice plants before and after elicitation with wounds treated with oral secretions from *M. loreyi* (WOS). Data are mean ± SE (n=4). Statistical differences between treatments (Control and WOS) were analyzed by Student's t-test (*P<0.05; **P<0.01; ***P<0.001 no symbol, not significant).

The *Lsi1* and *Lsi2* were only transiently induced in the roots 1 h after WOS elicitation (Figure 9A). In contrast, *Lsi6* and *SIET4* transcripts were clearly up-regulated 1 h after WOS treatment and gradually decreased, albeit still showing higher transcript levels relative to control untreated plants at time points between 3 and 10 h post treatment (Figure 9B). This data shows that distribution rather than additional uptake of Si may be regulated by herbivory stress to mount the effective plant defense.

### Primary metabolite changes after exposure to Si and/or herbivory

As Si-deprived rice plants grew significantly less in hydroponic media (Figure 7B), we hypothesized that Si supply may be altering the primary metabolic pathways in rice. We therefore performed an untargeted metabolomics screening by GC-MS, using N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA)-derivatized leaf extracts prepared from hydroponic rice plants. The plants kept for 7 weeks in the continuous presence or absence of Si, exposed to MYL feeding (or WOS), or left untreated for 24 hours, were used for analysis. The impact of Si and herbivory on primary metabolism was estimated from the levels of detectable sugars, amino acids, and organic acids. The local MYL- or WOS-treated mature leaves (second leaf), one stage younger developing, and one stage older mature leaves were used to estimate the overall effect of Si and herbivory on the aboveground metabolism of rice. Using a principal component analysis (PCA) tool in MetaboAnalyst, although obtaining a fairly complex picture, certain patterns could be depicted. The samples from Si-cultivated plants generally tended to differ in PCA plots from those kept in Si-free media, and separated along the PC1 axis (Figure 10). Interestingly, samples from the MYL-exposed local leaves in Si-free media (red dots) were well separated from the controls, following PC2 axis direction, however, MYL-treated and control leaves from Si-supplemented media (green dots) partly overlapped (Figure 10). It suggests that MYL feeding differentially affects metabolic pathways in Si-dependent manner. Similar trends were observed in WOS-treated plants (Figure 10), although WOS effects were less pronounced compared to direct MYL feeding. Notably, direct MYL herbivory affected primary metabolic profiles in the older systemic leaves, while this was not the case in WOS treatments (Figure 10), suggesting that a stronger systemic signaling requires real feeding stimulus.

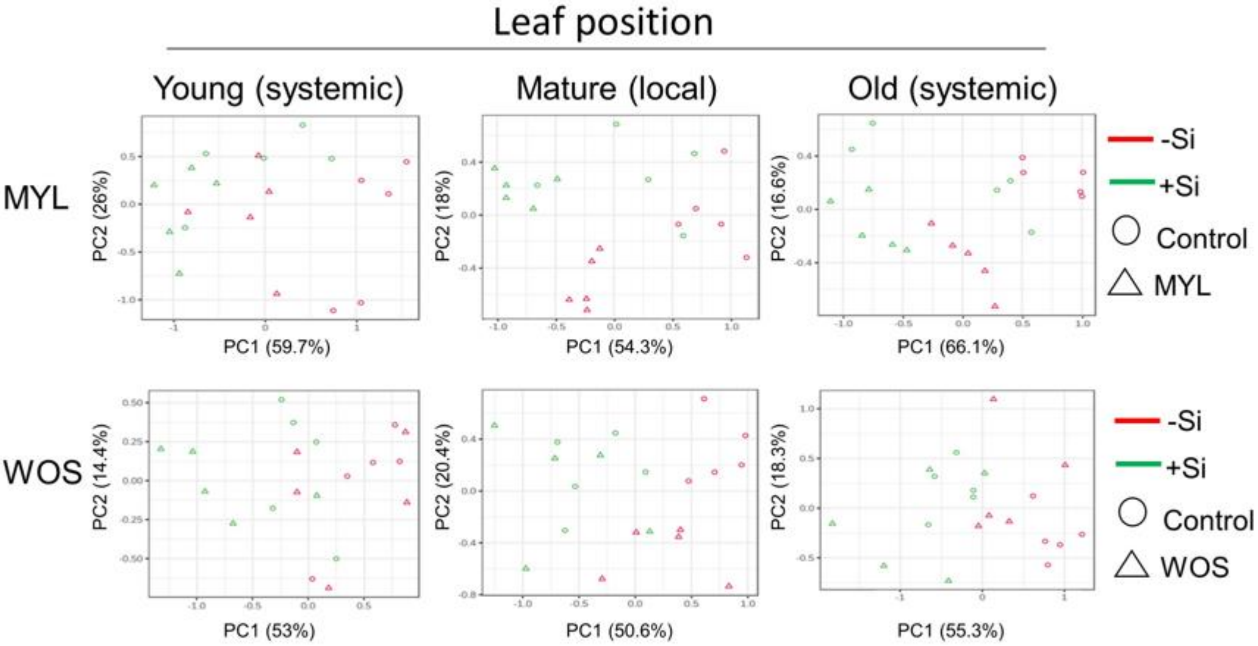

**Figure 10. Primary metabolite changes after exposure of rice plants to Si alone or in combination with herbivory stress.** Principal component analysis (PCA) of the primary metabolites accumulation in hydroponically grown WT rice plants supplied with or without Si and subjected to either *M. loreyi* feeding (MYL) or elicitation with wounds treated with oral secretions from *M. loreyi* (WOS) for 24 h.

Finally, we performed a Pearson's correlation analysis implemented in MetaboAnalyst to estimate the importance of each primary metabolite in separation of samples after herbivory exposure and/or Si +/- supplementation in rice. While the accumulation of sugars was generally positively correlated with the presence of Si, multiple amino acids showed a negative correlation with Si (Figure 11A). On the other hand, MYL treatment highlighted the importance of GABA as the main factor positively correlated with the exposure of rice to herbivory (Figure 11B). GABA accumulation in *Arabidopsis* was previously shown to be induced by simulated herbivory as well as by real *S. littoralis* Boisduval feeding (Scholtz et al., 2015).

In order to see if primary metabolic changes could be due to Si-dependent alteration of photosynthesis, we used a portable photosynthesis system (LI-COR) to determine the photosynthetic rates (Pn), transpiration rates (Tr), stomatal conductance (Gs), and intrinsic water use efficiency (iWUE) (Figure 12). The results show that Pn was not influenced by Si, however, it decreased after WOS treatment, similar to Gs and Tr (Figure 12). In addition, Gs significantly decreased in Si-supplemented plants treated with WOS, and as a result, iWUE was higher in these plants. Chlorophyll contents estimated with SPAD were not largely affected by affected by either Si supply or simulated herbivory treatments over the time period tested (Figure 13). However, regardless of Si or herbivory treatments, young, yet expanding leaves generally contained lower amounts of chlorophyll relative to the second (mature) and third (older mature) leaves (Figure 13A-C).

