The Role of Glycosylation in Modulating the Storage and Function of Volatile Organic Compounds: Case Studies of 1-Octen-3-ol and Linalool in Soybeans

(配糖体化がダイズ 1-オクテン-3-オールとリナロールの貯蔵と機能を調節する)

Juliano Mwenda Ntoruru

Graduate School of Sciences and Technology for Innovation

YAMAGUCHI UNIVERSITY

March 2025

Acknowledgments

I would first like to thank my thesis advisor Professor Matsui of the biological chemistry agriculture department at Yamaguchi University. The door to Prof. Kenji office was always open whenever I ran into a trouble spot or had a question about my research and writing. He consistently allowed this thesis to be my own work, but steered me in the right the direction whenever he thought I needed it.

I gratefully acknowledge the support and generosity of the Ministry of education, Culture, Sports, Science, and Technology of Japan, who supported my study by a scholarship for foreign students and by grants of priority areas without which the present study could not have been completed.

Dedication

To my mentor and a Family Host in Japan (Norio Sugita), Thank you for all your support along the way, I enjoyed a lot with you and your Family in Japan.

The Role of Glycosylation in Modulating the Storage and Function of Volatile Organic Compounds: Case Studies of 1-Octen-3-ol and Linalool in Soybeans

A dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Life Science at Yamaguchi University

By

Juliano Mwenda Ntoruru

MSc. Yamaguchi University, 2022

BSc. Meru University of Science and Technology, 2017

Supervisor: Professor Kenji Matsui

Yamaguchi University

March 2025

DIVISION OF LIFE SCIENCE

GRADUATE SCHOOL OF SCIENCES AND TECHNOLOGY

FOR INNOVATION

YAMAGUCHI UNIVERSITY

The Role of Glycosylation in Modulating the Storage and Function of Volatile Organic Compounds: Case Studies of 1-Octen-3-ol and Linalool in Soybeans

By

Juliano Mwenda Ntoruru

Supervisor: Professor Kenji Matsui

Judging committees:

Professor Jun'ichi Mano

Professor Shin-ichi Ozaki

Associate Professor Takao Koeduka

Assistant Professor Suzuka Kikuchi

Yamaguchi University

March 2025

CONFERENCES/SEMINARS/WORKSHOPS

Participation/Attendance

The 9th Asian-Oceanian Symposium on Plant Lipids: October 10 (Tue) ~ 13 (Fri), 2023 #11, Saint Ignatius House, Sogang University, Seoul, Korea https://aspl2023 seoul.kr. Presented: Linalool exposure enhanced defense against a herbivore and caused accumulation of linalyl diglycoside by Ntoruru, J. M.

The 2nd annual Meru University of Science and Technology International Conference (MUSTIC2023) held from 26th to 28th June 2023 at Meru University of Science and Technology Main campus in Meru. Presented: 1-Octen-3-ol is formed from its primeveroside after mechanical wounding of soybean leaves by Ntoruru, J. M.

The 17th Young Scientist Seminar held from 28th – 29th November, 2020. Yamaguchi University Japan. Presented: Study on the mechanism of biosynthesis of bioactive glycosides in soybean seeds. 28th/29th Nov seminar presentation by Ntoruru, J. M.

Post pregnancy Family planning training; PPFP Choices Research Ethics and Good Clinical Practices Training held from 26 February – March 2018, Nairobi Kenya by Jhpiego an affiliate of Johns Hopkins University.

Workshop on RT-PCR (extraction and thermocyling) held on the 17th & 18th April 2019 at Meru University of Science and Technology Main Campus.

Special seminar held in May 19th 2023 by distinguished professor Gregg Howe from Michigan State University at Yamaguchi University Japan, presented: Molecular mechanism of jasmonates signalling.

Regional conference by American society of plant Biologists (ASPB) on the plant cell webinar; Highlighting focus issue 'Plant Biotic Interactions` held on May 17th, 2022-Online.

Table of Contents

The Role of Glycosylation in Modulating the Storage and Function of Volatile Organic Compounds: Case Studies of 1-Octen-3-ol and Linalool in Soybeans

Introduction

Volatile organic compounds (VOCs) are critical in the biochemical communication, defense mechanisms, and sensory attributes of plants. Among these, compounds like 1-octen-3-ol and linalool are prominent in soybeans, contributing to their aroma profile and functional roles in plant physiology. However, these VOCs are not stored in their free form but are often glycosylated, a biochemical modification that attaches sugar moieties to the VOC molecules. Glycosylation modulates the storage, stability, and bioavailability of these compounds, allowing plants to control their release and function. Glycosylation involves the enzymatic addition of sugar moieties to hydrophobic molecules, altering their physical and chemical properties. In plants, glycosyltransferases (GTs) catalyze this process, using activated sugar donors such as UDP-glucose to conjugate Volatiles compounds.

Abstract

The glycosylation of 1-octen-3-ol and linalool results in glycosides like 1-octen-3-yl primeveroside and linalyl vicianoside, respectively. This report explores the role of glycosylation in modulating the storage and function of 1-octen-3-ol and linalool in soybeans, highlighting its biochemical significance and implications for plant science and agriculture.

Chapter 1, it was previously found that soybean (*Glycine max*) seeds contain 1-octen-3-yl *β*-primeveroside (pri). To elucidate the physiological significance and the biosynthesis of 1-octen-3-ol in plants, changes in the amount of 1-octen-3-yl pri during development of soybean plants was examined. A high 1-octen-3-yl pri level was found in young developing green organs, such as young leaves and sepals. Treatment of soybean leaves with methyl jasmonates resulted in a significant increase in the amount of 1-octen-3-yl pri; suggesting its involvement in defense responses. Although 1-octen-3-ol was below the detection limit in intact soybean leaves, mechanical damage to the leaves caused rapid hydrolysis of almost all 1-octen-3-yl pri to liberate volatile 1-octen-3-ol. Under the same conditions, the other glycosides, including isoflavone glycoside and linalool diglycoside, were hardly hydrolyzed. Therefore, the enzyme system to liberate aglycone from glycosides in soybean leaves should have strict substrate specificity. 1-Octen-3-yl pri might function as a storage form of volatile 1-octen-3-ol for immediate response against stresses accompanying tissue wounding.

Chapter 2, Linalool is anticipated to have significant ecological roles. In this study, linalyl 6-*O*-*β*-arabinopyranosyl-*β*-D-glucopyranoside (linalyl *β*-vicianoside: LinVic) was synthesized, and a linalool diglycoside purified from soybean leaves was identified as LinVic by using liquid chromatography-mass spectrometry. High levels of LinVic were detected in leaves and sepals during soybean plant growth. The LinVic content did not significantly increase following methyl jasmonate treatment of the leaves, indicating that its synthesis is independent of the jasmonic acid signaling pathway. In addition to LinVic, soybean also contains 1-octen-3-yl primeveroside. We treated soybean leaves with vaporized linalool and 1-octen-3-ol to determine whether the glycosylation system discriminates between these two volatile alcohols. Linalool treatment resulted in the accumulation of LinVic, while 1-octen-3-ol treatment caused little change in the amount of 1-octen-3-yl primeveroside, suggesting discrimination between these compounds. Linalool-treated soybean leaves exhibited increased resistance against common cutworms, indicating that LinVic may contribute to herbivore resistance.

Through glycosylation, soybeans have evolved a remarkable mechanism to amplify the utility of volatile compounds, ensuring their survival and resilience in dynamic ecosystems. Understanding and harnessing this mechanism holds promise for both scientific discovery and practical applications in crop science.

1 CHAPTER ONE

Study of biosynthetic pathways of 1-octen-3-ol and 1-octen-3-yl primeveroside in soybean

Abstract

Key message **Hydrolysis of 1-octen-3-yl *β*-primeveroside implemented by a system with high structure-specificity is accountable for the rapid formation of 1-octen-3-ol from soybean leaves after mechanical wounding.**

1-Octen-3-ol is a volatile compound ubiquitous in fungi; however, a subset of plant species also has the ability to form 1-octen-3-ol. Owing to its volatile nature, it has been anticipated that 1-octen-3-ol is associated with the effort of the emitter to control the behavior of the surrounding organisms; however, its ecological significance and the enzymes involved in its biosynthesis have not been fully elucidated, particularly in plants. It was previously found that soybean (*Glycine max*) seeds contain 1-octen-3-yl *β*-primeveroside (pri). To elucidate the physiological significance and the biosynthesis of 1-octen-3-ol in plants, changes in the amount of 1-octen-3-yl pri during development of soybean plants was examined. A high 1-octen-3-yl pri level was found in young developing green organs, such as young leaves and sepals. Treatment of soybean leaves with methyl jasmonates resulted in a significant increase in the amount of 1-octen-3-yl pri; suggesting its involvement in defense responses. Although 1-octen-3-ol was below the detection limit in intact soybean leaves, mechanical damage to the leaves caused rapid hydrolysis of almost all 1-octen-3-yl pri to liberate volatile 1-octen-3-ol. Under the same conditions, the other glycosides, including isoflavone glycoside and linalool diglycoside, were hardly hydrolyzed. Therefore, the enzyme system to liberate aglycone from glycosides in soybean leaves should have strict substrate specificity. 1-Octen-3-yl pri might function as a storage form of volatile 1-octen-3-ol for immediate response against stresses accompanying tissue wounding.

Keywords 1-octen-3-yl *β*-primeveroside, 1-octen-3-ol, isoflavone glycosides, methyl jasmonate, *Glycine max*

1.1 **Introduction**

Soybeans are consumed as vegetable, much like green peas or lima beans and when harvested at seed dry stages are used for different soy food products such as soy sauce, tofu, natto, kinako and soy milk. 1-Octen-3-ol is a volatile compound widely distributed among fungi and contributes to the fungal and earthy smell of fungi and their products (Inamdar et al. 2020). 1-Octen-3-ol is also found in plants, but in rather limited groups of plants, such as a subgroup of cyanobacteria (Brash et al. 2014), bryophytes (Croisier et al. 2010), and angiosperms belonging to Fabales and Lamilaes (Kigathi et al. 2009; Seo and Baek 2009; Matsui et al. 2018). Linoleic acid 10-hydroperoxide is the key intermediate in forming 1-octen-3-ol in fungi (Wurzenberger and Grosch et al. 1982), whereas arachidonic acid 12-hydroperoxide is involved in the formation of 1-octen-3-ol in bryophytes (Croisier et al. 2000; Kihara et al. 2014). However, the biosynthetic pathway involved in the formation of 1-octen-3-ol in vascular plants is not well understood.

Because 1-octen-3-ol is volatile, it has been anticipated that it plays a role in the interaction between the producers and the living organisms surrounding them. Flies and mosquitoes have specific odorant receptors for 1-octen-3-ol, and in most cases, use it as one of chemical cues to find their food (Potter et al. 2014; Holighaus et al. and Rohlfs et al. 2019). The effect of 1-octen-3-ol is preferable to one organism but sometimes evokes disadvantageous effects on another, and the latter situation would be preferable to the emitters if the receivers would be the enemies of the emitters. 1-Octen-3-ol is involved in repelling *Caenorhabditis elegans* (Ferrari et al. 2018), or even kills root-knot nematodes (*Meloidogyne hapla*) (Khoja et al. 2021). *Drosophila melanogaster* suffers from neurodegeneration by disrupting dopamine packaging by 1-octen-3-ol (Inamdar et al. 2013). 1-Octen-3-ol was found to suppress the infection of Arabidopsis by *Alternaria brassicicola*, the causal agent of cabbage leaf spot, essentially based on the activity of 1-octen-3-ol to inhibit the hyphal growth of the fungal pathogen (Fujita et al. 2021). The inhibition of hyphal growth of *Penicillium expansum*, a common food spoilage organism, has also been reported (Okull et al. 2003). 1-Octen-3-ol has hormone-like functions in

regulating fungal development (Holighaus and Rohlfs et al. 2019); thus, aberrant exposure to the volatile is thought to perturb the development of fungi. 1-Octen-3-ol in the rhizosphere attracted the entomopathogenic nematode *Steinernema diaprepesi* and repelled root-feeding larvae of root weevils (*Diaprepes abbreviatus*) (Wu and Duncan et al. 2020). Plants are also receivers of 1-octen-3-ol. For example, exposure to the vapor of 1-octen-3-ol induced a subset of defense genes in Arabidopsis (Kishimoto et al. 2007) and reduced the consumption of foliar tissues in maize plants (Contreras-Cornejo et al. 2019). In summary, the ecological and physiological functions of 1-octen-3-ol vary in a context-dependent manner depending on the interaction between organisms; however, the functions reported invoke a defensive role for 1-octen-3-ol in plants.

Mechanical wounding of plant tissues often induces the rapid formation of volatile compounds, such as green leaf volatiles or isothiocyanates. Quick formation is important to efficiently demonstrate direct or indirect defense facilitated by the volatiles. Green leaf volatiles are six-carbon aldehydes, alcohols, and their esters, and their rapid de novo formation after mechanical wounding is thought to be largely supported by the rapid activation of lipoxygenase, which catalyzes the dioxygenation of fatty acids or lipids (Mochizuki et al. 2016). In contrast, isothiocyanates are stored as glycosides, called glucosinolates, in a specified compartment in plant tissues. Mechanical damage results in the mixing of glucosinolates with a glycosidase enzyme called myrosinases that are stored in a different compartment, and subsequently, isothiocyanate is formed (Halkier and Gershenzon 2006). 1-Octen-3-ol is also rapidly formed when plant tissues would suffer stresses that are accompanied by mechanical wounding of the tissues (Ozawa et al. 2000; Leitner et al. 2010; Quintana-Rodriguez et al. 2015; Boggia et al. 2015). However, the mechanism underlying the rapid formation of 1-octen-3-ol in plant leaves after mechanical damage is not well understood.

Plants form a vast array of plant-specialized metabolites. Glycosides of volatile compounds are one of the major groups of plant glycosides. Glycosidically bound volatiles found in tea leaves are important in determining the quality of tea because volatile aglycones are liberated during tea

processing (Cui et al. 2016). We previously found that soybean seeds contained 1-octen-3-yl *β*-primeveroside (pri) and that the partial hydrolysis proceeded to form 1-octen-3-ol during processing soybean seeds for food manufacturing (Matsui et al. 2018); however, the distribution of the pri during growth of soybeans, the regulation of its biosynthesis, and its significance in the rapid formation of 1-octen-3-ol after mechanical wounding has not been fully studied. This study determined the amount of 1-octen-3-yl pri during development and after treating with methyl jasmonate and the fate of the pri after mechanical wounding. The results were compared with those obtained with other types of glycosides, such as glycosides of isoflavones and monoterpene alcohol.

1.2 Materials and methods

1.2.1 Chemicals

1-Octen-3-yl pri was purified from soybean seeds as described previously (Matsui et al. 2018). (*Z*)-3-Hexenal was obtained from Nippon Zeon (Tokyo, Japan). 1-Octen-3-ol, formononetin, and daidzin were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). Genistin was from LKT laboratory (St. Paul, MN).

1.2.2 Plant Materials

Soybeans (*Glycine max* 'Enrei') seeds harvested in Tsukuba City, Ibaraki Prefecture, Japan in 2019 were provided by Drs. Hiraga and Ishimoto (Institute of Crop Science, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan). The seeds were soaked on the surface of wet cotton for 24 h at 25°C under dim light. The seed coats, cotyledons, and embryonic axes were separated from the dry seeds and 1-day-imbibed seeds. The organs were stored at −20°C until analysis. To prepare the seedlings, 1-day-imbibed soybean seeds were sown with a 1:1 mixture of sterilized Takii Tanemakibaido (Takii, Kyoto, Japan) and vermiculite on August 17, 2020. Plants were grown in pots

in a growth chamber with 16 h light (fluorescent lights at 60 μmol m^{-2} s^{-1}) and 8 h dark regimen at 28°C until the emergence stage (VE), cotyledon stage (VC), or third trifoliate (V3) stages (Nleya et al. 2019). The seeds were sown in a planter (575 × 235 × 200 mm) filled with normal soil for a vegetable garden available at a local gardening store. The planters were placed at the experimental farm in the Faculty of Agriculture, Yamaguchi University, Japan, on May 29, 2020. The plants were grown under natural sunlight with daily irrigation until the full bloom (R2, harvested on July 17), full seed (R6, August 26), or full maturity stage (R8, 29 September).

1.2.3 Treatment with methyl jasmonate or linalool

Methyl jasmonate (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.05% (w/v) Tween 20 at 50 and 200 μM. Un-damaged soybean plants at the V3 stage were selected and randomly divided into three groups. The upper ground parts of the plants were uniformly sprayed with methyl jasmonate solution. As the control 0.05% Tween 20 was used. The first trifoliate leaves from the bottom were harvested immediately and at 1, 3, 5, and 7 days after treatment for analyses. Linalool (racemic) (FUJIFILM Wako Pure Chemicals) was dissolved in 0.05% Tween 20 at a concentration of 65 μM, and 1 mL of the volatile solution was adsorbed on cotton swabs and enclosed in a glass vessel (1000 cm^3) containing a soybean plant at V3 stage of growth. As the control, 0.05% Tween 20 containing no volatile was used. The vessels were kept under growth conditions described above for 24 h to allow for effective exposure of the plant to the volatile compound.