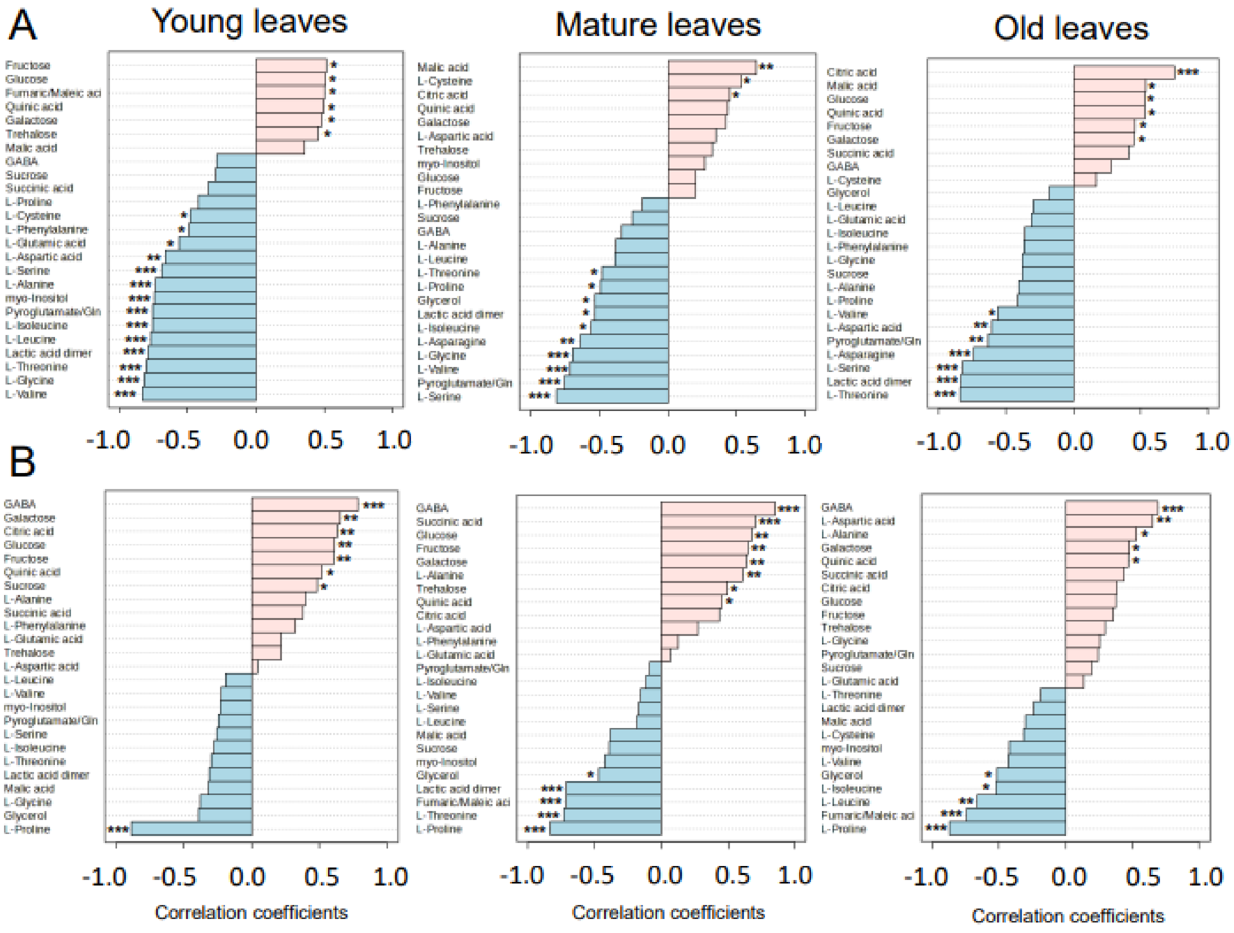

**Figure 11. Primary metabolite changes after exposure of rice plants to Si alone or in combination with herbivory stress.** Correlation plot showing association between primary metabolites and (A) Si amendment or (B) *M. loreyi* (MYL) herbivory treatment in rice leaves. Asterisks indicate p-values (*P<0.05; **P<0.01; ***P<0.001 no symbol, not significant).

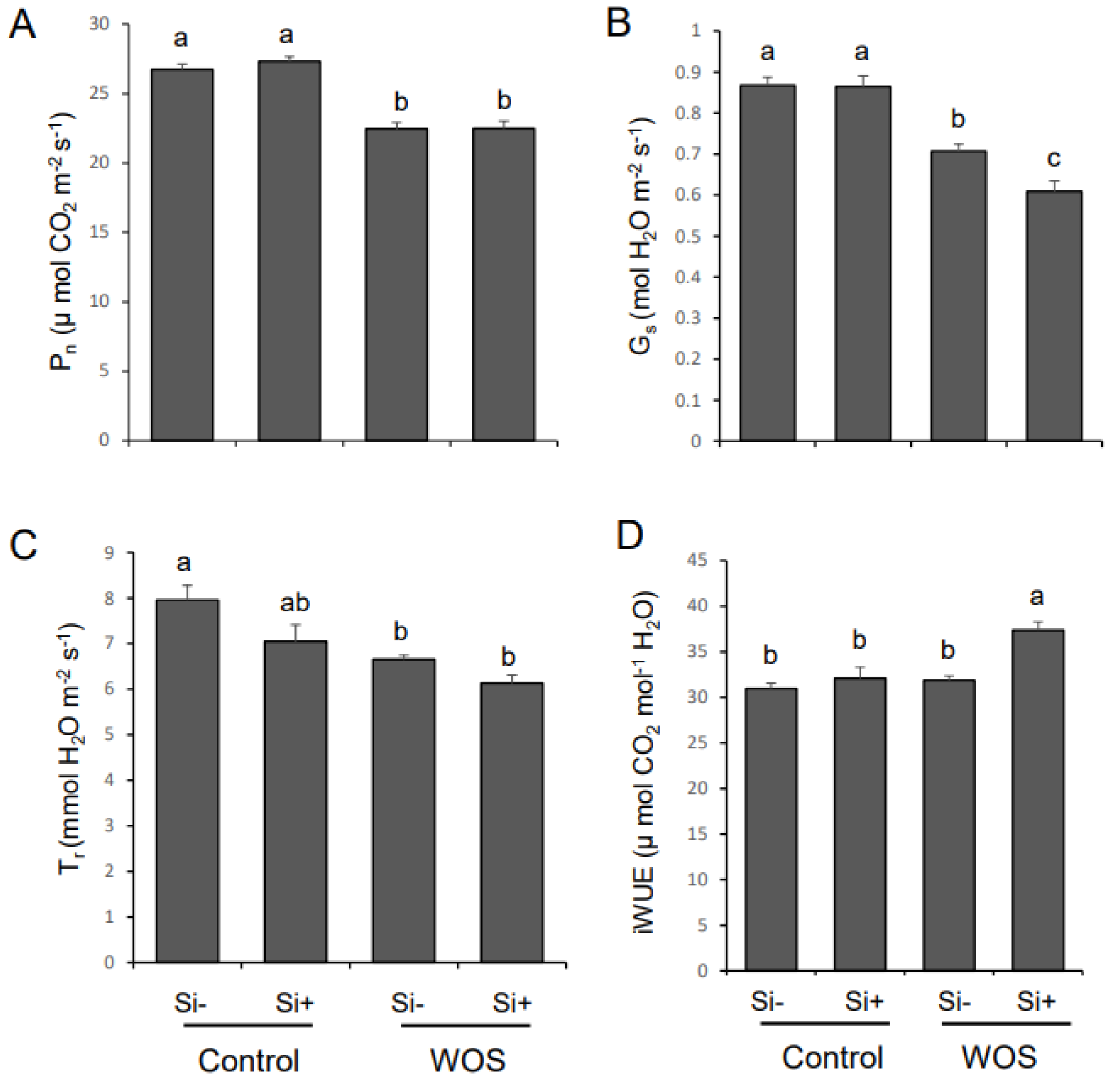

**Figure 12. Impact of Si on leaf photosynthetic parameters of rice exposed to herbivory stress.** (A) Net photosynthetic rates, (B) Stomatal conductivity, Gs (C) Transpiration rate, Tr (D) Intrinsic water-use efficiency, iWUE. Photosynthetic parameters were measured in mature leaves of 7-week-old WT rice plants before and after elicitation with wounds treated with oral secretions from *M. loreyi* (WOS). Data are means ± SE (n=5 for Si treatments, n=4 for WOS treatments). Data followed by different letters (a-c) indicate significant difference (P≤0.05) according to ANOVA followed by Tukey's HSD test.

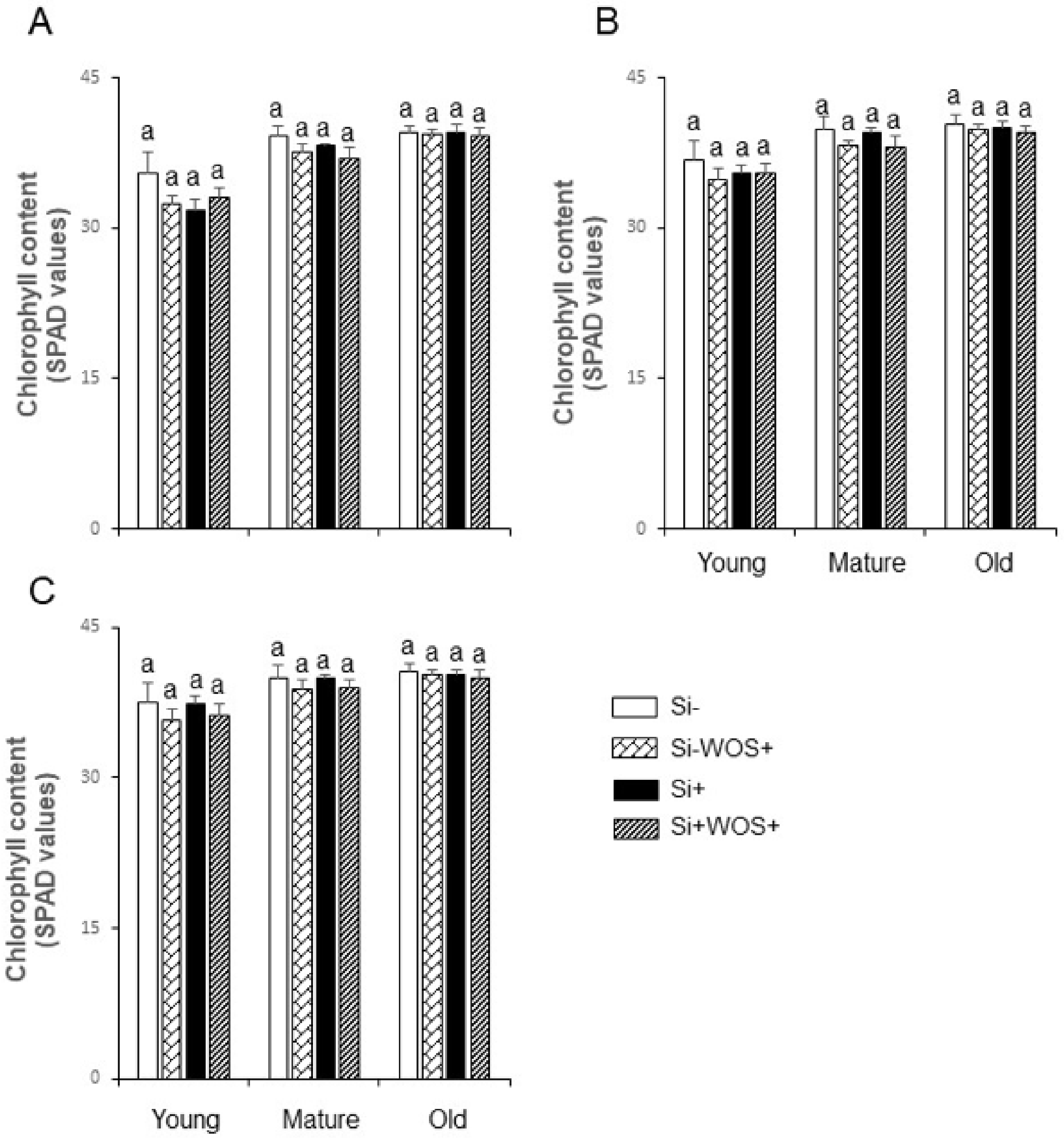

**Figure 13. Impact of Si on leaf chlorophyll content of rice exposed to herbivory stress.** Chlorophyll content in rice leaves as measured by SPAD after (A) 24 h, (B) 48 h, and (C) 72 h of elicitation of the mature leaves with wounds treated with oral secretions from *M. loreyi* (WOS) or untreated. Chlorophyll contents were measured in young, mature and old leaves of 7-week-old WT rice plants grown hydroponically with or without Si supply. Data are means ± SE (n=4). Similar letters within treatments in a leaf age indicate no significant difference (P≤0.05) according to ANOVA followed by Tukey's HSD test.

## Secondary metabolite levels in rice leaves exposed to Si and herbivory

We next examined the accumulation of defense-related specialized (secondary) metabolites in Si-supplemented and Si-deprived rice in hydroponic media, and *lsi1* mutants and the corresponding WT plants grown in pots with paddy field soil, exposed to either MYL infestation or WOS treatment. In the hydroponic experiments, Si supply did not affect the constitutive levels of IPA, but two PAs, CoP and FP in rice leaves increased (Figure 14A). In the soil experiments using rice mutants impaired in Si uptake, deficiency in Si transport system in the mutants did not affect the constitutive levels of IPA and FP, but levels of CoP were higher in the WT plants relative to the *lsi1* mutants (Figure 14B). When exposed to MYL feeding for 48 h, Si-supplied and WT rice leaves accumulated more IPA, CoP and FP relative to Si-deprived and *lsi1* mutant leaves, respectively (Figure 14). Similarly, WOS treatment induced more IPA, CoP and FP in Si-supplied compared to Si-deprived rice leaves (Figure 14). Overall, we detected more direct defense metabolites in Si-supplied leaves exposed to real or simulated herbivory stress, which was somewhat consistent with the more abundant hydrocarbon levels, such as sugars found in the Si-supplied rice leaves (Figure 11A). More so, the defense metabolites accumulation patterns in the WT and *lsi1* mutants grown in paddy soil with normal Si levels further suggest that Si may be mediating the contents of these metabolites in rice under herbivore attack.

## Impact of Si on herbivore-induced plant volatile (HIPV) emissions in rice

In addition to direct defense, plants resist herbivores indirectly, i.e., through emissions of VOCs that attract natural enemies of herbivores. Taking into account the changes in primary and secondary metabolite levels after Si supplementation, we decided to test whether Si can also modify the emissions of VOCs in rice under herbivory stress. We collected and analyzed both headspace and internal VOCs from Si-supplied and Si-deprived plants after WOS treatment by GC-MS method. Constitutive levels of VOCs were not largely affected by Si presence, except for indole and sesquiterpenes, β-elemene and β-caryophyllene, which showed higher constitutive levels in Si-supplied compared to Si-deprived plants (Figures 15&16). Similarly, constitutive levels of internal VOC pools were generally not affected by Si supply, but methyl salicylate (MeSA) showed higher constitutive levels in Si-supplemented relative to Si-deprived plants (Figures 15& 16). After WOS treatment, Si-supplemented plants emitted higher quantities of numerous VOCs, including monoterpenes (myrcene, trans-β-ocimene, linalool), sesquiterpenes (nerolidol, β-elemene, β-caryophyllene), indole, and methyl salicylate, relative to Si-deprived plants (Figures 15&16). On the other hand, Si supply did not affect the accumulation internal VOCs except for the higher levels of MeSA and β-caryophyllene in Si-supplied and Si-deprived plants, respectively after WOS treatments (Figure 16). These data suggest that volatiles in rice headspace can be promoted by Si, which could be either because of the higher release and/or biosynthesis of these compounds. Subsequently, the expression of several volatile-related genes in rice was examined.