1.2.4 Volatile analysis

Healthy and undamaged soybean leaves at the V3 stage were selected for headspace volatile collection. The trifoliate leaves were cut out at the petiole with a razor blade, weighed, and the cut surfaces were covered with wet cotton to avoid desiccation. Thereafter, the leaflets were carefully placed in a glass jar (6 cm internal diameter × 6.5 cm). The jar was closed tightly, and an SPME fiber (50/30-μm DVB/Carboxen /PDMS; Sigma-Aldrich) was exposed to the headspace of the jar for 30 min at 25°C

in a water bath. For partial mechanical wounding, the leaflets were damaged by rolling a roulette on the surface of the leaflet six times parallel to the midvein and then immediately placed into the glass jar. For the freeze-thaw treatment, the leaflets in a glass jar were frozen at −75°C for 1 h and then thawed in a water bath at 25°C. The volatiles formed by the partially wounded and freeze-thawed leaflets were collected with the SPME fiber at 25°C for 30 min. After the collection of volatiles, the leaflets were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

The SPME fiber was inserted into the injection port of a gas chromatography-mass spectrometry (GC-MS) instrument (QP-5050; Shimadzu, Kyoto, Japan) equipped with a 0.25 mm internal diameter × 30 m DB-WAX column (film thickness, 0.25 μm; Agilent, Santa Clara, CA). The column temperature was programmed as follows: 40°C for 1 min, increasing by 15°C min^{-1} to 180°C, and then 180°C for 1 min. The carrier gas (He) was delivered at a flow rate of 1 mL min^{-1}. The glass insert was an SPME Sleeve (Sigma-Aldrich), and splitless injections were performed with a sampling time of 1 min. To remove all the compounds from the matrix, the fiber was held in the injection port for 10 min. The injector and interface temperatures were 200° and 230°C, respectively. The mass detector was operated in electron-impact mode with ionization energy of 70 eV. Compounds were identified using retention indices and MS profiles of the corresponding authentic specimens, and quantitative analyses were performed with standard calibration curves constructed for each compound using an aqueous solution containing Tween 20. A given amount of standard compound with Tween 20 solution was placed on filter paper of the same size of leaflet used for analysis and placed in a glass jar for SPME GC-MS analysis, as described above.

1.2.5 High performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

After measuring the weight, the plant organs were homogenized completely with 10 μg mL^{-1} formononetin (IS) in 80% (v/v) methanol in a mortar and pestle. The cotyledons from the dry seeds were homogenized using a Polytron mixer. The suspension was centrifuged at 15,000 rpm (with a

rotor of T15A39, Hitachi Koki Co, Tokyo, Japan) for 10 min at 24 °C. The supernatant was filtered through a DISMIC-03JP (0.5 μm, Advantec, Tokyo, Japan) and used for LC-MS/MS analysis.

Glycosides were analyzed with LC−MS/MS (3200 Q-TRAP LC/MS/MS System; AB Sciex, Framingham, MA, USA) equipped with a Prominence UFLC (Shimadzu). The extracts were separated on a Mightysil RP18 column (2 mm inner diameter × 150 mm, 5 μm; Kanto Chemical, Tokyo, Japan) with solvent A (water/formic acid, 100:0.1, v/v) and solvent B (acetonitrile/formic acid, 100:0.1, v/v). The run consisted of 10% B for 5 min, a linear increase from 10% to 80% B over 17 min, and 80% B for 3 min, with a flow rate of 0.2 mL min^{-1}. The compounds were detected by MS/MS using enhanced MS scan mode with electrospray ionization in the negative ion mode [ion spray voltage, −4500 V; nitrogen as both the curtain gas (set to 40 arbitrary units) and collision gas (set to “high”); collision energy, −45 V; scan range, *m/z* 100−750; scan speed, 4000 Da s^{-1}; and declustering potential, −30 V]. Enhanced product ion scan mode analysis for linalyl diglycoside was carried out with 447.23 Da as the precursor ion with a collision energy of -25 V. Identification and quantification of the individual glycoside was based on comparisons with retention times, mass spectra, absorption spectra of standard molecules, and calibration curves constructed with them. The linalyl diglycoside was quantified with a calibration curve constructed with linalyl pri. The molecular formula and the range of *m/z* for the extracted ion chromatogram to detect each glycoside are summarized in Table 1-2. For better separation of glycosides, a long gradient program using a CAPCELL PAK C18 UG120 column (2.0 mm inner diameter × 150 mm; 5 μm) (Osaka Soda, Osaka, Japan) was used. The flow rate was 0.2 mL min^{-1} with 10% solvent B (0–25 min), followed by a slow increase in the hydrophobicity to 22% solvent B (25.1–75 min) (solvent A: water containing 0.05% (v/v) formic acid, B: acetonitrile). With this system, MS was analyzed using a Shimadzu LCMS-2020 system (Shimadzu) with negative electrospray ionization mode: dry temperature, 250 °C; flow rate, 0.2 mL min^{-1} and column oven temperature, 40 °C.

1.3 Data analysis

The results included three replicates measured separately and are represented as mean ± standard error. The data were analyzed statistically using analysis of variance (ANOVA) with Tukey's test ($P < 0.05$). Different alphabetical letters were assigned to demonstrate significant difference.

1.4 Results

1.4.1 Developmental regulation of 1-octen-3-yl primeveroside

It has been reported that 1-octen-3-ol is formed in the aerial parts of Fabaceae plants, such as *Lotus japonicus*, *Trifolium pratense* (red clover), and *Phaseolus lunatus* (lima bean), after herbivore or mechanical damage (Ozawa et al. 2000; Kigathi et al. 2009; Boggia e t al. 2015). These findings, along with our previous observation of the occurrence of 1-octen-3-yl pri in some of the aerial parts of 14-day-old soybean plants (Matsui et al. 2018), prompted us to examine the distribution of 1-octen-3-yl pri in soybean plants at various developmental stages (Table 1-1). In the dry soybean seeds, a high level of 1-octen-3-yl pri (7.88 ± 0.36 μg g FW^{-1}) was found in the embryonic axis (plumules plus radicles), whereas most daidzin and genistin were found in the cotyledons. The three glycosides were hardly detected in the seed coats. The amounts of 1-octen-3-yl pri, daidzin, and genistin in the embryonic axis significantly decreased during soaking, whereas the significant decrease in the amounts in cotyledons was found only with that of daidzin. At the emergence (VE) stages, the highest amount of 1-octen-3-yl pri (27.1 ± 1.82 μg g FW^{-1}) was found in the hypocotyls. At the later stages, that is, the cotyledon (VC) and the 3rd trifoliate (V3) stages, higher amounts were detected in the younger leaves. Among the organs analyzed, the small leaves at the V3 stage showed the highest level (more than 120 μg g FW^{-1}) of 1-octen-3-yl pri. At the full bloom (R2) stage high levels of 1-octen-3-

yl pri was found with the sepals, and the petals also contained a substantial amount of the pri. In the seeds and pods at the full seed (R6) stage, the level was not very high, and it tended to decrease with maturation. The distribution profiles of daidzin and genistin were completely different from that of 1-octen-3-yl pri. The highest amount of daidzin (22.0 ± 2.85 μg g FW^{-1}) was found in the roots at the 3rd trifoliate stage, as previously reported (Maeda et al. 2018), whereas the sepal was the organ where the highest amounts of genistin (374.3 ± 28.0 μg g FW^{-1}) were detected.

Table 1-1. Distribution of 1-octen-3-yl primeveroside, daidzin, and genistin in the organs of soybean plant at various developmental stages.

		Amount (μg/g FW)		
Development stages	Organs	1-Octen-3-yl primeveroside	Daidzin	Genistin
Dry seeds	Cotyledons	2.20 ± 0.53^{b}	132.4 ± 0.73^{a}	80.7 ± 20.91^{a}
	Embryonic axis	7.88 ± 0.36^{a}	8.61 ± 0.47^{b}	6.55 ± 0.53^{b}
	Seed coats	n.d.	n.d.	n.d.
Soaked seeds	Cotyledons	1.42 ± 0.12^{b}	56.4 ± 1.64^{a}	54.7 ± 0.61^{a}
	Embryonic axis	2.28 ± 0.28^{a}	1.13 ± 0.07^{b}	1.43 ± 0.09^{b}
	Seed coats	n.d.	n.d.	n.d.
Emergence stage (VE)	Cotyledons	0.53 ± 0.05^{a}	13.1 ± 1.49^{a}	12.5 ± 1.56^{a}
	Hypocotyl	27.1 ± 1.82^{b}	9.91 ± 0.73^{a}	12.6 ± 0.92^{a}
	Roots	1.12 ± 0.10^{a}	6.36 ± 3.67^{a}	8.07 ± 4.66^{a}
Cotyledon stage (VC)	Young Leaves	17.4 ± 2.03^{a}	4.62 ± 1.21^{b}	27.4 ± 6.91^{a}
	Cotyledon	0.36 ± 0.03^{b}	13.3 ± 0.82^{a}	24.8 ± 1.54^{a}
	Root	1.45 ± 0.33^{b}	3.72 ± 1.06^{b}	1.06 ± 0.31^{b}
	Stem	3.67 ± 1.27^{b}	2.32 ± 0.97^{b}	1.78 ± 0.75^{b}
3rd trifoliate stage (V3)	Leaves/Small	126.6 ± 12.8^{a}	2.46 ± 1.53^{b}	37.2 ± 9.45^{b}
	/Medium	70.1 ± 6.27^{b}	5.66 ± 0.52^{b}	163.1 ± 23.7^{a}
	/Large	10.18 ± 2.42^{c}	1.16 ± 0.32^{b}	18.0 ± 3.33^{b}
	Roots	n.d.	22.0 ± 2.85^{a}	5.41 ± 0.21^{b}
	Stem	6.72 ± 0.76^{c}	2.52 ± 0.68^{b}	4.18 ± 0.44^{b}
Full bloom stage (R2)	Petal	20.8 ± 0.94^{b}	8.57 ± 3.31^{a}	20.9 ± 4.52^{b}
	Sepal	109.0 ± 21.5^{a}	5.88 ± 1.35^{a}	374.3 ± 28.0^{a}
Full seed stage (R6)	Seeds/Small	5.46 ± 0.88^{a}	3.25 ± 1.90^{a}	9.07 ± 1.02^{a}
	/Medium	2.10 ± 0.22^{b}	0.90 ± 0.40^{a}	3.96 ± 1.71^{ab}
	/Large	1.17 ± 0.16^{b}	0.28 ± 0.02^{a}	4.05 ± 0.77^{ab}
	Pods/Small	0.77 ± 0.26^{b}	0.75 ± 0.44^{a}	2.67 ± 1.16^{b}
	/Medium	0.68 ± 0.15^{b}	1.09 ± 0.54^{a}	1.18 ± 0.47^{b}
	/Large	0.62 ± 0.25^{b}	0.39 ± 0.23^{a}	2.73 ± 1.61^{b}
Full maturity stage (R8)	Dry seed	25.9 ± 7.36	6.28 ± 1.01	81.4 ± 4.12

The mean ± standard error of the three biological replicates is shown. nd: not detected. Different letters indicate significant differences ($P < 0.05$) among the organs at a given stage (one-way analysis of variance, Tukey's test).

1.4.2 Effect of methyl jasmonate treatment

Metabolic pathways to form some defense compounds are regulated by jasmonate signaling (Wasternack and Strnad et al. 2019). A subset of glycosides of volatile compounds is known to function as a direct defense compound against herbivores (Sugimoto et al. 2014; Heiling et al. 2021). The aglycone, 1-octen-3-ol, also has insecticidal activity (Inamdar et al. 2020) and is considered to be one of the herbivore-induced plant volatiles responsible for the attraction of predatory mites to soybean plants; thus, it is involved in indirect defense (Ozawa et al. 2000). To examine whether the formation of 1-octen-3-yl pri was also associated with jasmonate-dependent defense responses, soybean plants at the V3 stage were treated with methyl jasmonate. Methyl jasmonate has been widely used to modulate secondary metabolites in plants (Wang et al. 2021, Wang et al. 2020). Spraying methyl jasmonate at 50 μM caused a slight effect on the amount of 1-octen-3-yl pri (Fig. 1-1); however, the treatment with 200 μM solution increase its amount 5 days after treatment, and a significant increase (ca 3-fold) was observed at 7 days after the treatment. The amount of daidzin was also enhanced with exogenous application of 200 μM methyl jasmonate; however, the significant increase in daidzin content was evident at 1 d after the treatment, faster than that observed with 1-octen-3-yl pri. In contrast, the amount of genistin was little affected by methyl jasmonate treatment.

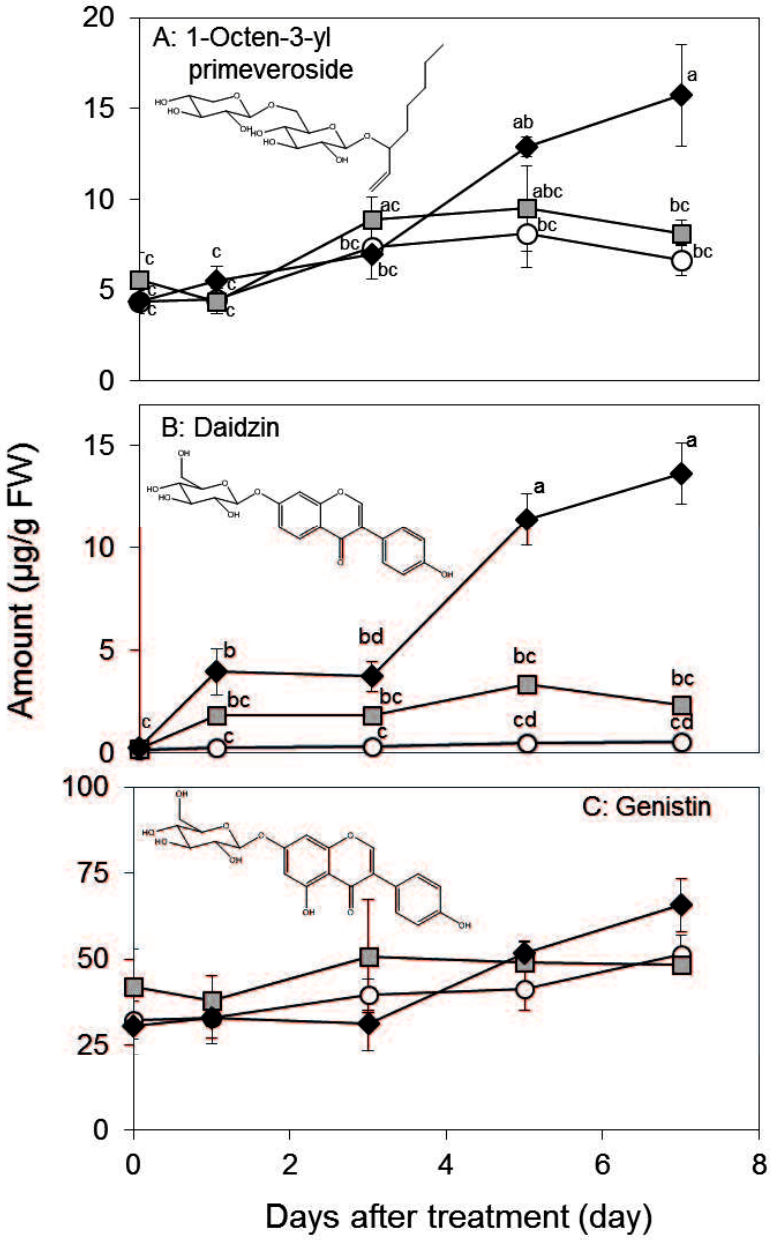


Fig 1-1: Effect of methyl jasmonate treatment on the amount of 1-octen-3-yl primeveroside (A), daidzin (B), and genistin (C). The soybean plants at the V3 stage were treated with 0 (circle), 50 (square), or 200 (diamond) µM of methyl jasmonate, and the trifoliate leaves were harvested at a given time for the glycoside analysis. The means ± standard errors ($n = 3$) are shown. Significant differences were identified using two-way analysis of variance ($P < 0.05$, Tukey's test). Different lowercase letters indicate significant differences

1.4.3 Correlation between 1-octen-3-yl pri and 1-octen-3-ol

Previously, it was suggested that the hydrolysis of 1-octen-3-yl pri accounted for the formation of 1-octen-3-ol with imbibed soybean seeds (Badenhop and Wilkens et al. 1969). To further confirm the

relationship in the soybean leaves, intact leaves at the V3 (3rd trifoliate) stage were partially mechanically damaged by rolling a roulette on the leaf or completely by freeze-thaw treatment, and the volatiles formed were analyzed. As observed in mechanically damaged leaves of angiosperms (Mochizuki et al. 2016), green leaf volatiles consisting of six carbon aldehydes, alcohols, and acetate were rapidly formed in the soybean leaves after partial mechanical wounding and complete tissue disruption with the freeze-thaw treatment (Fig. 1-2A). In addition to the green leaf volatiles, monoterpenes, such as *β*-myrcene, limonene, *β*-phellandrene, were also formed, particularly in the freeze-thaw treated leaves. A small linalool peak was also observed. The compositions of freeze-thaw-induced volatiles and partial wounding-induced volatiles were quite different from each other. Among the green leaf volatiles formed from the partially wounded leaves, (*Z*)-3-hexen-1-ol was the most abundant, followed by (*Z*)-3-hexen-1-yl acetate, and (*Z*)-3-hexenal was the most abundant green leaf volatile after complete disruption with freeze-thaw treatment. The peaks of 3-octanone and 3-octanol were observed after partial wounding, whereas their peaks detected in freeze-thaw treated leaves were quite small.