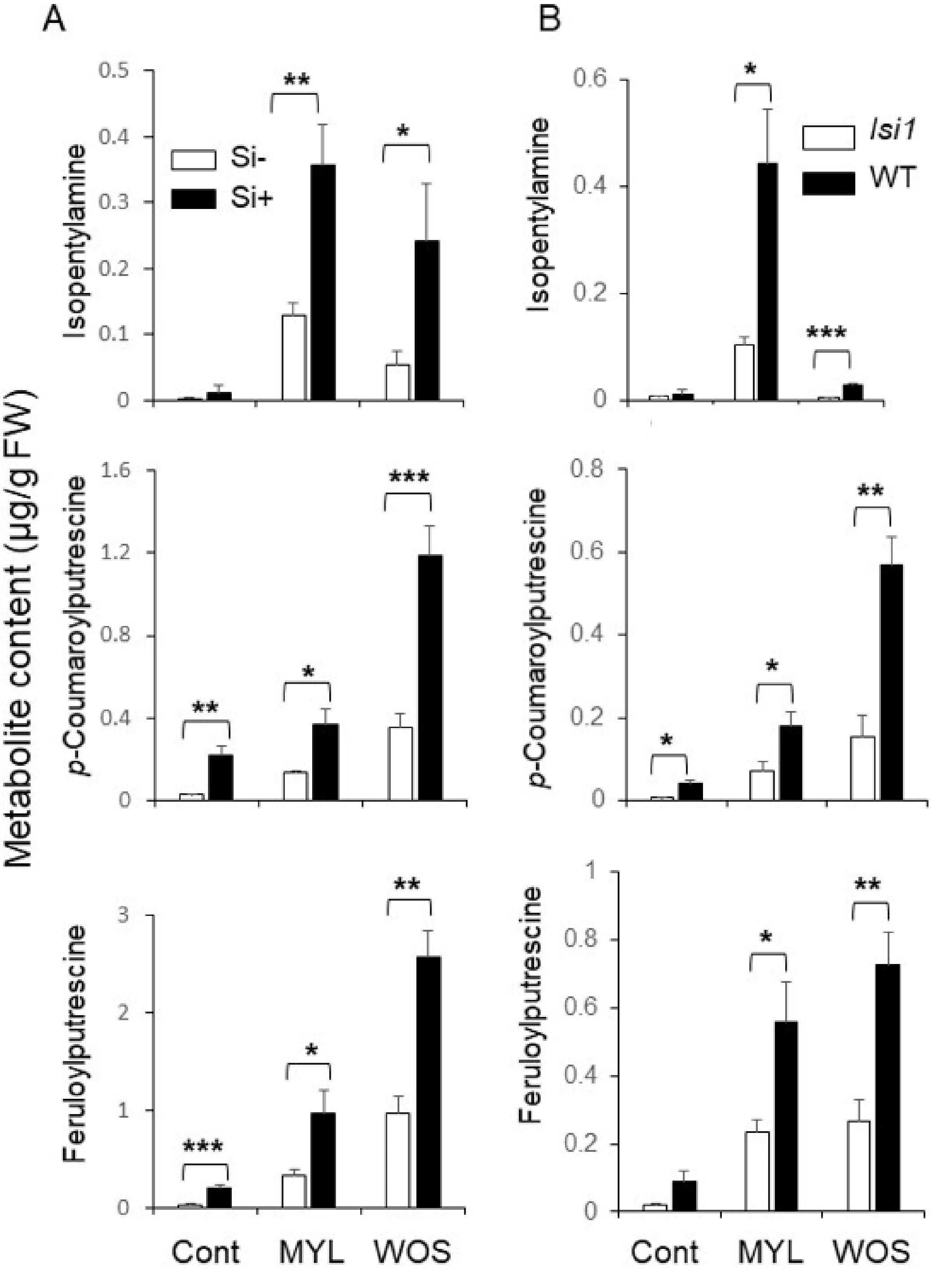

**Figure 14. Effect of Si application on constitutive and induced levels of secondary metabolites in rice subjected to herbivory stress.** Metabolite levels were determined with (A) WT rice plants grown hydroponically with or without 0.5 mM Si; (B) *lsi1* mutants and the corresponding WT rice plants grown hydroponically with continuous supply of 0.5 mM Si for 7 weeks (Cont., Control) and then subjected to either *M. loreyi* larvae feeding (MYL) or wounds treated with oral secretions from *M. loreyi* (WOS) for 48 h. Data are means ± SE (n=5 for A, n=4 for B). Statistical differences within treatments (with and without Si) were analyzed by Student's t-test (*P<0.05; **P<0.01; ***P<0.001; no symbol, not significant).

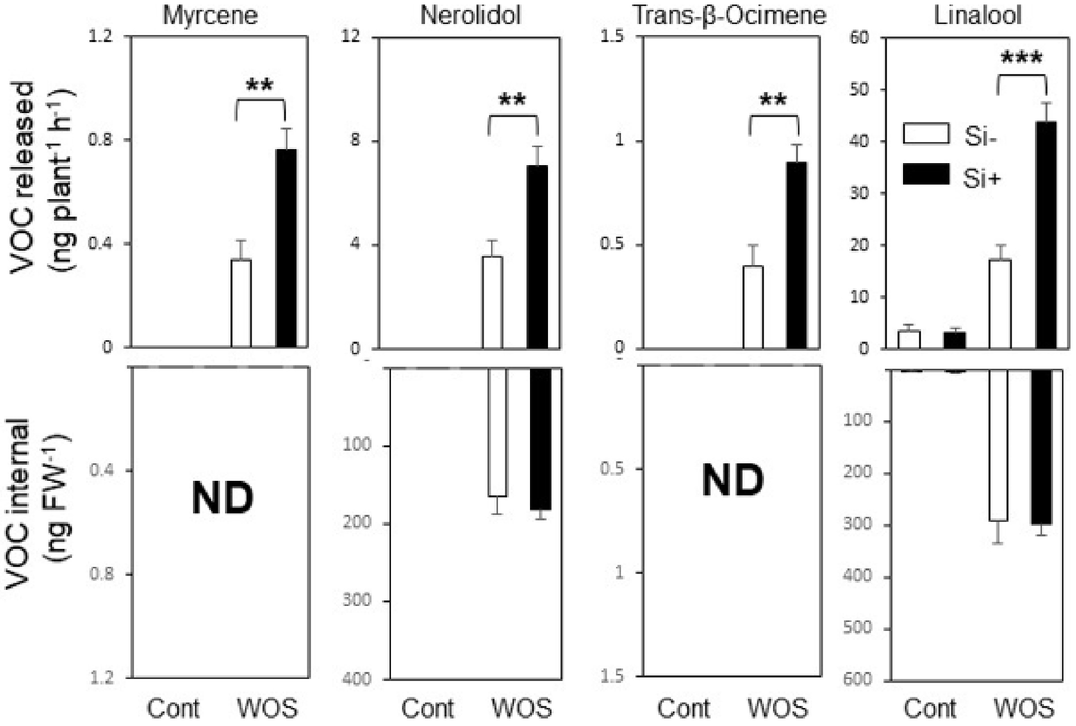

**Figure 15. Si and WOS-regulated accumulation and release of selected HIPVs from rice leaves.** Released and internal volatiles from 8-weeks old WT rice plants grown hydroponically with or without 0.5 mM Si (Cont., Control), and then subjected to wounds treated with oral secretions from *M. loreyi* (WOS) for 24 and 4 h, respectively were collected and analyzed by GC-MS. WOS treatment was performed on two fully expanded leaves (youngest and mature). Data are mean relative amount ± SE (peak normalized to tetralin) (n=6 for VOC released, n=8 for VOC internal). Statistical differences within treatments (Control and herbivory) were analyzed by Student's t-test (**P<0.01; ***P<0.001; no symbol, not significant).

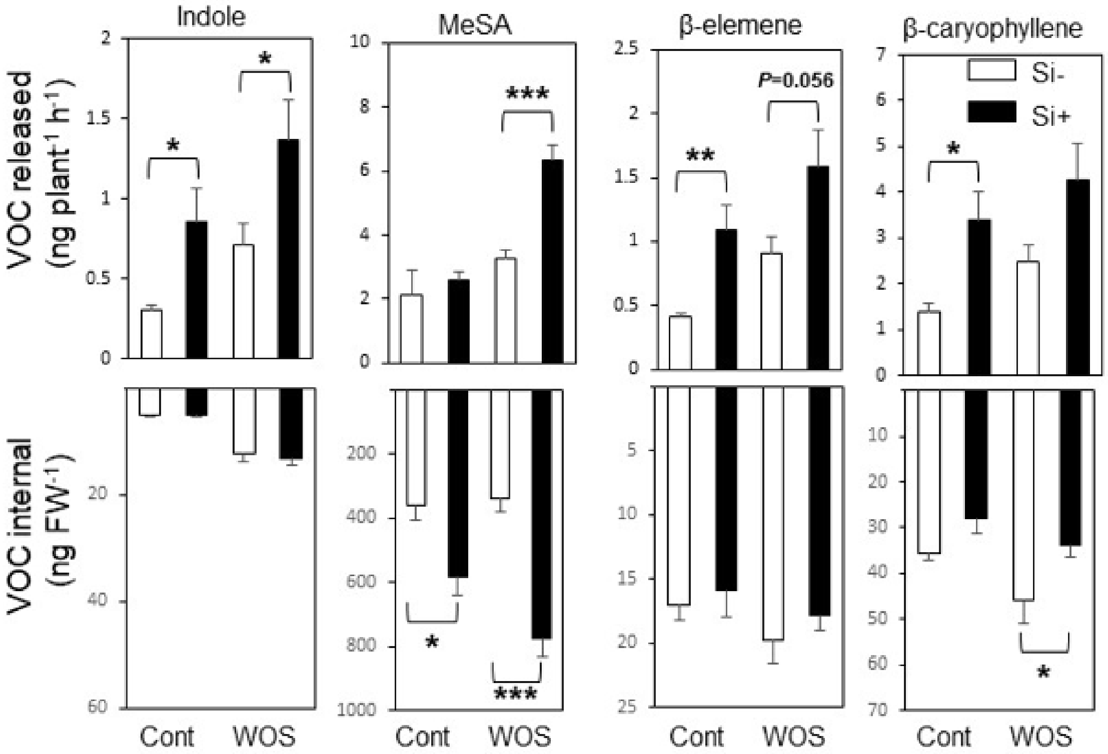

**Figure 16. Si and WOS-regulated accumulation and release of non-HIPVs from rice leaves.** Released and internal volatiles from 8-weeks old WT rice plants grown hydroponically with or without 0.5 mM Si (Cont., Control), and then subjected to wounds treated with oral secretions from *M. loreyi* (WOS) for 24 and 4 h, respectively were collected and analyzed by GC-MS. WOS treatment was performed on two fully expanded leaves (youngest and mature). Data are mean relative amount ± SE (peak normalized to tetralin) (n=6 for VOC released, n=8 for VOC internal). Statistical differences within treatments (Control and herbivory) were analyzed by Student's t-test (*P<0.05; **P<0.01; ***P<0.001; no symbol, not significant).