1-Octen-3-ol was one of the most prominent peaks on the chromatogram after the freeze-thaw treatment, and as much as ca. 1 μg/ g FW^{-1}, which was comparable with that of (*Z*)-3-hexenal (ca. 3.4 μg/g FW), was detected (Fig. 1-2B). During freeze-thaw damage, the amount of 1-octen-3-yl pri decreased from 1.7 to 0.1 μg /g FW^{-1}. In contrast to the remarkable change in the amount of 1-octen-3-yl pri after freeze-thaw treatment, a difference in the amounts of isoflavone glycosides after freeze-thaw treatment was hardly observed (Fig. 1-2C).

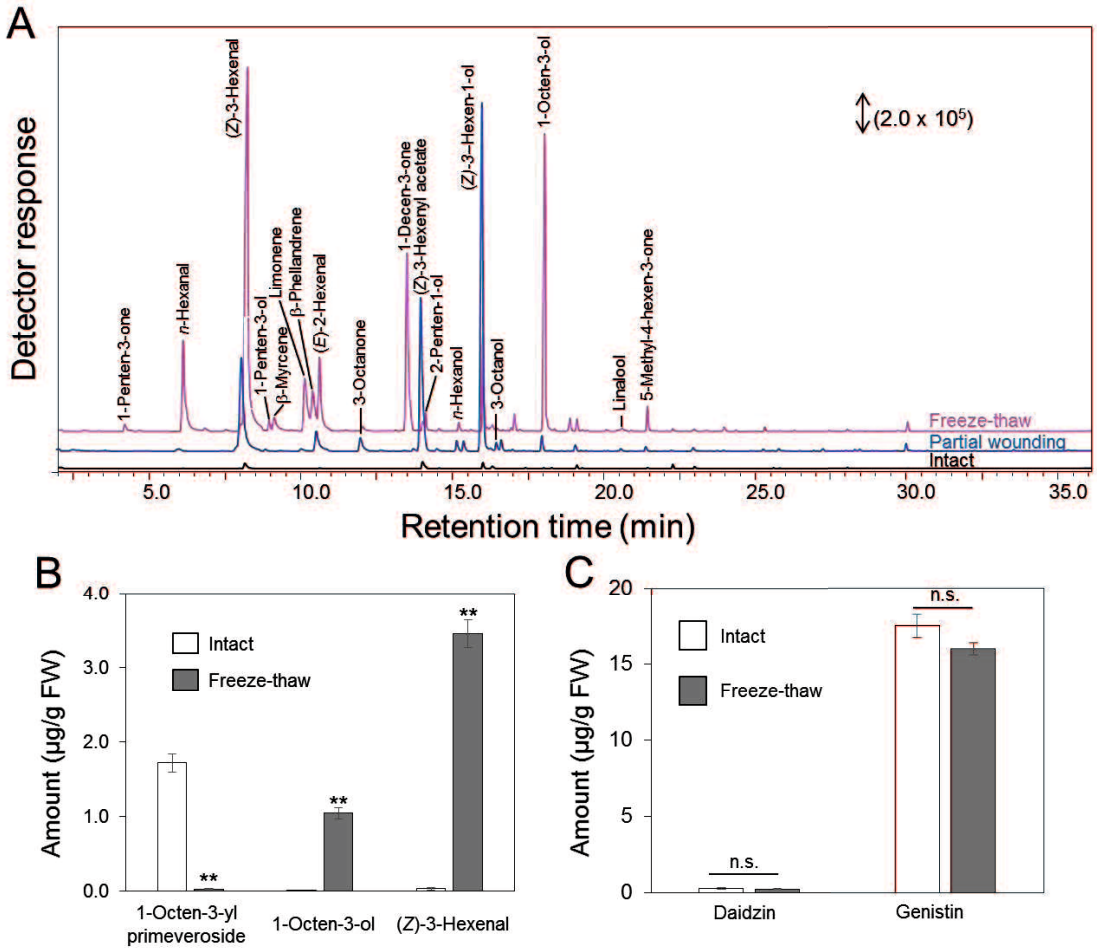


Fig 1-2: Formation of volatile compounds after partial wounding and freeze-thaw treatment on soybean trifoliate leaflets. A. Representative Chromatograms obtained with intact leaves (black chromatogram), partially wounded leaves (blue), and leaves disrupted with the freeze-thaw treatment (magenta). B. Changes in the amount of 1-octen-3-yl primeveroside, 1-octen-3-ol, and (*Z*)-3-hexenal after the freeze-thaw treatment on the leaflets. C. Changes in the amounts of daidzin and genistin after the freeze-thaw treatment of the leaflets. The means ± standard errors ($n = 3$) are shown. Significant differences were identified using Student's *t*-test (**; $P < 0.01$). n.s.; not significant.

1.4.4 Glycosides of the other volatile compounds

1-Octen-3-ol formed in the mechanically wounded soybean leaf tissues mostly derived from its primeveroside; therefore, it was assumed that the other volatiles with a hydroxyl group were also derived from their glycosides. As the volatile compounds harboring the hydroxyl group that might be stored as their glycosides, 1-penten-3-ol, 2-penten-1-ol, *n*-hexan-1-ol, (*Z*)-3-hexen-1-ol, and linalool were found in the mechanically wounded soybean leaves (Fig.1- 2A). The glycosides of benzyl alcohol,

2-phenylethanol, and geraniol were also examined, although these volatiles were not detected in damaged soybean leaves because their occurrence as glycosides in tea leaves has been reported (Ohgami et al. 2015). Data obtained from LC-MS/MS analysis of the extract prepared from intact soybean leaves were examined with the molecular ion related to the glucosides and pentopyranosylhexopyranoside of these volatile compounds (Supplemental Table 1-2), which yielded prominent peaks with MS profiles related to the pentopyranosylhexopyranoside of linalool (Fig. 1-3A, B). The peak size doubled when soybean leaves were exposed to linalool vapor (Fig. 1-4). With enhanced product ion scan mode with *m/z* 447.23 $[M\text{-}H^+]^-$ as the parent mass, MS peaks of *m/z* 315.2, corresponding to the fragment after removing the pentose moiety $[M\text{-}C_5H_9O_4]^-$ was detected (Fig. 1-3C), from which a structure of linalyl pentopyranosylhexopyranoside ($C_{21}H_{36}O_{10}$) was supported. The retention time of the linalyl pentopyranosylhexopyranoside was 2.1 min earlier than that of authentic linalyl pri (Fig. 1-3D). Therefore, the linalyl pentopyranosylhexopyranoside is not linalyl pri but the one composed of the glycone moiety different from pri. The glycosides of the other volatile compounds examined in this study, namely, benzyl alcohol, 2-phenylethanol, (*Z*)-3-hexen-1-ol, and 1-penten-3-ol, were not detected. When the amount of linalyl diglycoside in intact and freeze-thaw treated soybean leaves was determined with a calibration curve constructed with linalyl pri, a tendency ($P = 0.060$, Student's *t*-test) of a slight decrease in the amount of linalyl diglycoside (from 70.9 ± 8.6 to 54.9 ± 8.3 μg/ g FW^{-1}) was observed after freeze-thaw treatment (Fig. 1-3E). The decrease was small compared to the decrease observed with 1-octen-3-yl pri (Fig. 1-2B).

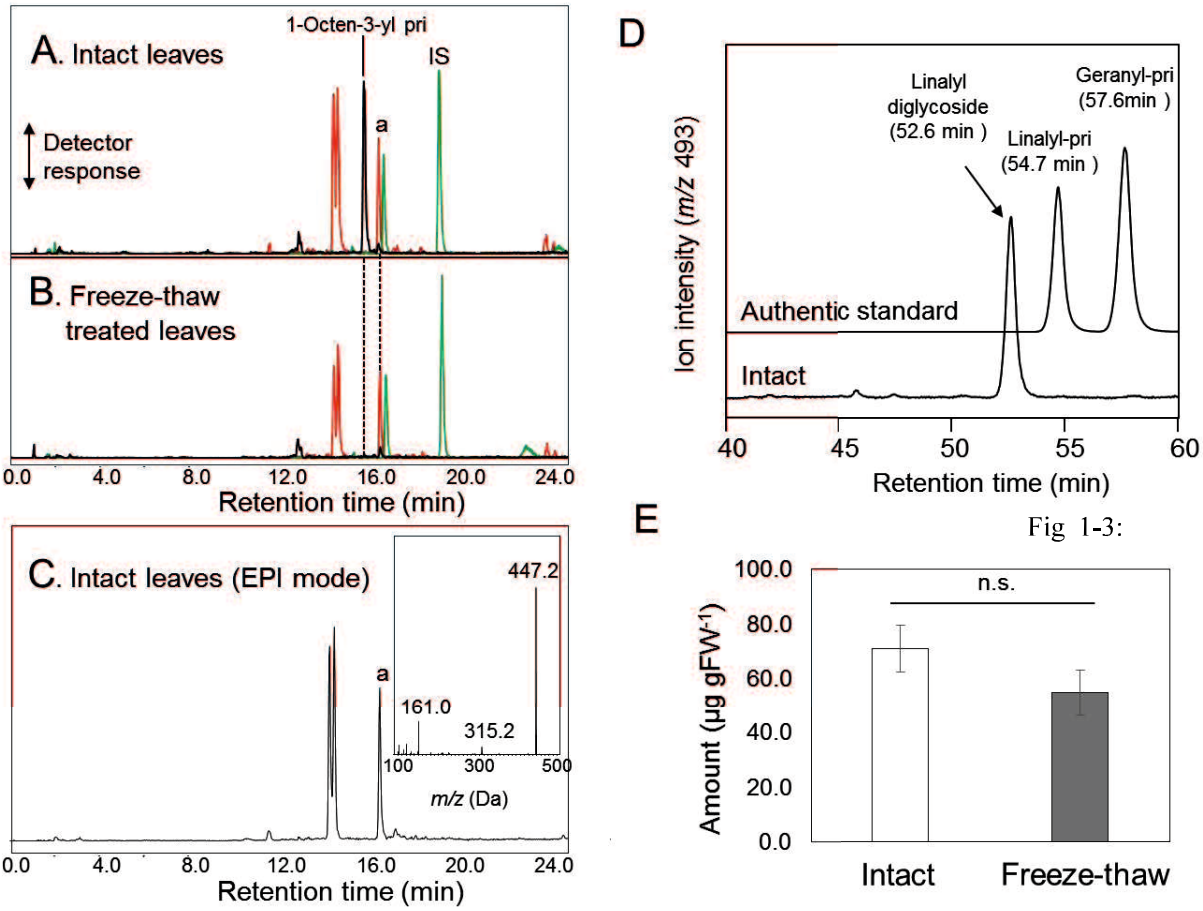


Fig 1-3:

Representative chromatograms for searching glycosides of volatile compounds in the extract prepared with intact (A) and freeze-thaw disrupted (B) soybean leaves. After EMS scan from m/z 100 to 750, signals for m/z of 267.6–268.6 (green, for formononetin), 420.8–421.8 (black, for 1-octen-3-yl primeveroside), and 446.7–447.7 [red, for linalyl diglycoside (pentose/hexose)] were extracted to draw selected ion chromatograms. The chromatogram obtained with the enhanced product ion analyses with m/z of 447.23 (peak b) are shown in C. MS profile of the peak as shown in inset. IS; internal standard (formononetin). D. Comparison of the retention time of the linalyl diglycoside with that of linalyl primeveroside. E. The amount of linalyl diglycoside determined with the standard curve constructed with linalyl primeveroside in intact and freeze-thaw-treated soybean leaves. The means ± standard errors (n = 3) are shown. Significant differences were identified using Student's t-test (**; P < 0.01). n.s.; not significant.

1.5 Discussion

Although 1-octen-3-yl pri was first found in dry seeds of soybean (Matsui et al. 2018), this study indicated that the young green organs, such as leaves and sepals, had much higher amounts of 1-octen-3-yl pri. This is largely consistent with the distribution of aroma primeverosides in tea plants, where

the amounts of pri composed of 2-phenylethanol, (*Z*)-3-hexen-1-ol, linalool, and geraniol were higher in the young leaves than in the mature leaves (Ohgami et al. 2015). Because the amounts of 1-octen-3-ol and 1-octen-3-yl glucoside in intact tissues of soybean plants were mostly negligible, 1-octen-3-ol formed from linoleic acid should be immediately converted into its pri through sequential glucosylation and xylosylation, as reported for tea leaves (Ohgami et al., 2015). With the moss *Physcomitrella patens*, a multifunctional lipoxygenase accounts for the formation of 1-octen-3-ol from arachidonic acid (Senger et al. 2005). In contrast, the seeds of soybean lipoxygenase-null mutants still have a substantial amount of 1-octen-3-ol, indicating that an oxygenase other than lipoxygenase is responsible for 1-octen-3-ol production (Kobayashi et al. 1995). In cyanobacteria, *Nostoc punctiforme*, a cyclooxygenase-related enzyme forms 10(*S*)-hydroperoxide of linoleic acid, and a catalase-related enzyme cleaves the hydroperoxide to yield 1-octen-3-ol (Brash et al. 2014). The involvement of cyclooxygenase-related genes in the formation of 1-octen-3-ol in fungal species belonging to Ascomycota has also been reported (Ferrari et al. 2018; Kataoka et al. 2020). Based on Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html), the soybean genome contains four genes tentatively annotated as prostaglandin G/H synthase (cyclooxygenase). Correlation analysis of the amount of 1-octen-3-yl pri and the transcript levels of the candidate genes must give a useful clue to identify the genes responsible for the formation of 1-octen-3-yl pri.

Because 1-octen-3-yl pri was hydrolyzed in preference to the other glycosides, such as isoflavone glycosides or linalyl diglycoside, immediately after the tissues would be damaged, it is likely that 1-octen-3-yl pri is a storage form of volatile 1-octen-3-ol for quick formation of volatiles upon tissue damage. This reminds us of the myrosinase-glucosinolate system in Brassicaceae. The physiological significance of 1-octen-3-ol in plants has not been completely clarified; however, it has been assumed that 1-octen-3-ol is involved in inter- and intraspecies communication (Inamdar et al. 2020). 1-Octen-3-ol is formed from *Phaseolus vulgaris* (common beans) leaves after infection by the fungal pathogen *Colletotrichum lindemuthianum* (Quintana-Rodriguez et al. 2015). 1-Octen-3-ol

failed to inhibit the mycelial growth of *C. lindemuthianum* but inhibited the growth of other fungal pathogens, such as *Fusarium oxysporum* and *Botrytis cinerea* (Quintana-Rodriguez et al. 2018). Herbivore damage on *Phaseolus lunatus* and *Lotus japonicus* leaves caused induction of 1-octen-3-ol formation (Ozawa et al. 2000; Boggia et al. 2015). 1-Octen-3-ol might be involved in the suppression of oviposition by *Maruca vitrata* on cowpea (*Vigna unguiculat*a) plants (Osei-Owusu et al. 2020), attracting predatory mites onto herbivorous mite-infested *L. japonicus* leaves (Ozawa et al. 2000), or functions as an airborne signal to induce defense responses in *Arabidopsis thaliana* plants (Kishimoto et al. 2007). In situations where the defensive functions of volatile compounds should be effectively demonstrated, timely and at-the-right-place formation of volatile compounds is important. The myrosinase-glucosinolate system is one way to quickly form defensive isothiocyanate at the right place (Halkier and Gershenzon et al. 2006). Green leaf volatiles are also rapidly formed, but through de novo formation, depending on the rapid and local activation of a lipoxygenase (Mochizuki et al. 2016). The quick formation of 1-octen-3-ol after mechanical damage to plant organs through hydrolysis of its glycoside is another example of the quick formation of defense volatiles immediately after mechanical damage. The timely and local formation of 1-octen-3-ol is accomplished by the rapid hydrolysis of its pri upon disruption of tissues. This must be supported by a primeverosidase that is specific to 1-octen-3-yl pri. The primeverosidase should be compartmentalized from its substrate or kept latent until tissue disruption.