### Expression of terpene and phenylpropanoid-related genes

Stimulation of VOC release from rice is dependent on the increased expression of specific biosynthetic genes, such as *linalool synthase* (LIS) and *S-adenosyl methionine methyltransferase* (SAMT), involved in the rice linalool and methyl salicylate biosynthesis, respectively (Mujiono et al., 2020). When we measured the expression of *LIS*, induction of this gene was more profound in Si-containing plants relative to Si-deprived ones. However, this trend was not reflected in the expression of DXS3 located upstream in terpenoid biosynthetic pathway (Figure 17). The transcript levels of *caryophyllene synthase* (CAS) were not induced by WOS as reported previously (Mujiono et al., 2020), and Si showed no consistent effect on expression of this gene. The transcript levels of phenylalanine ammonia lyase (*PAL*) and *SAMT* for MeSA were both elevated by Si, providing a support for the observed higher levels of MeSA in Si-supplemented rice headspace. In contrast to linalool and MeSA, increased levels of β-caryophyllene in rice headspace could not be explained by corresponding gene transcript data, suggesting that Si may be promoting release of β-caryophyllene by another mechanism.

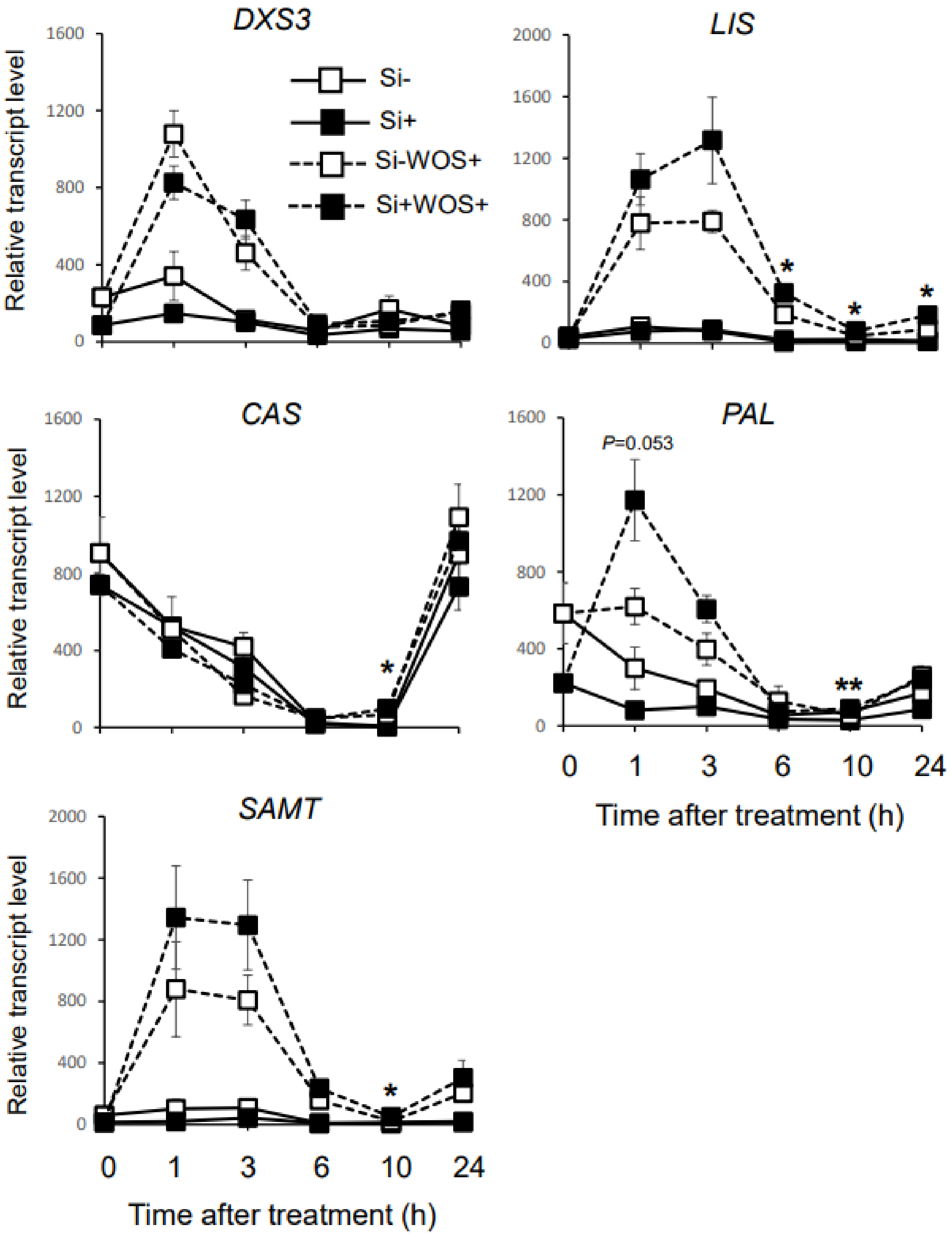

**Figure 17. Relative transcript levels of VOC-related genes in rice plants after Si supplementation and WOS treatments.** WT rice plants were grown hydroponically with or without 0.5 mM Si (Control), subjected to wounds treated with oral secretions from *M. loreyi* (WOS). Gene expression was determined in control and WOS-treated fully expanded young and mature rice leaves by qRT-PCR. Data are means ± SE (n=4). Statistical differences within pairs of treatments (Control or herbivory) were analyzed by Student's t-test (*P<0.05; **P<0.01; ns, not significant).

### Impact of Si on JA accumulation in rice exposed to herbivory stress

Since jasmonic acid is a key factor in regulation of plant defense responses against insect herbivores, including specialized metabolism and VOC release, we examined the accumulation of JA and JA-Ile in Si-supplied and deprived rice plants exposed to MYL infestation (Figure 18A). Si supplementation alone did not significantly alter the basal JA and JA-Ile levels in the observed 24 h period (Figure 18A). As expected, both hormones were strongly induced by MYL feeding, which surprisingly showed a more profound but non-significant trend in Si-deprived compared to their Si-supplemented plants (Figure 18A). This trend was in contrary to our initial hypothesis that more specialized metabolites and VOCs could be due to elevation of defense signaling, based on the Si-mediated modulation of jasmonate levels. As we suspected that more feeding activity in Si-free plants (Figure 6) could be directly promoting jasmonates, we followed with the more standardized WOS treatment to test this possibility. Indeed, the levels of JA and JA-Ile were similar in most examined time points, except for 1 h post elicitation, where JA was still higher in Si-deprived plants exposed to WOS (Figure 18B). These results generally rule out the possibility that higher metabolite levels in Si-supplemented plants could be due to higher levels of jasmonates after Si supplementation. In other hormones, abscisic acid (ABA) levels increased after MYL infestation and WOS treatment, however, Si supply did not cause any further changes (Figure 19). Salicylic acid (SA) levels tended to be higher in Si-deprived media, showing a transient increase induced by MYL feeding 1 h post infestation (Figure 19).