We found that soybean leaves contained substantial amounts of linalyl diglycoside. Neither linalyl pri nor 1-octen-3-yl vic was detected. Linalool and 1-octen-3-ol are secondary alcohols that share the 1-alkene-3-ol structure. 1-Octen-3-yl pri should be formed by UDP-xylosyl transferase from 1-octen-3-yl glucoside, and linalyl diglycoside should be formed by UDP-pentosyl transferase from linalyl glucoside. The enzyme responsible for this second step of pentosylations should have strict substrate specificity against either the sugar acceptors (linalyl or 1-octen-3-yl glucoside) or the sugar donor (UDP-pentoses). Furthermore, the enzyme involved in the hydrolysis of 1-octen-3-yl pri should

also have a strict substrate specificity and showed no or low activity against linalyl diglycoside because its amount decreased only slightly, while that of 1-octen-3-yl pri decreased considerably after disruption of the soybean leaf tissues. This might be due to the high substrate specificity of a soybean glycosidase. The β-primeverosidase isolated from tea leaves accepts only primeverose as the glycone substrate, even though it broadly accepts various aglycones (Ma et al., 2001). As the biosynthesis and degradation of these two glycosides are distinct, their physiological functions might be distinct as well. 1-Octen-3-yl pri releases volatile 1-octen-3-ol upon mechanical damage on leaf tissues, while linalyl diglycoside exist without being decomposed. A subset of glycosides, such as 17-hydroxygeranyllinalool diterpene glycosides or (*Z*)-3-hexenyl vicianoside, exert direct defense against herbivores (Heiling et al. 2010; Sugimoto et al. 2014). Based on these reports, it might be possible that linalyl diglycoside play a role as a non-volatile direct defense chemical. To confirm this hypothesis, the enzymes involved in the biosynthesis of the two diglycosides and the primeverosidase specific to 1-octen-3-yl pri should be carried out. As the structure of the glycone moiety has not been completely determined, complete structure elucidation of the linalyl diglycoside is also essential. In addition, further research should focus in understanding the specific factors affecting glycosides content in soybeans since the content of glycosides during developmental stages varied among the location of specific tissues, thus demonstrating that the distribution and the identities of glycosides are tissue specific.

References

Badenhop AF, Wilkens WF (1969) The formation of 1-octen-3-ol in soybeans during soaking. J. Am Oil Chem Soc 46:179-182. https://doi.org/10.1007/BF02635729

Boggia L, Sgorbini B, Bertea CM, Cagliero C, Bicchi C, Maffei ME, Rubiolo P (2015) Direct contact-sorptive tape extraction coupled gas chromatography-mass spectrometry to reveal volatile topographical dynamics of lima bean (*Phaseolus lunatus* L.) upon herbivory by *Spodoptera littoralis* Boids. BMC Plant Biol 15:102. https://doi.org/10.1186/s12870-015-0487-4

Brash AR, Niraula NP, Boeglin WE, Mashhadi Z (2014) An ancient relative of cyclooxygenase in cyanobacteria is a linoleate 10*S*-dioxygenase that works in tandem with a catalase-related protein with specific 10*S*-hydroperoxide lyase activity. J Biol Chem 289:13101-1311.

https://doi.org/10.1074/jbc.M114.555904

Contreras-Cornejo HA, Marcías-Rodríguez L, del-Val E, Larsen J (2019) The root endophytic fungus *Trichoderma atroviride* induces foliar herbivory resistance in maize plants. Appl Soil Ecol 124:45-53. https://doi.org/10.1016/j.apsoil.2017.10.004

Croisier E, Rempt M, Pohnert G (2010) Survey of volatile oxylipins and their biosynthetic precursors in bryophytes. Phytochemistry 71:574. https://doi.org/10.1016/j.phytochem.2009.12.004

Cui, J., Katsuno, T., Totsuka, K. et al (2016) Characteristic fluctuations in glycosidically bound volatiles during tea processing and identification of their unstable derivatives. J Agric Food Chem 64:1151-7. https://doi.10.1021/acs.jafc.5b05072.

Ferrari R, Lacaze I, Le Faouder P, et al (2018) Cyclooxygenase and lipoxygenases are used by the fungus *Podospora anserina* to repel nematodes. Biochim Biophys Acta 1862:2174-2182. https://doi.org/10.1016/j.bbagen.2018.07.012

Fujita R, Yokono M, Ube N, Okuda Y, Ushijima S, Fukushima-Sakuno E, Ueno K, Osaki-Oka K, Ishihara A (2021) Suppression of *Alternaria brassicicola* infection by volatile compounds from spent mushroom substrates. J Biosci Bioeng 132: 25-32. https://doi.org/10.1016/j.jbiosc.2021.03.003

Halkier BA, Gershenzon, J (2006) Biology and biochemistry of glucosinolates. Ann Rev Plant Biol 57:303-333. https://doi.org/10.1146/annurev.arplant.57.032905.105228

Heiling S, Llorca LC, Li J, et al (2021) Specific decorations of 17-hydroxygeranyllinalool diterpene glycosides solve the autotoxicity problem of chemical defense in *Nicotiana attenuata*. Plant Cell 33: 1748–1770 in press. https://doi.org/10.1093/plcell/koab048

Holighaus G, Rohlfs M (2019) Volatile and non-volatile fungal oxylipins in fungus-invertebrate interactions. Fungal Ecol 38:28-36. https://doi.org/10.1016/j.funeco.2018.09.005

Inamdar AA, Hossain MM, Bernstein AI, Miller GW, Richardson JR, Bennett JW (2013) Fungal-derived semiochemical 1-octen-3-ol disrupts dopamine packaging and causes neurodegeneration. Proc Natl Acad Sci USA 110: 19561-19566. https://doi.org/10.1073/pnas.1318830110

Inamdar AA, Morath S, Bennett JW (2020) Fungal volatile organic compounds: more than just a funky smell? Ann Rev Microbiol 74:101-116. https://doi.org/10.1146/annurev-micro-012420-080428

Kataoka R, watanabe T, Yano S, Mizutani O, Yamada O, Kasumi T, Ogihara J (2020) *Aspergillus luchuensis* fatty acid oxygenase *ppoC* is necessary for 1-octen-3-ol biosynthesis in rice koji. J Biosci Bioeng 129:192-198. https://doi.org/10.1016/j.jbiosc.2019.08.010

Kigathi RN, Unsicker SB, Reichelt M, Kesselmeier J, Gershenzon J, Weisser WW (2009) Emission of volatile organic compounds after herbivory from *Trifolium pratense* (L.) under laboratory and field condition. J Chem Ecol 35:1335-1348. https://doi.10.1007/s10886-009-9716-3

Kihara, H., Tanaka, M., Yamato, K.T., Horibata, A., Yamada, A., Kita, S., Ishizaki, K., Kajikawa, M., Fukuzawa, H., Kohchi, T., Akakabe, Y., Matsui, K. (2014) Arachidonic acid-dependent carbon-eight volatile synthesis from wounded liverwort *(Marchantia polymorpha).* Phytochemistry 107:42-49. https://doi.org/10.1016/j.phytochem.2014.08.008

Kishimoto K, Matsui K, Ozawa R, Takabayashi J (2007) Volatile 1-octen-3-ol induces a defensive

response in *Arabidopsis thaliana*. J Gen Plant Pathol 73:35-37. https://doi.org/10.1007/s10327-006-0314-8

Kobayashi A, Tsuda Y, Hirata N, Kubota K, Kitamura K (1995) Aroma constituents of soybean [*Glycine max* (L.) Merril] milk lacking lipoxygenase isozymes. J Agric Food Chem 43, 2449-2452. https://doi.org/10.1021/jf00057a025

Leitner M, Kaiser R, Hause B, Boland W, Mithöfer A (2010) Does mycorrhization influence herbivore-induced volatile emission in *Medicago trancatula*? Mycorrhiza 20:89-101. https://doi.org/10.1007/s00572-009-0264-z

Ma SJ, Mizutani M, Hiratake J, Hayashi K, Yagi, K, Watanabe N, Sakata K (2001) Substrate specificity of □-premeverosidase, a key enzyme in aroma formation during ooling tea and black tea manufacturing. Biosci Biotechnol Biochem 65, 2719-2729. https://doi.org/10.1271/bbb.65.2719

Maeda R, Waki T, Kawai Y, Takahashi S, Nakayama T (2018) Involvement of chalcone reductase in the soybean isoflavone metabolon: identification of GmCHR5, which interacts with 2-hydroxyisoflavonone synthase. Plant J 96:56-74. https://doi.org/10.1111/tpj.14014

Matsui K, Takemoto H, Koeduka T, Ohnishi T (2018) 1-Octen-3-ol is formed from its glycoside during processing of soybean [*Glycine max* (L.) Merr.] Seeds. J Agric Food Chem 66:7409-7416. https://doi.org/10.1021/acs.jafc.8b01950

Mochizuki S, Sugimoto K, Koeduka T, Matsui K (2016) Arabidopsis lipoxygenase 2 is essential for formation of green leaf volatiles and five-carbon volatiles. FEBS Lett 590:1017-1027. https://doi.org/10.1002/1873-3468.12133

Potter CJ (2014) Stop the biting: targeting a mosquito's sense of smell. Cell 156:878-881. https://doi.org/10.1016/j.cell.2014.02.003

Nleya T, Sexton P, Gustafson K, Miler JM (2013) Soybean growth stages. In: Clay, David E.; Carlson, Charles Gregg; Clay, Sharon A.; Wagner, Larry; Deneke, Darrell L.; and Hay, Christopher H., "iGrow Soybeans: Best Management Practices for Soybean Production" (2013). *Agronomy, Horticulture, and Plant Science Books*. 2. https://openprairie.sdstate.edu/plant_book/2

Ohgami S, Ono E, Horikawa M et al (2015) Volatile glycosylation in tea plants: sequential glycosylations for the biosynthesis of aroma *β*-primeverosides are catalyzed by two *Camellia sinensis* glycosyltransferases. Plant Physiol 168:464-477. https://doi.org/10.1104/pp.15.00403

Okull DO, Beelman RB, Gourama H (2003) Antifungal activity of 10-oxo-*trans*-8-decenoic acid and 1-octen-3-ol against *Penicillium expansum* in potato dextrose agar medium. J Food Prot 66:1503-1505. https://doi.org/10.4315/0362-028X-66.8.1503

Osei-Owusu J, Vuts J, Caulfield JC, Woodcock CM, Withall DM, Hooper AM, Osafo-Acquaah S, Birkett MA (2020) Identification of semiochemicals from cowpea, *Vigna unguiculata,* for low-input management of the legume pod borer, *Maruca vitrata*. J Chem Ecol 46**,** 288-298. https://doi.org/10.1007/s10886-020-01149-7

Ozawa R, Shimoda T, Kawaguchi M, Arimura G, Horiuchi J, Nishioka Y, Takabayashi J (2000) *Lotus japonicus* infested with herbivorous mites emits volatile compounds that attract predatory mites. J Plant Res 113:427-433. https://doi.org/10.1007/PL00013951

Quintana-Rodriguez E, Morales-Bargas AT, Molina-Torres J, Adame-Alvarez RM, Acosta-Gallegos JA, Heil M (2015) Plant volatiles cause direct, induced and associational resistance in common bean to the fungal pathogen *Colletotrichum lindemuithianum.* J Ecol 103:250-260. https://doi.org/10.1111/1365-2745.12340

Quintana-Rodriguez E, Rivera-Macias LE. Adame-Alvarez RM, Torres JM, Heil M (2018) Shared weapons in fungus-fungus and fungus-plant interactions? Volatile organic compounds of plant or fungal origin exert direct antifungal activity in vitro. Fungal Ecol 33:115-121. https://doi.org/10.1016/j.funeco.2018.02.005

Senger T, Wichard T, Kunze S, Göbel C, Lerchl J, Pohnert G, Feussner I (2005) A multifunctional lipoxygenase with fatty acid hydroperoxide cleaving activity from the moss *Physcomitrella patens*. J Biol Chem 280, 7588-7596. https://doi.org/10.1074/jbc.M411738200

Seo WH; Baek HH (2009) Characteristic aroma-active compounds of Korean perilla (*Perilla f rutescens* Britton) leaf. J Agric Food Chem 57, 11537−11542. https://doi.org/10.1021/jf902669d

Sugimoto K, Matsui K, Iijima Y, et al (2014) Intake and transformation to a glycoside of (*Z*)-3-hexenol from infested neighbors reveals a mode of plant odor reception and defense. Proc Natl Acad Sci USA 111:7144-7149. https://doi.org/10.1073/pnas.1320660111

Wang R, Ren C, Dong S, Chen C, Xian B, Wu Q, Wang J, Pei J, Chen J (2021) Integrated metabolomics and transcriptome analysis of flavonoid biosynthesis in safflower (*Carthamus tinctorius* L.) with different colors. Front. Plant Sci. http://doi.org/10.3389/fpls.2021.712038.

Wang T, Wang S, Wang Y, Li J, Yan F, Liu Y, Zhao L, Wang Q (2020) Jasmonic acid-induced inhibition of root growth and leaf senescence is reduced by GmbHLH3, a soybean bHLH transcription factor. Can J. Plant Sci. http://doi.org/10.1139/cjps-2019-0250.

Wasternack C, Strnad M (2019) Jasmonates are signals in the biosynthesis of secondary metabolites – pathways, transcription factors and applied aspects – a brief review. New Biotechnol 48: 1-11. https://doi.org/10.1016/j.nbt.2017.09.007

Wu SY, Duncan LW (2020) Recruitment of an insect and its nematode natural enemy by olfactory cues from a saprophytic fungus. Soil Biol Biochem 144:107781. https://doi.org/10.1016/j.soilbio.2020.1007781

Wurzenberger M, Grosch W (1982) The enzymic oxidative breakdown of linoleic acid in mushrooms (*Psalliota bispora*). Z Lebensm Unters Forsch 175:186-190. https://doi.org/10.1007/BF01139769

Appendix

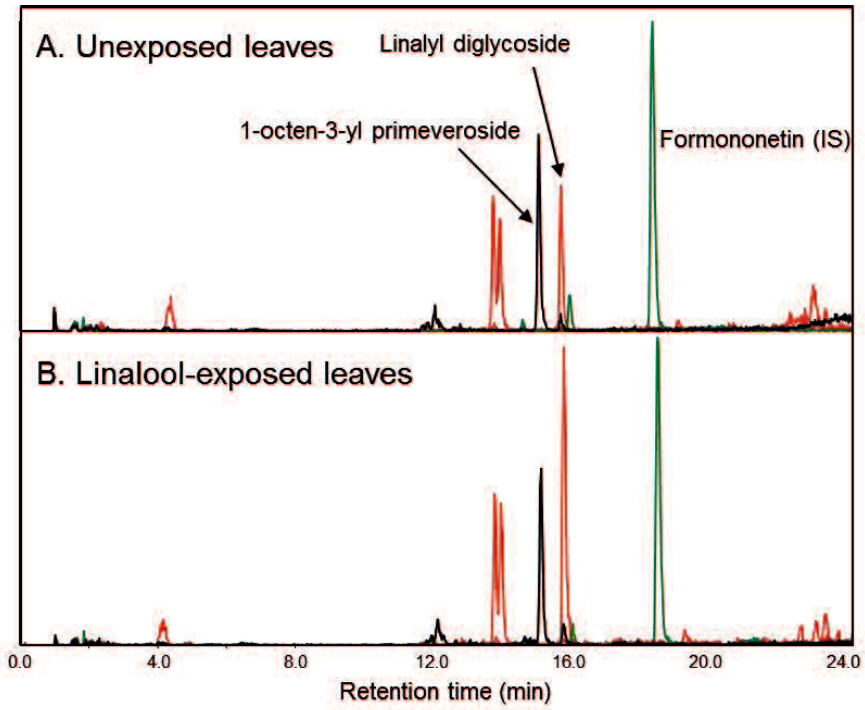


Fig. 1-4. Effect of exposure of intact soybean leaves with linalool vapor on the chromatograms of linalyl diglycoside. A. Chromatogram of unexposed leaves. B. Chromatogram of linalool-exposed leaves. Only the peak at a retention time of 16.0 (corresponding to peak in Fig. 1-3) increased after exposure.

Table 1-2. The range of *m/z* extracted for molecular ion chromatograms.

Compounds	Hill notation	Formula weight	Monoisotopic mass	*m/z* extracted for molecular ion chromatogram [M-H$^+$]
Linalyl pentopyranosylhexopyranoside	$C_{21}H_{36}O_{10}$	448.51	448.23	446.7-447.7
Geranyl primeveroside	$C_{21}H_{36}O_{10}$	448.51	448.23	447.0-448.0
Benzyl primeveroside	$C_{18}H_{26}O_{10}$	402.39	402.15	400.9-401.9
2-Phenyethyl primeveroside	$C_{19}H_{28}O_{10}$	416.42	416.17	414.9-415.9
n-Hexyl primeveroside	$C_{17}H_{32}O_{10}$	396.43	396.20	394.9-395.9
(*Z*)-3-Hexenyl primeveroside	$C_{17}H_{30}O_{10}$	394.41	394.18	392.9-393.9
1-Octen-3-yl primeveroside	$C_{19}H_{34}O_{10}$	422.47	422.22	420.8-421.8
1-Penten-3-yl primeveroside	$C_{16}H_{28}O_{10}$	380.39	380.17	378.9-379.9
2-Penten-1-yl primeveroside	$C_{16}H_{28}O_{10}$	380.39	380.17	378.9-379.9
Octan-3-yl primeveroside	$C_{19}H_{36}O_{10}$	424.48	424.23	423.0-424.0
Daidzin	$C_{21}H_{20}O_9$	416.38	416.11	414.9-415.9
Genistin	$C_{21}H_{20}O_{10}$	432.38	432.11	430.9-431.9

2 CHAPTER TWO

Exposing soybean leaves to linalool vapor resulted in accumulation of linalyl vicianoside and higher defense against herbivore

Abstract

Linalool is anticipated to have significant ecological roles. In this study, linalyl 6-*O*-α-arabinopyranosyl-β-D-glucopyranoside (linalyl β-vicianoside: LinVic) was synthesized, and a linalool diglycoside purified from soybean leaves was identified as LinVic by using liquid chromatography-mass spectrometry. High levels of LinVic were detected in leaves and sepals during soybean plant growth. The LinVic content did not significantly increase following methyl jasmonate treatment of the leaves, indicating that its synthesis is independent of the jasmonic acid signaling pathway. In addition to LinVic, soybean also contains 1-octen-3-yl primeveroside. We treated soybean leaves with vaporized linalool and 1-octen-3-ol to determine whether the glycosylation system discriminates between these two volatile alcohols. Linalool treatment resulted in the accumulation of LinVic, while 1-octen-3-ol treatment caused little change in the amount of 1-octen-3-yl primeveroside, suggesting discrimination between these compounds. Linalool-treated soybean leaves exhibited increased resistance against common cutworms, indicating that LinVic may contribute to herbivore resistance.

Key words: linalool, β-vicianoside, soybean (*Glycine max*), plant-herbivore interaction

2.1 Introduction

Plants produce a wide array of secondary metabolites, and their diversity is further increased through modification with sugars (Kyutidou *et al*. 2020). Glycosylation of plant metabolites involves multiple outcomes. Storage of defensive compounds that might be harmful to producers as a less self-toxic state is sometimes accomplished by glycosylation. For example, cyanogenic glycosides are the safe storage forms of hydrogen cyanide (Cressey and Reeve *et al*. 2019). Hydrolysis of cyanogenic glycosides can be mediated by the gut microbiome of herbivores during digestion of the plant materials; as such, the glycosides are the potential toxins for the plants' enemies. Too much secondary metabolites might be also detrimental to the producers; therefore, surplus metabolites are sometimes converted into their glycosides as, for example, reported with transgenic petunia plants overexpressing linalool synthase (Lücker *et al*. 2001). Glycosylation of phytohormones such as abscisic acid, auxin, cytokinins, among others, leads to their inactive storage forms that can be activated through hydrolysis when it is needed (Gachon *et al*. 2005). Taken together, these examples suggest that part of the role of glycosylation is to provide a temporary storage form for chemically or physiologically toxic or active compounds. It has been reported that the glycosides thus stored in plants can also contribute to the functionality of the food when that plant is consumed by humans. For example, a portion of flavor compounds is stored as its glycoside, and hydrolysis that proceeds during the processing of plant materials contributes to the flavor characteristics of green tea (Ohgami *et al*. 2015) and soymilk (Matsui *et al*. 2018).

In these examples, the structure of the sugar moiety of the glycoside does not seem to be so important. However, to think that the addition of the sugar merely suppresses the activity of the aglycon moiety does not seem to fully explain the fact that the structures of the sugar moiety are quite diverse. 17-Hydroxygeranyllinalool diterpene glycosides found in *Nicotiana* species function in resistance against herbivores (Heiling *et al*. 2010). Specific glycosylation of the corresponding aglycon is essential for the plants to overcome the autotoxicity problem; but at the same time, the

glycosylation is shown to be critical for the defensive function against herbivores (Heiling *et al*. 2021). It is also reported that 4-methoxy-3-indolylylmethyl glucosinolate is required to activate Arabidopsis response against pathogen attack (Clay *et al*. 2009). (*Z*)-3-Hexenyl β-vicianoside accumulated in tomato leaves that had been exposed to the vapor of (*Z*)-3-hexenol exerted its defensive function against common cutworms (Sugimoto *et al*. 2014). From these findings, it appears that the addition of sugars to metabolites not only mask the biological and/or chemical activity of the metabolite but may also confer a function as a glycoside per se, depending on the structure of the sugar to be added. In this case, the overall structure of the glycoside generated via the addition of specific sugars should be important.

Recently we have found 1-octen-3-yl β-primeveroside in soybean plants. At that time, we found a compound that appeared to be a disaccharide glycoside of linalool, but its structure could not be determined (Matsui *et al*. 2018; Ntoruru *et al*. 2022). In this study, we synthesized linalyl β-vicianoside. Comparison of retention times and mass spectra of the linalyl disaccharide extracted from soybean leaves and the synthesized authentic compound determined by using liquid chromatography-mass spectrometry (LC-MS) revealed that the linalyl disaccharide was linalyl β-vicianoside. It seems that soybeans have two pathways to glycosylate volatile alcohols, one to lead β-primeveroside and the other to lead β-vicianoside. Soybean plants employ these two systems specifically depending on the structure of the volatile alcohols. We also examined the possibility that this diglycoside contributes to defense against herbivores by using soybean leaves exposed to linalool vapor.

2.2 Materials and Methods

2.2.1 Chemicals

The reference chemicals used for identification or quantitation were 1-octen-3-ol, linalool, nonanyl acetate, and formononetin, purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). Linalyl

β-vicianoside was synthesized as described below. Chemicals used for synthesis were reagent grade and were purchased from Nacalai Tesque (Kyoto, Japan) and Tokyo Chemical Industry (Tokyo, Japan). Linalyl primeveroside was prepared as described (Guo *et al*. 1994).

2.2.2 Synthesis of linalyl β-vicianoside

A solution of (2,3,4-tri-*O*-benzoyl-α-L-arabinopyranosyl)-(1→6)-2,3-di-*O*-benzyl-4-*O*-acetyl-D-glucopyranosyl 2,2,2-trifluoro-*N*-phenyl-acetimidate (S1) (0.30 g, 0.29 mmol) prepared as described (Yu *et al*. 2002; Gu et al., 2013; Sugimoto *et al*. 2023), molecular seive 4Å (0.30 g) and (*RS*)-linalool (44.0 mg, 0.29 mmol) in dichloromethane (2.9 mL) was stirred at 0 °C. Trifluoromethanesulfonic acid (1.3 μL, 14 μmol) was added to the mixture and the mixture was stirred at 0 °C for 30 min. The reaction mixture was quenched with triethylamine and filtered through the membrane filter with glass filter, then the filtrate was concentrated *in vacuo*. The obtained crude product was purified by flash column chromatography (hexane : ethyl acetate = 4 : 1 to 3 : 1) to give the desired product (*RS*)-linalyl-(2,3,4-tri-*O*-benzoyl-α-L-arabinopyranosyl)-(1→6)-2,3-di-*O*-benzyl-4-*O*-acetyl-β-D-glucopyranoside (S2) (0.24 g, 82%); ^{1}H nuclear magnetic resonance (NMR) (400 MHz, $CDCl_3$): δ (in ppm) = 8.07─7.83 (10H, m, aromatic), 7.60─7.30 (15H, m, aromatic), 5.77─5.43 (5H, m), 5.39─5.31 (1H, m), 5.19─4.95 (3H, m), 4.91─4.81 (2H, m), 4.68 (1H, d, *J* = 8 Hz), 4.35 (1H, dd, *J* = 4.9, 12.5 Hz), 4.04─3.95 (1H, m), 3.91 (1H, d, *J* = 12.5 Hz), 3.87─3.76 (1H, m), 3.76 (1H, m), 1.92 (3H, s), 1.87─1.62 (2H, m), 1.61─1.55 (3H, m), 1.48─1.28 (5H, m), 1.11─1.01 (3H, m).; ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.8 (minor), 169.7, 165.9, 165.9 (minor), 165.8, 165.7, 165.3, 164.9, 142.2, 141.5 (minor), 133.6, 133.5, 133.5, 133.5, 133.4, 133.2, 133.2, 131.5 (minor), 131.4, 130.0, 129.9, 129.8, 129.6, 129.6, 129.5, 129.4, 129.3, 129.2, 129.2, 129.1, 128.7, 128.6, 128.6, 128.5, 128.5, 128.4, 124.3, 124.2 (minor), 116.5 (minor), 115.1, 100.5 (minor), 100.3, 96.4 (minor), 96.1, 80.9, 80.9 (minor), 73.5, 73.4 (minor), 73.3 (minor), 73.1, 71.9,

71.8 (minor), 70.6 (minor), 70.3, 69.9, 69.4, 68.4 (minor), 68.3, 68.2, 41.9 (minor), 40.5, 25.7, 25.7 (minor), 22.8 (minor), 22.3, 22.3, 22.1 (minor), 20.7, 17.7, 17.6 (minor).; high resolution mass spectrometry-electrospray ionization (HRMS-ESI) (*m/z*) : $[M+NH_4]^+$ calculated for $C_{58}H_{64}NO_{16}$, 1028.4063, found 1028.4082.

A solution of S2 (0.21 g, 0.21 mmol) in methanol (0.8 mL) and tetrahydrofuran (1.6 mL) was stirred at room temperature. Sodium methoxide (0.5 M) in methanol (42 μL, 21 μmol) was added to the mixture and the mixture was stirred at room temperature for overnight. The reaction mixture was quenched with DOWEX MAC-3 ion exchange resin (Sigma-Aldrich, St. Louis, MO, USA) and filtered through the membrane filter with glass filter, then the filtrate was concentrated *in vacuo*. The obtained crude product was purified by flash column chromatography ($CHCl_3$: methanol = 3 : 1) with Silica Gel 60 mesh 230-400 (Nacalai Tesque) and preparative high performance liquid chromatography (HPLC) with a Triart C18 column (YMC, Kyoto, Japan), with the solvent system of acetonitrile/H_2O, 9 to 90%) to give the desired product (*RS*)-linalyl-β-vicianoside (49.8 mg, 53%) ^{1}H NMR (400 MHz, CD_3OD): δ (in ppm) = 6.06 (0.7H, dd, *J* = 11.0, 17.6 Hz, H-2, major:*3R*-isomer), 5.92 (0.3H, dd, *J* = 10.9, 17.7 Hz, H-2, major:*3S*-isomer), 5.28─5.14 (2H, m), 5.14─4.06 (1H, m), 4.38─4.27 (2H, m), 4.05─3.96 (1H, m), 3.86 (1H, dd, *J* = 3.6, 12.2 Hz), 3.83─3.78 (1H, m), 3.71 (1H, dd, *J* = 4.9, 11.3 Hz), 3.64─3.57 (1H, m), 3.57─3.48 (2H, m), 3.40─3.26 (3H, m), 3.20─3.11 (1H, m), 2.09─1.97 (2H, m), 1.71─1.55 (8H, m), 1.37 (0.9H, s, CH_3-10, minor:*3S*-isomer), 1.32 (2.1H, s, CH_3-10, major:*3R*-isomer).; ^{13}C NMR (100 MHz, CD_3OD): δ 144.4 (minor), 144.3, 132.1 (minor), 132.1, 125.8, 125.7 (minor), 115.9 (minor), 115.2, 104.9 (minor), 104.8, 99.6 (minor), 99.3, 81.5 (minor), 81.5, 78.1, 76.4 , 75.2 (minor), 75.0, 74.1, 72.3, 71.6, 69.3 (minor), 69.3, 69.2 (minor), 69.2, 66.3 (minor), 66.3, 42.7 (minor), 41.7, 25.9, 23.7, 23.5, 23.2 (minor), 17.8, 17.7.; HRMS-ESI (*m/z*) : $[M+NH_4]^+$ calcd for $C_{21}H_{40}NO_{10}$, 466.2647, found 466.2646.

^{1}H and ^{13}C NMR spectra were recorded at 298 K on AVANCE III HD 400 spectrometer

(Bruker, Billerica, MA, USA). Chemical shifts are reported in ppm relative to the solvent peak: $CDCl_3$ δ = 7.26 ppm (^{1}H NMR), 77.16 ppm (^{13}C NMR); CD_3OD δ = 3.31 ppm (^{1}H NMR), 49.00 ppm (^{13}C NMR). HRMS-ESI was carried out in positive and negative mode on Shimadzu (Kyoto, Japan) LCMS-IT-TOF (ion trap-time of flight) with aqueous acetonitrile containing 10 mM ammonium acetate as the mobile phase and sodium trifluoroacetate as an external standard.

2.2.3 Plants and insects

Soybean (*Glycine max* 'Enrei') seeds were harvested in Tsukuba City, Ibaraki Prefecture, Japan in 2019. The seeds were soaked on wet cotton for 24 h at 25°C under dim light. The seed coats, cotyledons, and embryonic axes were separated from the dry seeds and 1-day-imbibed seeds. To prepare the seedlings, 1-day imbibed soybean seeds were sown with a 1:1 mixture of sterilized Takii Tanemakibaido (Takii, Kyoto, Japan) and vermiculite. Plants were grown in pots in a growth chamber with 16 h light (fluorescent lights at 60 μmol m^{-2} s^{-1}) and 8 h dark regimen at 28°C until the emergence stage (VE), cotyledon stage (VC), or third trifoliate (V3) stages (growth stage classification followed the criteria of Clay *et al*. 2013). The seeds of *Mucuna pruriens*, *Vigna angularis, Phaseolus vulgaris* were purchased from a local market, and grown as shown above for soybean for 21 days. A portion of the soybean seeds were sown in a planter (575 × 235 × 200 mm) filled with normal soil for a vegetable garden available at a local gardening store on May 29, 2020. The planters were placed at the experimental farm in the Faculty of Agriculture, Yamaguchi University, Japan. The plants were grown under natural sunlight with daily irrigation until the full bloom (R2, harvested on July 17), full seed (R6, August 26), or full maturity stage (R8, 29 September).

Eggs and larvae of tobacco cutworm (*Spodoptera litura*) were purchased from Sumika Techno Service Co. Ltd. (Takarazuka, Hyogo, Japan). They were incubated in a climate-controlled

room at 24±1°C with a photoperiod of 16 h. The hatched larvae were reared on artificial diet (Insecta LFS, Nihon Nosan Kogyo, Tokyo, Japan) in a plastic container (0.9 L) with a lid with mesh. Feces in the plastic case were removed and pieces of an artificial diet was added three times a week. Third instars of *S. litura* larvae with an average body weight of 86.9 ± 3.17 mg were used for the feeding assay.

2.2.4 Purification of linalyl β-vicianoside

The soybean leaves on the 28th day of growth were harvested, weighed (428.2 g), and soaked into 2.4 L of methanol. The leaves were homogenized with a Polytron mixer (PT20, Kinematica Ag., Malters, Switzerland) at the maximum speed for 10 min, and the suspension was further stirred at 25°C with a magnetic stirrer for 24 h. The suspension was vacuum-filtered through filter paper (grade no. 2, qualitative, Advantec Toyo, Tokyo, Japan), and the residue was homogenized with 1.3 L of methanol with a Polytron mixer for 10 min, followed by a second filtration with the filter paper. The filtered extracts were combined, and methanol was removed from the extract under vacuum. The aqueous solution was diluted with water to 500 mL and washed with 500 mL of hexane. The aqueous solution was subjected to freeze drying for 12 h. The residues were dissolved with water to 550 mL and applied to a column (13.5 × 4.6 cm inner diameter) with Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan) that had been washed with 500 mL of isopropanol/0.05N NaOH (1:1, v/v) and subsequently equilibrated with 500 mL of water. The column was washed with 500 mL of water, and the glycosides were eluted with 300 mL of methanol. Methanol was removed from the eluate with a rotary evaporator under vacuum, and the remaining solution was diluted with water to 400 mL. The aqueous solution was applied to a column (15.0 × 2.3 cm inner diameter) with YMC GEL ODS-A (75 μm, YMC) that had been washed with 200 mL of methanol and equilibrated with 200 mL of water. The column was then washed with 100 mL of water. Subsequently, the column

was washed with 200 mL each of methanol solution of 20, 40, 50, and 100% (v/v) in water. The linalyl diglycoside was recovered with 50% methanol. Methanol was removed from the glycoside fraction, and the aqueous solution was dried with a freeze-dryer. The residue (7.0 mg) was dissolved in 5 mL of acetonitrile and fractionated with a semi-preparative HPLC system (Yamazen Smart Flash, Yamazen Co., Tokyo, Japan). The fractionation was carried out with a silica gel column (2.3×12.3 cm, 16 g) at 25˚C with monitoring absorbance at 254 nm. The solvent system of 100% acetonitrile (solvent A) and 80% methanol (solvent B) was used. The run consisted of 100% A for 1 min, a linear increase from 100% A to 100% B over 5 min, and 10% B for 2 min with a flow rate of 10 mL min^{-1}. The glycoside was eluted at 8−12 min under the condition (2.6 mg).