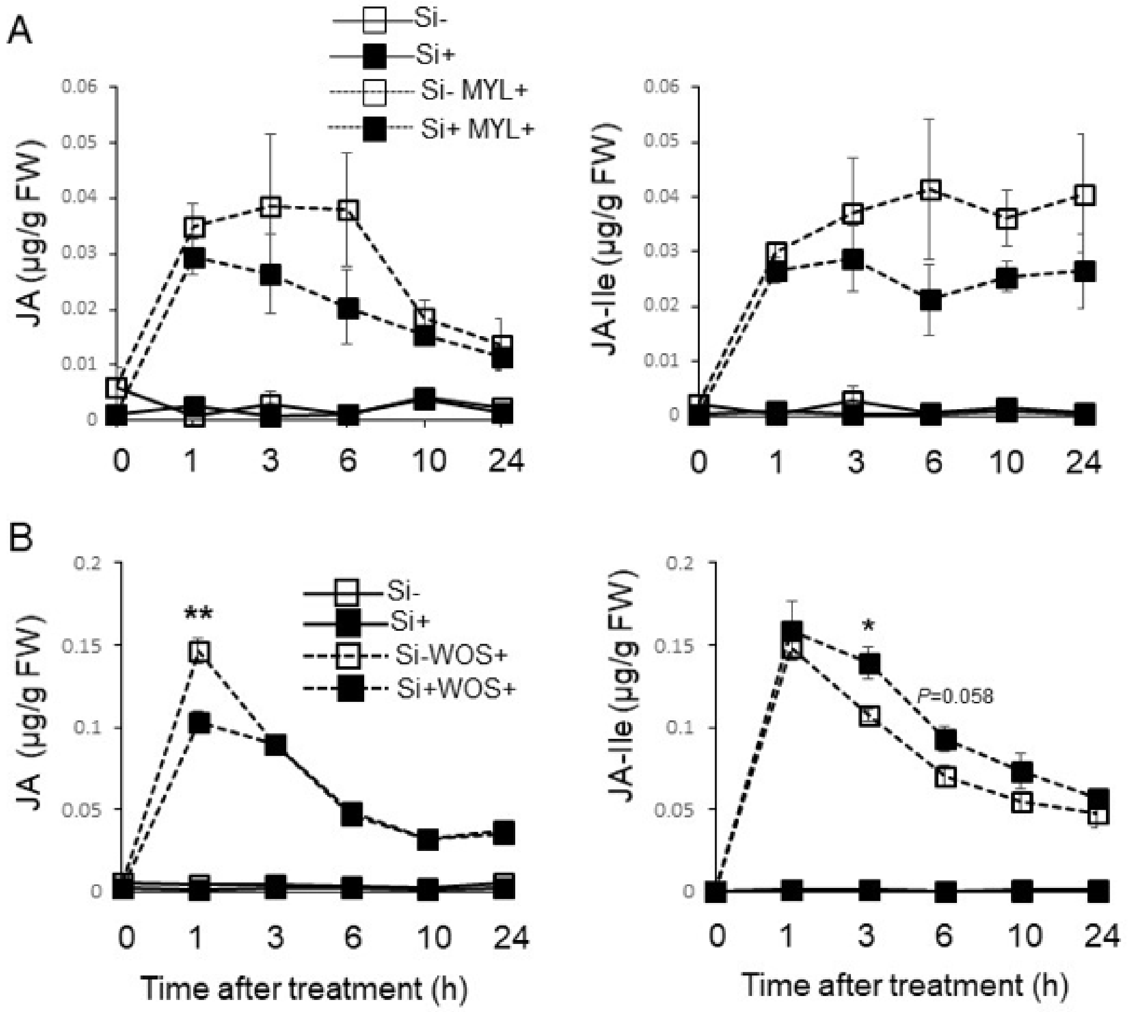

**Figure 18. Si and herbivory-regulated accumulation of phytohormones in rice leaves.** Jasmonic acid (JA) and jasmonoyl-L-isoleucine (JA-Ile) levels in mature rice leaves exposed to *M. loreyi* feeding (MYL; A) or in fully expanded young and mature rice leaves elicited with wounds treated with oral secretions from *M. loreyi* (WOS; B). Data are means ± SE (n=4). Statistical differences within pairs of MYL or WOS treatments were analyzed by Student's t-test (*P<0.05; **P<0.01; no symbol, not significant).

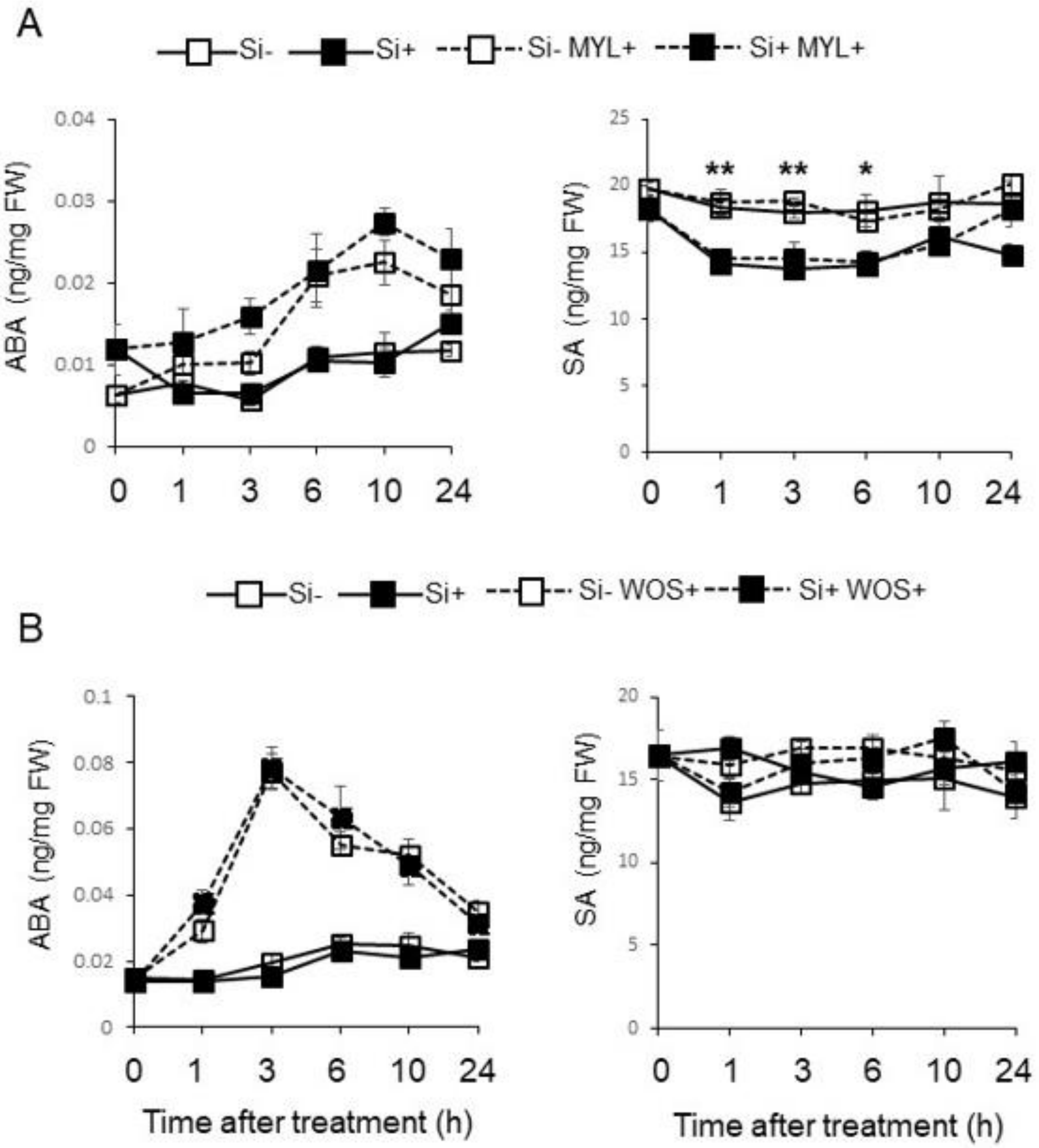

**Figure 19. Si and herbivory-regulated accumulation of phytohormones in rice leaves.** Abscisic acid (ABA) and salicylic acid (SA) levels in mature rice leaves exposed to *M. loreyi* feeding (MYL; A) or in fully expanded young and mature rice leaves elicited with wounds treated with oral secretions from *M. loreyi* (WOS; B). Data are means ± SE (n=4). Statistical differences within pairs of MYL or WOS treatments were analyzed by Student’s t-test (*P<0.05; **P<0.01; no symbol, not significant).

### Ecological relevance of Si-regulated VOC releases

VOCs serve as danger as well as host-location cues for ovipositing females of herbivores (Achhami et al., 2021). We therefore investigated if differential emission of VOCs from Si-supplemented and Si-deprived rice plants, untreated or exposed to simulated herbivory, could change the oviposition behavior of MYL females. In cage choice-type experiments, Si alone did not affect the oviposition choice of MYL, even after 2 d of interaction with the plants (Figures 20A, B). However, in WOS-treated plants, more eggs were deposited on Si-deprived (67%) compared to Si-supplemented plants (31%) at 1 d after plant exposure to gravid moths (Figure 20A), which remained significant until two days (Figure 20B). Preference for Si-deprived rice plants treated with WOS, which generally released less volatiles (Figures 15&16), suggests that MYL females may be choosing a more suitable host plant based on the lower amounts of volatiles, which may signal a lower defense capacity of the host to herbivores. On the other hand, silicon impregnation of leaves in control set of plants without herbivory did not seem to discourage MYL from laying eggs, although, presumably, the neonates would need to face a stronger feeding challenge after hatching from the eggs.