2.2.5 Linalool exposure and feeding assay

Uniform and undamaged soybean plants (at V3 stage, 21-day-old) were chosen. Linalool was dissolved with 0.1% (w/v) Tween 20 at the concentration of 50, 100, and 200 μg mL^{-1}. The aqueous solution of linalool (1 mL) was adsorbed on a cotton ball and enclosed in an 8-L glass jar containing two pots with two plants each (total four plants). Tween 20 solution (0.1%) was used as control. 1-Octen-3-ol solution at 200 μg mL^{-1} in 0.1% Tween 20 was also used. The leaves were harvested immediately and after 1, 3, 5, and 7 days. The leaves were stored at −20°C for the glycoside and volatile analysis. For the bioassay with herbivores, the soybean plants at the V3 stage exposed to linalool vapor (0, 50, and 200 μg mL^{-1}) in the 8-L jar for 3 days as described above were used. The first trifoliate leaves were cut out, and the cut surface of the petiole was carefully covered with wet cotton to avoid desiccation. Five third instars of *S. litura* larvae were placed on the leaf placed inside a plastic container overnight. The weight of larvae was recorded before and after feeding. The experiment was performed in triplicate.

2.2.6 Analyses of glycosides and phytohormones

Plant organs were homogenized with 10 volumes (v/w) of 10 μg mL^{-1} formononetin (as an internal standard) in 80% (v/v) methanol in a mortar and pestle. The cotyledons from the dry seeds were homogenized using a Polytron mixer. The suspension was centrifuged at 20,000 × g for 10 min at 24°C. The supernatant was filtered through a DISMIC-03JP (0.5 μm, Advantec, Tokyo, Japan) and used for LC-MS/MS analysis (3200 QTRAP LC/MS/MS System; AB Sciex, Framingham, MA, USA) equipped with a Prominence UFLC (Shimadzu). The extracts were separated on a Mightysil RP18 column (2 mm inner diameter × 150 mm, 5 μm; Kanto Chemical) with solvent A (water/formic acid, 100:0.1, v/v) and solvent B (acetonitrile/formic acid, 100:0.1, v/v). The run consisted of 10% B for 5 min, a linear increase from 10 to 80% B over 17 min, and 80% B for 3 min, with a flow rate of 0.2 mL min^{-1}. To accomplish a base-line separation of linalyl β-vicianoside and linalyl β-primeveroside, the run consisted of 10% B for 5 min, a linear increase from 10 to 30% over 120 min, and subsequently from 30 to 80% over 5 min, and 80% B for 5 min, with a flow rate of 0.2 mL min^{-1}. The compounds were detected by MS/MS using enhanced MS scan mode with electrospray ionization in the negative ion mode [ion spray voltage, −4500 V; nitrogen as both the curtain gas (set to 40 arbitrary units) and collision gas (set to “high”); collision energy, −45 V; scan range, *m/z* 100−750; scan speed, 4000 Da s^{-1}; and declustering potential, −30 V]. Quantification of the glycosides was carried out with calibration curves constructed with the authentic glycosides by using formononetin as the internal standard. Ultra-high-performance liquid chromatography quadrupole-orbitrap mass spectrometry (UPLC-Q-Orbitrap HRMS) analysis was performed on a Thermo QExactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were separated on a Thermo Hypersil Gold VANQUISH C18 column (2.1 × 100 mm, 3 μm). The mobile phases were solvent A (0.1% formic acid in water, v/v) and solvent B (acetonitrile, v/v). The elution conditions applied were: 0–5 min, 5% B; 5–12 min, 25–80% B; 12–18 min, 80–99% B; 18–20 min, 99–5% B. The flow rate was 0.2 mL min^{-1}, and the sample injection volume was 3 μL. The

column was maintained at 40°C. The negative ionization mode conditions were as follows: capillary voltage, 3.00 kV; carrier gas, nitrogen; sheath gas pressure, 3.5 MPa; auxiliary gas pressure, 1.0 MPa; capillary temperature: 320°C; auxiliary gas heating temperature: 320°C; primary resolution: 70,000. The full scan mode was used. The scanning range of the negative ion spectra recorded were 80–1200 *m/z*. The levels of jasmonic acid and salicylic acids in the leaves subjected to linalool exposure were determined with LC-MS/MS analyses as described above but with MRM scan mode. Mass transitions for jasmonic acid and salicylic acid are 209.9/58.5 and 136.9/93.0, respectively. The conjugates of these acids were not examined.

2.2.7 Analysis of endogenous linalool

To examine the amount of linalool in soybean leaves when exposed to authentic linalool, the exposed leaves were cut at the base of the petiole, weighed and immediately grounded with liquid nitrogen using pestle and mortar. Approximately 300 mg of frozen powder was further grounded with 2 mL of acetonitrile containing 10 μg mL^{-1} nonayl acetate (internal standard) and vortexed for 10 min. To the mixture, 2 mL of distilled water was added followed by 1 g of ammonium sulfate to separate organic and aqueous phases. The suspension was centrifuged at 15,000 rpm for 10 min at 25°C. The volatile compounds in the upper organic phase were analyzed with a gas chromatography-mass spectrometry (GC–MS) instrument (QP-5050; Shimadzu, Kyoto, Japan) with a 0.25 mm internal diameter × 30 m DB-WAX column (film thickness, 0.25 μm; Agilent, Santa Clara, CA). The column temperature was programmed as follows: 40°C for 1 min, increasing by 15°C min^{-1} to 180°C, and then 180°C for 1 min. The carrier gas (He) was delivered at a constant pressure of 86.1 kPa. The mass detector was operated in electron-impact mode with an ionization energy of 70 eV.

2.3 Data analysis

The results included at least three replicates measured separately and are represented as mean ± standard error. The data were analyzed statistically using analysis of variance (ANOVA) with the Tukey's test ($P < 0.05$). Different alphabetical letters were assigned to demonstrate significant differences.

2.4 Results

2.4.1 Purification of linalyl vicianoside

When a crude methanol extract prepared with soybean leaves was subjected to LC-MS/MS analysis (at negative mode), a prominent peak was detected by monitoring the ion of *m/z* corresponding to 447.2 ± 0.5 (Fig. 2-S1A). The retention time of the peak was different from that of an authentic linalyl β-primeveroside (LinPri) that was detected by monitoring the same ion (Ntoruru *et al*. 2022). The compound was purified from methanol extracts of soybean leaves through ion-exchange chromatography, reversed-phase chromatography, and preparative reversed-phase HPLC. HRMS analysis (at negative mode) showed a *m/z* value of 448.2067 with reasonable correspondence to $C_{21}H_{36}O_{10}$ (calculated *m/z* value of 448.20737) corresponding to the linalyl pentopyranosylhexopyranoside.

Because it was expected that the compound was linalyl 6-*O*-α-arabinopyranosyl-β-D-glucopyranoside (linalyl β-vicianoside: LinVic), we synthesized it according to the scheme shown in Scheme 1 in Supplementary data. ^{1}H- and ^{13}C-NMR, as well as HRMS of synthesized compound unambiguously supported the structure of LinVic (Fig. 2-S2 and 2-s3, and the data shown in Materials and Methods) (Pabst *et al*. 1991; Watanabe *et al*. 1994; Sugimoto *et al*. 2023). When the synthesized

LinVic was subjected to the LC-MS/MS analysis, the retention time and MS profile reasonably coincided with those found with the compound extracted from the soybean leaves (Fig. 2-1). Accordingly, it is confirmed that the compound found in the soybean leaf was LinVic.

2.4.2 Developmental changes in the amount of linalyl β-vicianoside in soybean plants

The amount of LinVic was determined with soybean organs of several developmental stages, ranging from germinated seeds to the seed-ripening stages (Clay *et al*. 2013). Among the organs analyzed, the leaves showed a substantial amount of LinVic, and the medium leaves at V3 stage showed the highest (Fig. 2-1). Next to the leaves, the flowers; either the petals or sepals, showed a high amount of LinVic. With the roots, stems, seeds, and pods at the full seed (R6) stage, the level of LinVic was not very high. Previously, we found that methyl jasmonate treatment resulted in a higher amount of 1-octen-3-yl Pri in soybean leaves (Ntoruru *et al*. 2022). The data obtained at that time were reanalyzed to quantify LinVic, and it was found that the amount of LinVic was little affected until at least 7 days after treating soybean plants with methyl jasmonate (Fig. 2-S4), suggesting the physiological functions of these two diglycosides are different.

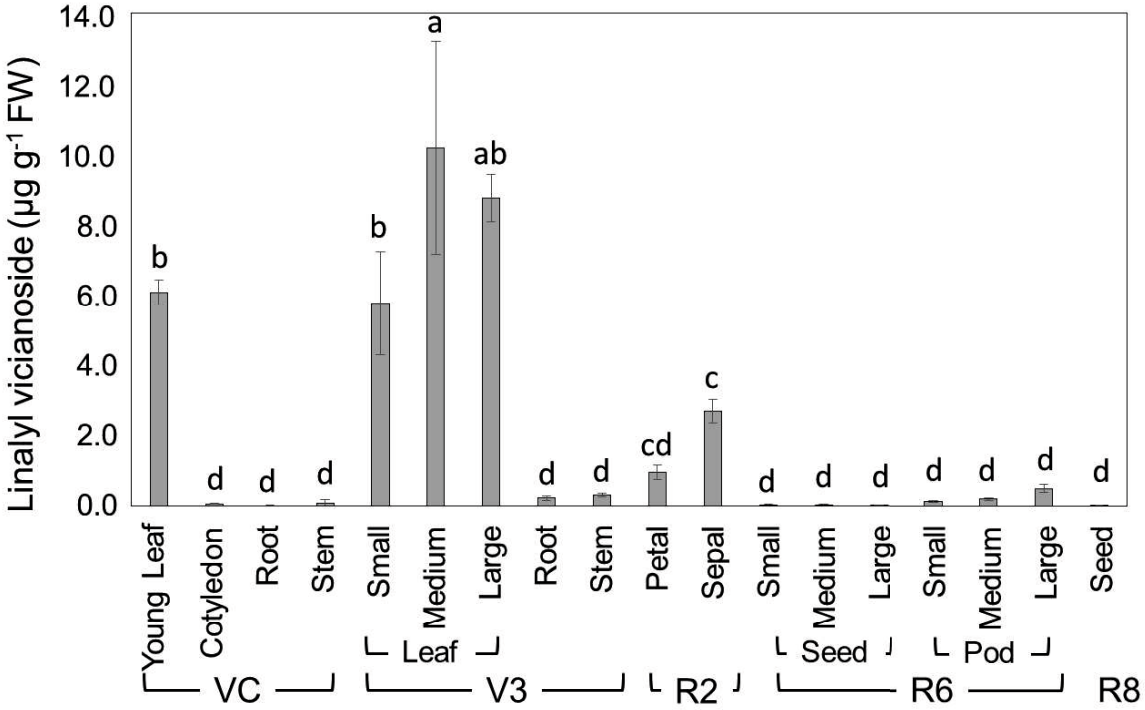


Fig. 2-1. Amount of linalyl β-vicianoside in soybean. The growth stages of soybean plants were grouped into VC (vegetative stage with emergence), V3 (vegetative stage with three trifoliates fully unfolded), R2 (reproductive stage with full bloom), R6 (reproductive stage with full green seeds), and R8 (reproductive stage with fully matured seeds) (Clay *et al*., 2013). Leaves at the V3 stage, and seeds and pods at the R6 stage were further grouped into three sizes (small, medium, large). The content of linalyl β-vicianoside was determined using LC–MS/MS analysis. The mean ± standard error with three biological replicates (n = 3) is shown. Different letters above each bar indicate significant differences among the materials analyzed ($P < 0.05$; one-way ANOVA; Tukey's test).

2.4.3 Effects of linalool vapor on LinVic

Several plants have abilities to take airborne compounds harboring a hydroxy group into their organs and convert them to their glycosides (Sugimoto *et al*. 2014, 2015; Tingting *et al*. 2019). We exposed soybean plants to the vapor of linalool vaporized from 1 mL of 0, 50, and 200 μg mL^{-1} solution, in an 8-L enclosed cylindrical glass container kept under the growth conditions. There was no significant difference in the amount of LinVic in each treatment at 24 hours (day 1) (Fig.2- 2). At day 3, the amount of LinVic was significantly increased with the leaves treated with 200 μg mL^{-1} of linalool solution, but not with 50 μg mL^{-1} solution. Based on the total fresh weight of soybean leaves in the jar (ca. 3 g FW), total amount of LinVic increased after treatment with 200 μg mL^{-1} of linalool

solution was calculated to be 54 μg (0.12 μmol), which accounted for ca. 9% of linalool placed in the jar (1.30 μmol). The high level of LinVic persisted at least until day 7 with the leaves treated with 200 μg mL^{-1} of linalool solution. In another experiment, soybean plants were treated with 0, 50, 100, and 200 μg mL^{-1} of linalool for 3 days. The accumulation of LinVic was hardly detected with 0, 50, and 100 μg mL^{-1} of linalool solution (Fig. 2-S5). Neither linalyl glucoside (LinGlu) nor LinPri was detected throughout these experiments. The amount of linalool in the linalool-exposed soybean leaves little increased depending on the amount of linalool used for exposure (Fig.2- S6). Exposure of plants to volatile compounds sometimes induces jasmonic acid- and/or salicylic acid-dependent defense responses (Engelberth *et al*. 2004; Yao *et al*. 2023). In order to examine if such defense responses would be activated or not, we determined the amounts of these phytohormones in the soybean leaves exposed to the linalool vapor. The amounts of jasmonic acid and salicylic acid showed no significant changes in the soybean leaves exposed to linalool vapor (Fig. 2-S7).

When soybean plants were exposed to the vapor of 1-octen-3-ol vaporized from 1 mL of 200 μg mL^{-1} solution as they were with linalool, the amount of 1-octen-3-yl Pri showed no increase (Fig.2-S8). Accumulation of 1-octen-3-yl Vic was neither observed in the 1-octen-3-ol-exposed soybean leaves.

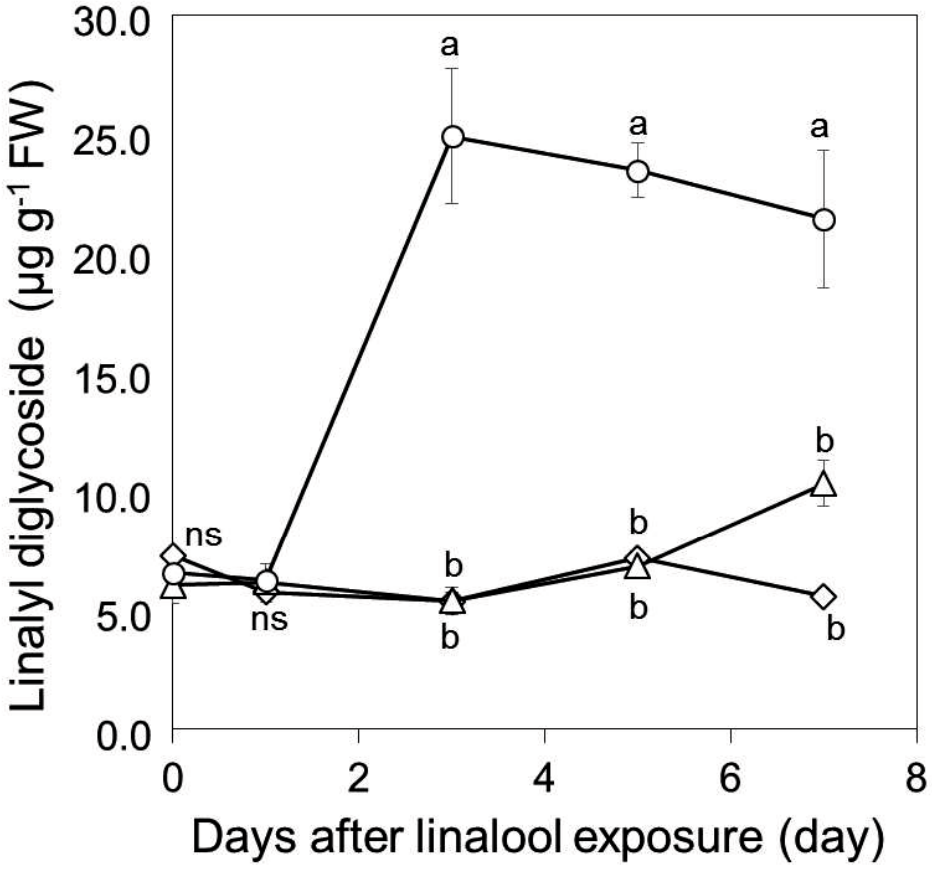


Fig. 2- 2. Effect of exposing soybeans to linalool vapor on the amount of linalyl β-vicianoside in the leaves. The soybean plants at the V3 stage were exposed to vapor derived from 1 mL of 0 (diamond), 50 (triangle), or 200 (circle) μg mL^{-1} of linalool solution impregnated into a cotton ball placed at the bottom of an 8-L glass jar. The trifoliate leaves were harvested at specified times for glycoside analysis. The means ± standard errors (n = 3) are shown. Significant differences were identified using two-way analysis of variance ($P < 0.05$, Tukey's test). Different lowercase letters indicates significant differences. ns: not significant.