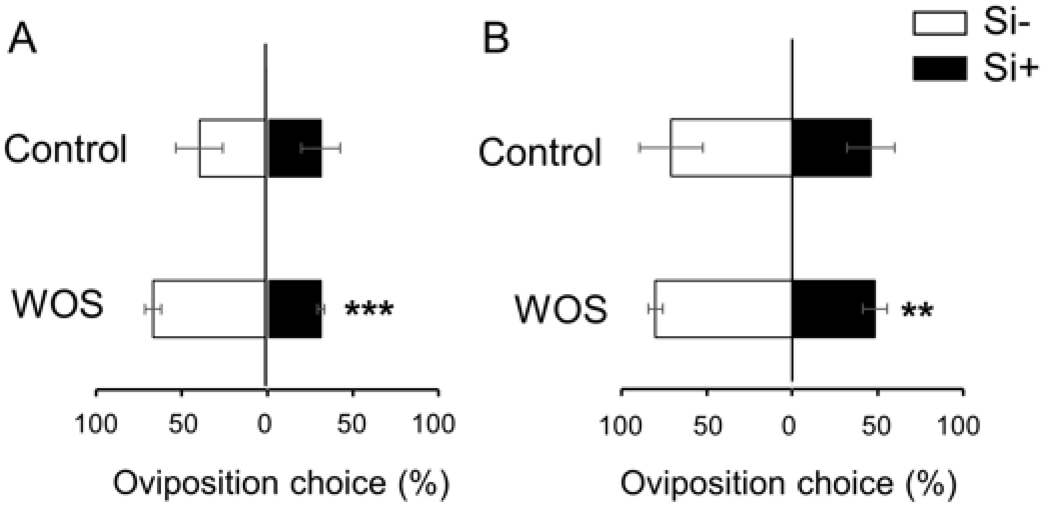

**Figure 20. Effects of Si application on oviposition preference of gravid *M. loreyi* moths on rice plants.** Oviposition choice of gravid *M. loreyi* moths (n=20) after 1 d (A) or 2 d (B) of release on WT rice plants grown hydroponically with or without 0.5 mM Si (Control) or subjected to wounds treated with oral secretions from *M. loreyi* (WOS) (n=12). WOS treatment was performed on fully expanded young and mature leaves. Data are means ± SE of four independent biological replicates. Statistical differences within pairs of treatments (with and without Si) were analyzed by Student's t-test (**P<0.01; ***P<0.001; no symbol, not significant).

# Discussion

## Si accumulation and rice defense against chewing herbivores

Rice accumulates up to 10% of Si in the shoots on dry weight basis, which is known to improve the grain yield, in addition to mitigating damages caused by environmental stresses (Tamai and Ma, 2008; Ma 2004). In this study, Si supplementation increased the resistance of rice to larvae of MYL, which is a generalist chewing herbivore, relative to Si-deprived plants. However, Si-deprived rice supplied with Si 3 d before exposure to larvae could restore their resistance to the level comparable with the plants continuously grown in the Si-containing media (Figure 6B). This was consistent with the feeding performance of MYL larvae on cv. Nipponbare mutant *lsi1*, which is impaired in Si uptake (Suzuki et al., 2012), grown in the paddy field soil with normal Si levels in the laboratory (Figure 6A). Previously, extensive field damage was reported in similar *lsi1* mutants with cv. Oochikara genetic background in the field (Ma et al., 2006; Tamai and Ma, 2008). Interestingly, *lsi1* mutants tend to accumulate more lignin, possibly as a structural counter-response to gravity force (Suzuki et al., 2012), which could be also partially reverting the lack of structural toughness against herbivores in the Si-deprived plants.

The accumulation of Si was reported as part of the inducible defense arsenal in plants (Massey et al., 2007; Johnson et al., 2020). While the accumulation of Si differed with position (age) of rice leaves, directly insect-fed leaves contained significantly more Si compared to unfed controls (Figures 7C, 8A). Si is acquired from soil as $Si(OH)_4$ through the cooperation of specific influx and efflux root transporters, Lsi1 and Lsi2 (Ma et al., 2006; Ma et al., 2007b). Subsequently, Si is translocated to shoots by transpiration stream

(Mitani et al., 2005), and then unloaded from xylem into leaf tissues by Lsi6 (Yamaji et al., 2008). Recently, Mitani-Ueno et al., (2023) identified SIET4 as a novel transporter required for proper deposition of Si on the rice leaf surface. Following these current updates, we found that the expression of *Lsi1* and *Lsi2* in the roots was only slightly altered by simulated herbivory (WOS) treatment (Figure 9A). In contrast, transcripts of *Lsi6* and *SIET4* were clearly upregulated in WOS-treated leaves at 1 h post elicitation (Figure 9B), suggesting that distribution and/or deposition rather than *de novo* uptake of Si by roots may be accounted for the higher levels of Si in herbivore attacked leaves. On the other hand, the expression of Lsi1 in roots of rice infested by a specialist chewing herbivore, rice leaffolder (*C. medinalis*), was induced by approximately 2-fold (Lin et al., 2019), suggesting that stimulation of root Si uptake at transcriptional level may be dependent on herbivore species, or in particular, specialist vs. generalist features of the attackers.

## Impact of Si and herbivory stress on rice metabolic profiles

Earlier studies proposed that Si amendment alters basal gene expression in rice, which may be subsequently changing metabolic profiles, possibly as means of priming prior to and/or during stress responses (Brunings et al., 2009; Van Bockhaven et al., 2015; Frew et al., 2018; Jiang et al., 2022). In plant-pathogen interactions, application of Si to rice plants mediated differential expression of genes involved in nitrogen metabolism and transport, glycolysis and cell wall biosynthesis and degradation, while the amino acid metabolism-related genes were down-regulated (Van Bockhaven et al., 2015). In this study, we revealed a strong impact of Si on primary metabolites (e.g., soluble sugars, amino acids), which serve as structural and energy resources in plants, among others, used for the biosynthesis of specialized defense metabolites during herbivory stress (Wari et al., 2022). Soluble sugars tended to accumulate more in plants with Si, while amino acids content decreased with Si application (Figure 11A). Against our assumption, leaf photosynthetic activity could not explain the observed Si-associated metabolic changes, such as sugar levels (Figures 12&13). Nonetheless, it is possible that availability of sugars could, at least in part, contribute to increased growth rate of Si-supplemented relative to Si-deprived rice plants (Figure 7B). Previously, rice growth enhancement by Si under hydroponic conditions was reported by Lin et al. (2019) but particular mechanisms were not elucidated. Considering the significantly higher intrinsic water use efficiency (iWUE) in Si-supplemented plants subjected to herbivory stress (Figure 12), it appears that Si amendment positively affects the water usage in rice. Similar to our findings, Jiang et al. (2022) showed that Si application increased the iWUE of rice plants under drought stress.

Previous studies demonstrated that sugars in the absence of pathogens can trigger the expression of pathogenesis-related (PR) proteins (Moghaddam and Van den Ende, 2012; Rojas et al., 2014). In tobacco, leaf disks floated on sugars (glucose, fructose and sucrose) induced *PR* gene expression (Herbers et al., 1996). Thus, it appears that, in addition to higher Si accumulation, the increased levels of soluble sugars in Si-supplied rice may be partly preconditioning plants for a more efficient defense after herbivore attack. Interestingly, it was reported that reduced concentrations of Si and soluble sugars in Lsi1 mutant rice affected resistance of mutants to *Bipolaris oryzae* (Breda de Haan) Shoemaker (Dallagnol et al., 2013). Furthermore, regardless of Si supply, we found a higher level of sugars in herbivore-infested plants relative to the controls (Figure 11B). This is not surprising as plants have been reported to respond to herbivore attack by activating local catabolism of energy storage compounds like sucrose in order to recruit carbon sources for the production of defense-related metabolites (Zhou et al., 2015). In *Arabidopsis*, Ferrieri et al. (2012) showed that radioactively labeled carbohydrates were translocated to wounded or methyl-jasmonate-treated leaves, and used for the production of defense-related compounds such as phenolic glycosides and cinnamic acid. Moreover, the ability of *Populus* foliage to respond to herbivory or JA elicitation with elevated concentrations of phenolic compounds has been demonstrated to depend on the leaf's ability to import carbohydrates from other plant modules (Arnold et al., 2004). Thus, the elevated accumulation of specialized defense metabolites in Si-supplied rice plants relative to the Si-deprived plants exposed to herbivory stress (Figure 14) could be, at least in part, due to a higher level of sugars in the rice leaves (Figures 11A&B).

### Regulation of HIPVs in rice by Si

While energy metabolism and direct defenses are closely related, indirect plant defenses, which depends on volatile-mediated attraction of natural enemies of herbivores to attacked plants (Dudareva et al., 2006; Maffei et al., 2011) also demand a constant supply of plant energy. For instance, rice HIPVs are mainly emitted during light photoperiod and strongly decline at night (Mujiono et al., 2021). Previously, plant HIPV emissions responded to Si supply (Liu et al., 2017; de Oliveira et al., 2020; Leroy et al., 2019), which is highly consistent with our current data (Figures 15&16). Si supplementation significantly elevated the headspace levels of several major HIPVs in rice, including linalool and methyl salicylate, compared to Si-deprived plants (Figures 15&16). However, it still remains unclear whether Si is altering the synthesis or emissions of HIPVs in rice.