To confirm whether LinVic accumulation is a special phenomenon observed only in soybean plants or whether it can be observed in other legumes in general, we examined whether absorption of airborne linalool and its glycosylation proceeded in other Fabaceae plants: *Mucuna pruriens*, *Vigna angularis*, and *Phaseolus vulgaris.* The plants were exposed to linalool vapor derived from 1 mL of 200 μg mL^{-1} of linalool solution in the 8-L glass container for 3 days under the same conditions as soybean plants, and the amount of LinVic was measured. The three Fabaceae plants used here contained a low but detectable amount of LinVic even before exposure (Fig. 2-3), and all the three plants more or less accumulated higher amounts of LinVic after exposed to linalool vapor. Among them, *M. pruriens* plants showed the largest increase in LinVic. Neither LinPri nor LinGlu was detected with the plants used here.

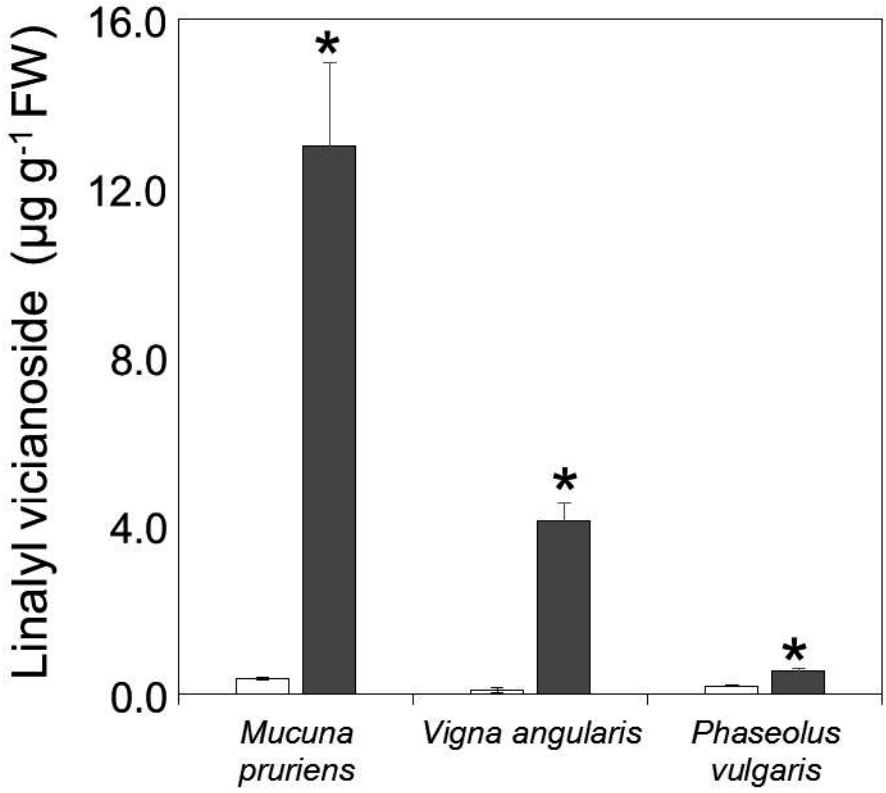


Fig. 2-3. Effect of exposing *Mucuna pruriens, Vigna angularis*, and *Phaseolus vulgaris* plants to linalool vapor on the amount of linalyl β-vicianoside in the leaves. Each Fabaceae plant at a growth stage equivalent to the V3 stage of soybean was exposed to linalool vapor generated from 200 μg mL^{-1} of linalool solution for three days, and the amount of linalyl β-vicianoside in the leaves was determined (black bars). As a control, 0.1% (w/v) Tween 20 solution was used instead of the linalool solution, and the amount of linalyl vicianoside was determined (white bars). Significant differences within each Fabaceae species were identified using Student's *t*-test (*$P < 0.05$). Data are mean ± standard error ($n = 3$).

2.4.4 Performance of *Spodoptera litura*

Previously, we observed that tomato plants exposed to the (*Z*)-3-hexenol vapor induced their resistance to an herbivore (Sugimoto *et al*. 2014). The detailed mechanism of this induced resistance is still unclear, but it has been suggested that a glycoside of (*Z*)-3-hexenol is involved (Sugimoto *et al*. 2014, 2023). Based on this background, we examined whether linalool-exposed soybeans also acquired higher resistant to an herbivore. Five third instar larvae of *Spodoptera litura* were reared on soybean leaves that had been treated with linalool vapor (derived from 1 mL of 0, 50, and 200 μg

mL^{-1} solution) for 3 days. The weight gain of the insect over the next 24 hours was measured (Fig. 2-4). With the leaves exposed to linalool vapor derived from 1 mL of 50 μg mL^{-1} solution, the larval body weight gain was not significantly suppressed from it observed with the control plants. With the leaves exposed to linalool vapor from 200 μg mL^{-1}, the weight gain was significantly suppressed.

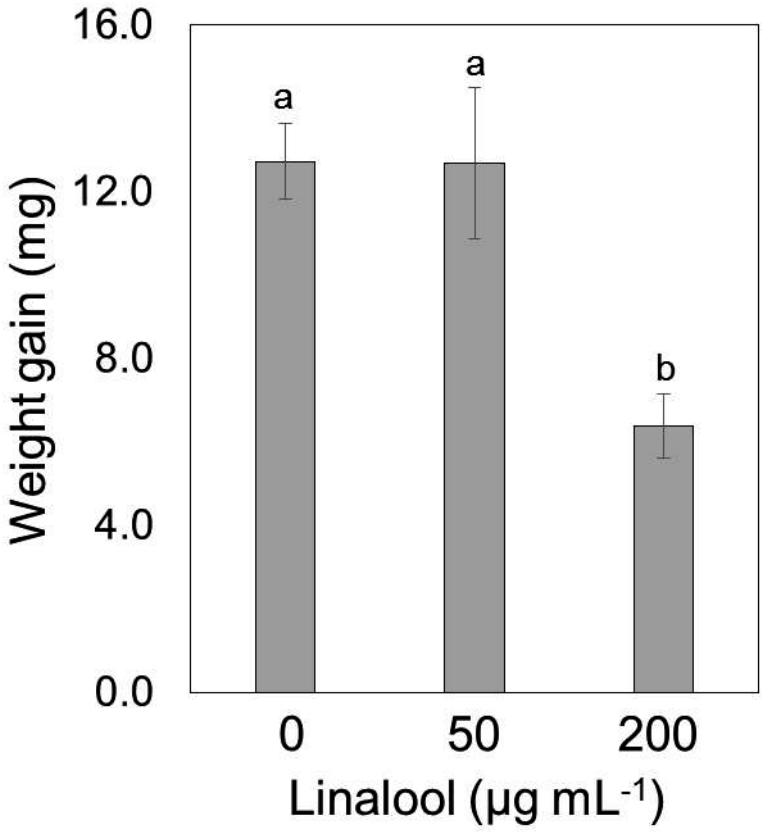


Fig. 2-4. Fumigation of soybean plants with linalool vapor enhances defense against herbivores (*Spodoptera litura*). Soybean plants were exposed to vapor generated from 0, 50, or 200 μg mL^{-1} linalool solution for three days. Subsequently, leaves cut from the petioles were fed to the third instar larvae of *S. litura*. The weight gains of the larvae on the detached exposed leaves are shown. The mean ± standard error (n = 3) is displayed. Different letters above each bar indicate significant differences ($P < 0.05$; one-way ANOVA; Tukey's test).

2.5 Discussion

We purified linalyl diglycoside whose structure was previously unknown, and determined its structure to be linalyl 6-*O*-α-arabinopyranosyl-β-D-glucopyranoside (linalyl β-vicianoside: LinVic)

using the synthesized compound. LinVic was first identified in raspberry (*Rubus idaeus*) fruits (Pabst *et al*. 1991), and subsequent isolations have only been reported from flower buds of *Gardenia jasminoides* (Watanabe *et al*. 1994), leaves of *Tarenna gracilipes* (Zhao *et al*. 2008), and leaves of *Ilex paraguariensis* (Sugimoto *et al*. 2009). This is the first report of LinVic in Fabaceae plants. Because glycoside of volatile monoterpene alcohols including linalool has been mostly found as its primeveroside in plants (Yazaki *et al*. 2017; Su *et al*. 2021), and because 1-octen-3-ol was found as its primeveroside in soybean (Ntoruru *et al*. 2021), it was a little surprising to us that the diglycoside turned out to be LinVic. The results shown in this study unambiguously indicate that 1-octen-3-ol is converted solely into its Pri while linalool is converted solely into its Vic in soybean plants. LinPri has never been detected in soybeans. Linalool and 1-octen-3-ol have a distinct structural difference, being tertiary and secondary alcohols, respectively, albeit they share the common structure of allyl alcohols with the backbone of 1-octene. Therefore, glycosyltransferases involved in the biosynthesis of LinVic and 1-octen-3-yl Pri from their aglycons should have a distinctive substrate specificity enough to distinguish these two alcohols. Tea leaf glycosyl transferase 1, CsGT1 (UGT85K11), catalyzes glucosylation to aglycons of volatile compounds. CsGT1 showed high activity with geraniol, eugenol, and (*Z*)-3-hexenol but only slight activity with linalool (Ohgami *et al*. 2015). The glycosyltransferase involved in the addition of the second sugar unit in tea leaves, CsGT2 (UGT94P1), also showed a relatively narrow substrate specificity preferring geranyl Glu but not linalyl Glu. As assumed from this, the modes of organ distribution of aroma glycosides, such as 2-phenylethyl Pri, hexenyl Pri, and geranyl Pri, which are most likely produced by CsGT1 and CsGT2, in tea plants are different from that of LinPri, which is expected to be formed by GTs other than CsGT1 and CsGT2 (Ohgami *et al*. 2015). In a way consistent with this, the distribution of LinVic by the organ of soybean plants is also distinctive. LinVic is basically leaf-specific and is hardly found in the cotyledons, stems, roots, pods, and seeds. This mode of organ distribution is somehow different from that of 1-octen-3-yl Pri (Ntoruru *et al*. 2022). 1-Octen-3-yl Pri is abundant in leaves at the 3rd

trifoliate stage as LinVic is, but a substantial amount of 1-octen-3-yl Pri is also detected in the organs other than leaves such as hypocotyls and fully matured seeds. Accordingly, the differences in the distribution of soybean LinVic from that of 1-octen-3-yl Pri should be due to the distinct pathway to biosynthesize them. The availability of UDP-xylose and UDP-arabinose should also affect how each is distinctively produced. It is assumed that they are independently biosynthesized because each has a distinctive physiological and ecological function.

Defensive hydroxyl geranyllinalool diterpene glycosides accumulated in wild tobacco plants (*Nicotiana attenuata*) in response to feeding by the hornworm *Manduca sexta* and to jasmonic acid (JA) treatment (Heiling *et al*. 2010). Spraying methyl jasmonate onto soybean leaves also caused an accumulation of 1-octen-3-yl Pri and isoflavone glycosides (Ntoruru *et al*. 2022). However, methyl jasmonate treatment caused little effect on the amount of LinVic in soybean leaves. This fact also supports our assumption that LinVic and 1-octen-3-yl Pri are formed through different pathways and have different functions. In soybeans, in particular, Pri and Vic are produced separately depending on the type of volatile alcohols, i.e., linalool and 1-octen-3-ol. The biochemical and physiological reasons why these two alcohols have distinctive fates in soybean leaves should shed light on the reason for the structural diversity of glycosides in nature. Further study is awaited to clarify them.

Previously, we have shown that the intake and glycosylation of exogenous (*Z*)-3-hexenol proceeds in a wide array of plant species (Sugimoto *et al*. 2014). With the plant species examined at that time, glucosylation to form hexenyl Glu was common but the fate of hexenyl Glu varied from plant species to plant species. Hexenyl Glu stayed as it is in almost half of the plant species examined, but some of the rest converted hexenyl Glu exclusively to either hexenyl Vic or hexenyl Pri, and yet another species produced both hexenyl Vic and hexenyl Pri. With Arabidopsis, linalool was taken in and converted to LinGlu, but further glycosylation was not detected (Sugimoto *et al*.

2015). Exposure of soybean plants to the vapor of linalool resulted in accumulation of LinVic, and at least under the experimental conditions employed here, increases in the amounts of LinGlu were not detected. Thus, there must be a high UDP-arabinosyltransferase activity that quickly converts the generated LinGlu to LinVic. It appears that plants absorb volatile alcohols from the atmosphere and convert them into glycosides, but each plant species seems to intentionally lead to its own unique disaccharide glycosides. Under the experimental condition employed here, treating soybean plants with 50 or 100 μg mL^{-1} of linalool solution did not lead to LinVic accumulation, and 200 μg mL^{-1} was needed for effective LinVic accumulation. The reason for this apparent threshold-like response is totally obscure. Further detailed study is needed.

Suppression of growth of *S. litura* larvae reared on soybean leaves that had been treated with linalool vapor implies that LinVic accumulated in the exposed soybean leaves accounted for the induced defense. A subset of glycosides of volatile compounds is known to function as a direct defense compound against herbivores (Sugimoto *et al*. 2014; Heiling *et al*. 2010, 2021). The possibility that linalool exposure activated the other defense systems in soybean plants cannot be ruled out. For example, when maize seedlings and hybrid poplar were exposed to the vapor of green leaf volatiles, the amounts of jasmonic acid and 12-oxophytodienoic acid increased (Engelberth *et al*. 2004; Frost *et al*. 2008). Exposing soybean plants to mint volatiles consisting of 1, 8-cineole, menthone, and menthol, among others, induced the expression of the trypsin inhibitor gene (Sukegawa *et al*. 2018), possibly through activation of the jasmonic acid signaling pathway as reported with tomato leaves (Farmer *et al*. 1992). Accordingly, it is assumed that exposing soybean plants to linalool activates the jasmonic acid signaling pathway, resulting in suppression of larval growth. Unexpectedly, exposing soybean plants to the vapor of linalool little affected the levels of jasmonic acid as well as salicylic acid. Therefore, the higher defense observed with linalool-exposed soybean plants seems to employ a different defense response system than the canonical jasmonic acid-mediated induced defense. The feeding experiment with longer and more detailed observation

of insect behavior is necessary to clarify the mechanism underlying the suppression of insect weight gain observed in the present study.

Linalool is a naturally occurring monoterpene alcohol found in a wide array of flowers and herbs. The involvement of linalool in interaction between plants and insects and between plants and micro-organisms has been repeatedly reported (Kessler and Baldwin 2001; Gershenzon and Dudareva 2007). It is expected that the use of natural compounds such as linalool to enhance crop resistance will enable the development of pest management techniques that do not burden the environment. It has also been shown that soybean makes Vic and Pri differently, and moreover, the hydrolysis rates of LinVic and 1-octen-3-yl Pri presumably catalyzed by endogenous glycosidases after tissue disruption are also very different (Ntoruru *et al.* 2022). Even though further studies are needed to know what enzymes and genes are involved in the metabolic system to form and degrade these glycosides and how they are regulated, by clarifying these issues, we expect to be able to develop a more effective method for applicable usage of linalool and LinVic functions in crop production and food production.

Conclusion

The glycosylation of VOCs such as 1-octen-3-ol and linalool represents a vital biochemical adaptation in soybeans, enabling the plants to store these volatile compounds efficiently and control their bioavailability. This process not only safeguards the plant from potential autotoxicity but also ensures the timely release of VOCs in response to environmental cues. By deepening our understanding of glycosylation and its impact on VOC functionality, we can unlock new pathways for improving crop resilience, optimizing plant-insect interactions, and enhancing the agricultural value

of soybeans. The interplay between glycosylation and VOCs exemplifies the sophistication of plant metabolic regulation and its potential applications in science and industry.

References

Clay NK, Adio AM, Denoux C *et al*. Glucosinolate metabolites required for an Arabidopsis innate immune response. *Science* 2009;323:95-101.

Clay DE, Carlson, CG, Clay SA *et al*. *iGrow Soybeans: Best Management Practices for Soybean Production.* South Dakota State University, 2013.

Cressey P, Reeve J. Metabolism of cyanogenic glycosides: a review. *Food Chem Toxicol* 2019;125:225–32.

Engelberth J, Alborn HT, Schmelz EA *et al*. Airborne signals prime plants against insect herbivore attack. *Proc Natl Acad Sci USA* 2004;101:1781-85.

Farmer EE, Johnson RR, Ryan CA. Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. *Plant Physiol* 1992;98:995-1002.

Frost CJ, Mescher MC, Dervinis C *et al*. Priming defense genes and metabolites in hybrid poplar by the green leaf volatile *cis*-3-hexenyl acetate. *New Phytol* 2008;180:722-34.