To this end, we found an elevated level of transcripts of *LIS*, *PAL*, and *SAMT* in WOS-challenged rice growing in the presence of Si, suggesting that Si may be promoting the emissions of linalool and methyl salicylate by amplifying their biosynthesis through transcriptional mechanisms (Figure 17). Previously, defense genes including CAT, SOD, PPO and POD were also more expressed in Si-supplemented relative to Si-deprived rice infested with a leaffolder (Lin et al., 2019). As defense genes, such as *LIS* and *SAMT* are known to be controlled by jasmonic acid signaling upon wounding and herbivory, we purported the involvement of these hormones. However, a decreased level of jasmonates in Si-supplied plants under herbivory was found (Figure 18), which is not consistent with the more abundant transcripts of *LIS* and *SAMT*. In support of our data, Kim et al. (2014) also found that wounding stress resulted in higher JA accumulation in Si-deprived relative to Si-supplied rice plants. Similarly, in a conceptual model proposed for Si-JA relationship, Hall et al. (2019) suggested that Si-enriched plants generally show low levels of JA induction upon herbivore attack, possibly due to the available physical protection by Si. On the other hand, another study showed a higher expression of jasmonate biosynthesis genes (LOX, AOS2) in rice supplied with Si relative to Si-deprived plants (Lin et al., 2019), suggesting that these points need further clarifications.

# Chapter 4

# Conclusions and future perspectives

Rice is an important staple crop that is usually threatened by the attacks of insect herbivores in the field. Until now, considerable progress has been made in studying the biological systems for the biosynthesis of anti-herbivore defense metabolites in rice (Alamgir et al., 2016; Gu et al., 2024; Wang et al., 2018). Improvements of basal innate defense systems in plants against herbivores *via* the elevation of the production of defense metabolites have widely been suggested to be an environmentally friendly alternative to chemical-based pesticides. Seemingly, increased synthesis of defense metabolites by plants when attacked by herbivores would require the allocation of sufficient resources in the form of mineral nutrients for their production. Nevertheless, the inherent plant mechanisms for the mobilization of such mineral resources required for defense under herbivore-triggered biotic stress is poorly understood. In this thesis, using hydroponically grown rice plants subjected to herbivory stress, I showed that herbivory induced the differential accumulation of elements at both spatial (different plant parts) and temporal (different time intervals) levels in rice. Although, the accumulation of several micronutrients including Ni and Co were found to be higher in herbivore exposed rice tissues, however, their specific functions in rice anti-herbivore defense responses remain to be fully elucidated. For example, it is still unclear whether the higher accumulation of

Ni in rice exposed to herbivory stress is used for direct poisoning of the attacker insect or indirectly for the production of other defense-related metabolites through the nitrogen metabolism pathway. Previous reports suggest that higher concentrations of some trace elements in plant tissues may provide plants with a new type of chemical defense against insect herbivores commonly referred to as elemental defense (Boyd, 2007; Martens and Boyd, 1994). Plants containing high concentrations of Ni in the leaf tissues are reportedly less eaten by chewing herbivores and thus resistant to herbivore attacks (Boyd, 2007). Nevertheless, whether the concentration of Ni accumulating in rice exposed to herbivory in this study is sufficient to directly protect them against damage by insect still needs to be investigated. On the other hand, Ni is an essential component of the enzyme urease, which catalyzes the conversion of urea to ammonia (Polacco, 1977; Dixon et al., 1975; Polacco et al., 2013). Ni-deprivation has been reported to result in alterations in nitrogen (N) and carbon metabolites pools pointing to the potential important role of Ni in plant primary metabolism (Polacco et al., 2013). For instance, Walker et al. (1985) showed that Ni deprivation in cowpea (*Vigna unguiculata*) altered the metabolism of amino acids. Similarly, in *Cucurbita pepo* convar. *giromontiina*, Ni supply resulted in increased contents of the amino acid, glutamine in urea-grown plants suggesting that the efficient use of urea N is Ni dependent (Gerendas and Sattelmacher, 1997). Apparently, it appears that Ni may play crucial roles in N metabolism via urease activation. To clarify the possible indirect roles of Ni in rice defense against herbivores, it would be necessary to identify the metabolic sinks for Ni during rice anti-herbivore defense by measuring the changes in the profile of several Ni associated amino acids such as glutamine, asparagine, and urea cycle intermediates including arginine (Polacco et al., 2013) in rice exposed to herbivory stress.

Uptake of silicon is essential for defense against chewing herbivores in numerous plant species (Liang et al., 2015). In addition to physical deterrence due to incorporation of Si into plant tissues, other possible mechanisms of plant resistance against insect herbivory regulated by Si remain unclear. In this study, I comprehensively examined the interactions between Si, and defense and metabolic traits in rice, which collectively revealed a potentially highly pleiotropic nature of Si involvement in plant-herbivores interactions. For example, herbivore feeding resulted in the alteration of rice primary metabolism in an Si-dependent manner. Previous transcriptional evidence obtained using the Arabidopsis-powdery mildew plant-pathogen system suggests that Si alleviates the down regulation of genes involved in primary metabolism during the fungal infection process (Fautex et al., 2006). However, how herbivory mediates the observed Si-dependent changes in primary metabolic pathways in rice still remains unclear. Given the availability of well characterized Si-transporter-deficient rice mutant, it would be interesting to comprehensively perform transcriptome analysis of both WT and mutant rice plants after exposure to herbivory stress to gain unique insights on the impact of Si on rice primary metabolic pathways during anti-herbivore defense responses.

As described in chapter 3 above, Si-supplied rice plants when exposed to herbivory stress generally emitted higher levels of several VOCs relative to Si-deprived plants. Given the higher β-caryophyllene levels in headspace of rice grown in the presence of Si, that was not supported by transcriptional data and internal β-caryophyllene contents in leaves, it is likely that Si facilitates the release of volatiles by multiple independent mechanisms. For instance, plant cuticle may largely affect the release of volatiles from plants by passive diffusion mechanisms (Widhalm et al., 2015; Liao et al., 2021). As we did not examine the cuticle of rice leaves grown in the presence or absence of Si, it cannot be excluded that Si may be actually altering the cuticle thickness, similar to lignin deposition (Suzuki et al., 2012). Alternatively, Si could be altering the conductivity of apoplastic routes used for volatiles to escape from plants (Widhalm et al., 2015). The actual mechanisms involved in volatile production, release and gene expression changes mediated by Si remain as one of the most important tasks for future studies, fully acknowledging the important fact that volatiles and Si play crucial roles not only in defense but also in the host plant selection by ovipositing moth females.

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## Supplementary Table 1

Oligonucleotide primers used in the study

| | **RAP-DB** | **Forward primer (5’ -> 3’)** | **Reverse primer (5’ >3’)** |
|---|---|---|---|
| *LOW SILICON RICE 1 (OsLsi1)* | *Os02g0745100* | GCCAGCAACAACTCGAGAACAA | CATGGTAGGCATGGTGCCGT |
| *LOW SILICON RICE 2 (OsLsi2)* | *Os03g0107300* | ATCACCTTCCCCAAGTTCCT | CAGCTCCCTCCAGTACATGC |
| *LOW SILICON RICE 6 (OsLsi6)* | *Os06g0228200* | ACATGATGTTCGTCACCTGC | GAAGTAGATCCAGAGGCCGG |
| *SILICON EFFLUX TRANSPORTER 4 (OsSIET4)* | *Os03g0147400* | AAGCAGACGGTGATTGAGAAGG | GCATGTGCAGTTGTACAAACACC |
| *1-DEOXY-D-XYLULOSE 5-PHOSPHATE SYNTHASE 3 (OsDXS3)* | *Os07g0190000* | GGGGGAGGTTCCAGTAAGAA | TCATTTTGCATTTGGAAGCA |
| *LINALOOL SYNTHASE (OsLIS)* | *Os02g0121700* | CCAGGATGGTCGGCGTCATG | CACGCCATTATGCATGGACGATG |
| *CARYOPHYLLENE SYNTHASE (OsCAS)* | *Os08g0139700* | ATCGCCGGGGAGTGTCTC | GGAGTGTATTGTATCCTTGAGCG |
| *PHENYLALANINE AMMONIA-LYASE (OsPAL)* | *Os02g0626600* | CTACCCGCTGATGAAGAAGC | CTACCCGCTGATGAAGAAGC |
| *S-ADENOSYL-L-METHIONINE: SALICYLIC ACID CARBOXYL METHYLTRANSFERASE (OsSAMT)* | *Os02g0719600* | CTCATCGCTCGTCATTTCGG | ACACACTGGCACGCTACTTA |