Gachon CMM, Langlois-Meurinne M, Saindrenan P. Plant secondary metabolism glycosyltransferases: the emerging functional analysis. *Trends Plant Sci.* 2005;10:542–9.

Gershenzon J, Dudareva N. The function of terpene natural products in the natural world. *Nat Chem Biol* 2007; 3:408-14.

Gu G, Adabala PJP, Szczepina MG, *et al*. Synthesis and immunological characterization of modified hyaluronic acid hexasaccharide conjugates. *J Org Chem* 2013; 78:8004－19.

Guo W, Hosoi R, Sakata K *et al*. (*S*)-Linalyl, 2-phenylethyl, and benzyl disaccharide glycosides isolated as aroma precursors from oolong tea leaves. The function of terpene natural products in the natural world. *Biosci Biotechnol Biochem* 1994; 58:1532-4.

Heiling S, Schuman MC, Schoettner M *et al*. Jasmonate and ppHsystemin regulate key malonylation

steps in the biosynthesis of 17-hydroxygeranyllilnalool diterpene glycosides, and abundant and effective direct defense against hebivores in *Nicotiana attenuata*. *Plant Cell* 2010;22:273-92.

Heiling S, Llorca LC, Li J *et al*. Specific decorations of 17-hydroxygeranyllinalool diterpene glycosides solve the autotoxicity problem of chemical defense in *Nicotiana attenuata*. *Plant Cell* 2021; 33:1748-70.

Kessler A, Baldwin IT. Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 2001; 291:2141-44. DOI: 10.1126/science.291.5511.2141

Kytidou K, Artola M, Overkleeft HS *et al*. Plant glycosides and glycosidases: a treasure-trove for therapeutics. *Front Plant Sci* 2020, DOI: 10.3389/fpls.2020.00357.

Lücker J, Boumeester HJ, Schwab W *et al*. Expression of *Clarkia S*-linalool synthase in transgenic petunia plants results in the accumulation of *S*-linalyl-β-D-glucopyranoside. *Plant J*. 2001;27,315-24.

Matsui K, Takemoto H, Koeduka T *et al*. 1-Octen-3-ol is formed from its glycoside during processing of soybean [*Glycine max* (L.) Merr.] seeds. *J Agric Food Chem* 2018;66:7409-16.

Moon J-H, Watanabe N, Ijima Y *et al*. *cis*- and trans-Linalool 3,7-oxides and methyl salicylate glycosides and (*Z*)-3-hexenyl -β-D-glucopyranoside as aroma precursors from tea leaves for oolong tea. *Biosci Biotechnol Biochem* 1996; 60:1815-9.

Ntoruru JM, Ohnishi T, Katsumata F *et al*. 1-Octen-3-ol is formed from its primeveroside after mechanical wounding of soybean leaves. *Plant Mol Biol* 2022;109:551-61.

Ohgami S, Ono E, Horikawa M *et al*. Volatile glycosylation in tea plants: sequential glycosylations for the biosynthesis of aroma *β*-primeverosides are catalyzed by two *Camellia sinensis* glycosyltransferases. *Plant Physiol* 2015;168:464–77.

Pabst A, Barron D, Sémon E *et al*. Isolation of a novel linalool disaccharide glycoside from raspberry fruit. *Tetrahedron Lett* 1991;32:4885-8.

Someya Y, Kobayashi A, Kubota K. Isolation and identification of *trans*-2- and *trans*-3-ydroxyl-1-8-cineole glucosides from *Alpinia galangal*. *Biosci. Biotechnol. Biochem* 2001;65:950-3.

Su D, Zheng Y, Chen Z *et al*. Simultaneous determination of six glycosidic aroma precursors in pomelo by ultra-high performance liquid chromatography-tandem mass spectrometry. *Analyst* 2021;146:1698-704.

Sugimoto S, Nakamura S, Yamamoto S *et al*. Brazilian natural medicines. III. Structures of triterpene oligoglycosides and lipase inhibitors from mate, leaves of *Ilex paraguariensis*. *Chem Pharm Bull* 2009; 57:3257-61.

Sugimoto K, Matsui K, Iijima Y *et al*. Intake and transformation to a glycoside of (*Z*)-3-hexenol from infested neighbors reveals a mode of plant odor reception and defense. *Proc Natl Acad Sci USA* 2014; 111:7144-9.

Sugimoto K, Matsui K, Takabayashi J. Conversion of volatile alcohols into their glucosides in Arabidopsis. *Commun Integr Biol* 2015, DOI: 10.4161/19420889.2014.992731

Sugimoto K, Ono E, Inaba T *et al*. Identification of a tomato UDP-arabinosyltransferase for airborne

volatile reception. *Nat Commun* 2023, DOI: 10.1038/s41467-023-36381-8.

Sukegawa S, Shiojiri K, Higami T *et al*. Pest management using mint volatiles to elicit resistance in soy: mechanism and application potential. *Plant J* 2018; 96:910-20.

Tingting J, Na Z, Ting G *et al*. Glucosylation of (*Z*)-3-hexenol informs intraspecies interactions in plants: a case study in *Camellia sinensis*. *Plant Cell Environ* 2019; 42:1352-67.

Watanabe N, Nakajima R, Watanabe S *et al*. Linalyl and bornyl disaccharide glycosides from *Gardenia jasminoides* flowers. *Phytochemistry* 1994; 37:457-9.

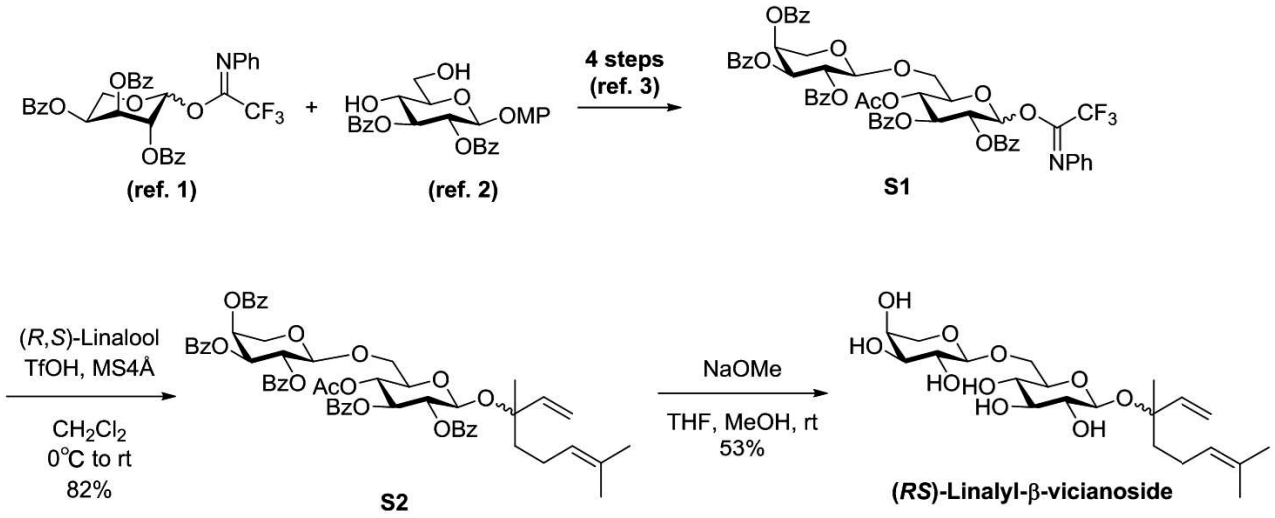
Yazaki K, Arimura GI, Ohnishi T. 'Hidden' terpenoids in plants: their biosynthesis, localization and ecological roles. *Plant Cell Physiol* 2017; 58:1615-21.

Yao C, Du L, Liu Q *et al*. Stemborer-induced rice plant volatiles boost direct and indirect resistance in neighboring plants. *New Phytol* 2023; 237:2375-87.

Yu B, Tao H. Glycosyl trifluoroacetimidates. 2. Synthesis of dioscin and xiebai saponin I. *J Org Chem* 2002;67:9099-9102.

Zhao Z, Matsunami K, Otsuka H *et al*. Tareciliosides A-G: Cycloartane glycosides from leaves of *Tarenna gracilipes (Hay.)* OHWI. *Chem Pharm Bull* 2008;56:1153-8.

Appendix



Scheme 1. Synthesis of (*RS*)-linalyl-β-vicianoside.

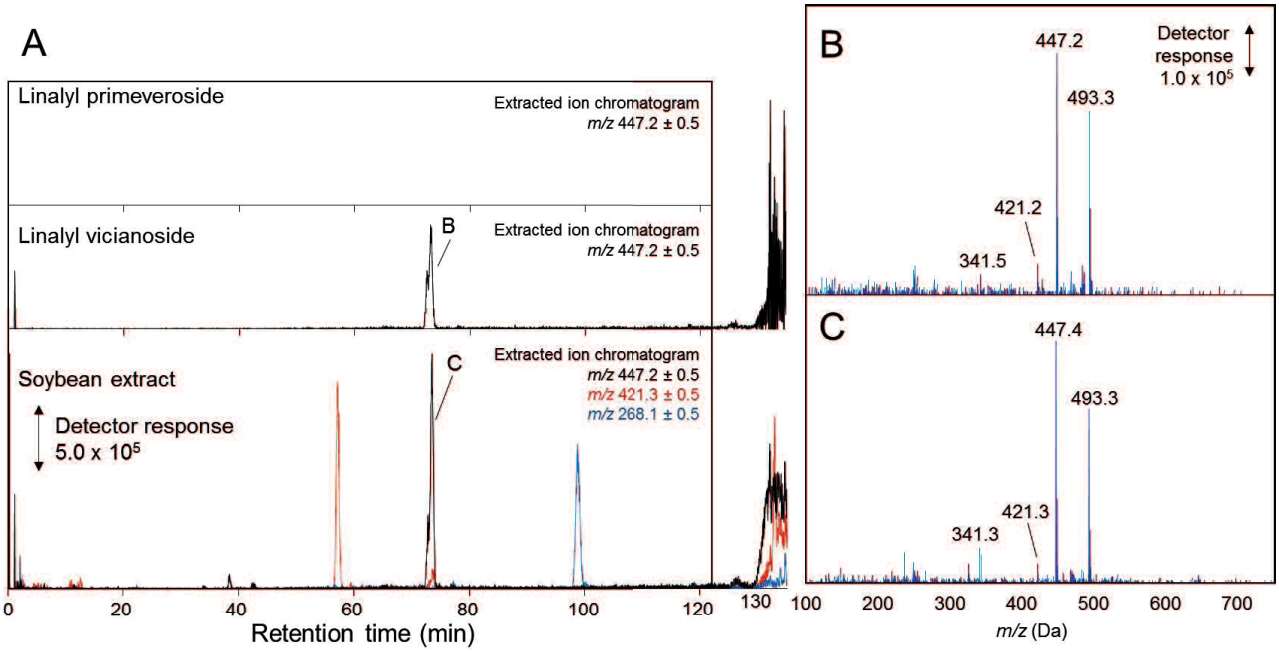


Fig. 2-S1. Separation of linalyl vicianoside and linalyl primeveroside with the LC-MS/MS system used in this study. Linalyl primeveroside and linalyl vicianoside prepared by organic synthesis (upper two chromatograms) and crude extract prepared from soybean leaves (lower chromatogram) were subjected to LC-MS/MS analysis as described in the Method section. For detection of linalyl pentopyranosyl-hexopyranoside, ions ranging from m/z 447.2± 0.5(shown in black), for detection of 1-octen-3-yl primeveroside, ions ranging from m/z 421.3± 0.5(shown in red), and for detection of formononetin (internal standard), ions ranging from m/z 268.1 ± 0.5(shown in blue) were extracted to draw extracted ion chromatograms. MS profiles of peaks A and B detected with linalyl vicianoside and soybean extract, respectively, were shown to the right.

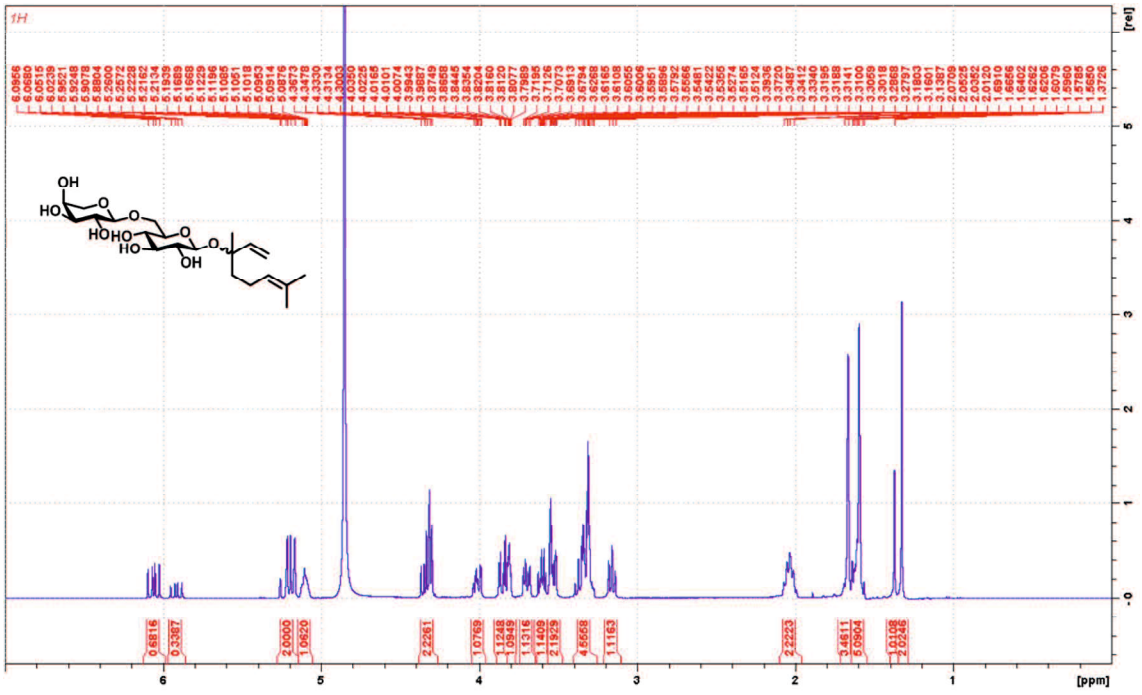


Fig. 2-S2. ^{1}H NMR (400 MHz, CD_3OD) spectrum of (*RS*)-linalyl-β-vicianoside

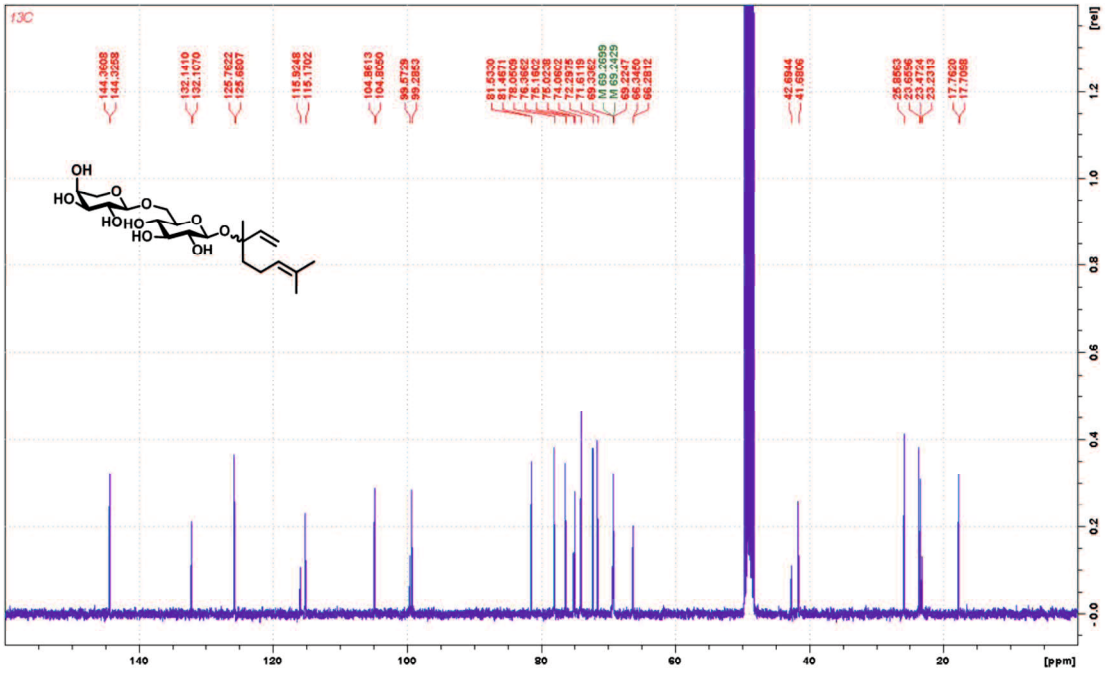


Fig. 2-S3. ^{13}C NMR (100 MHz, CD_3OD) spectrum of (*RS*)-linalyl-β-vicianoside.

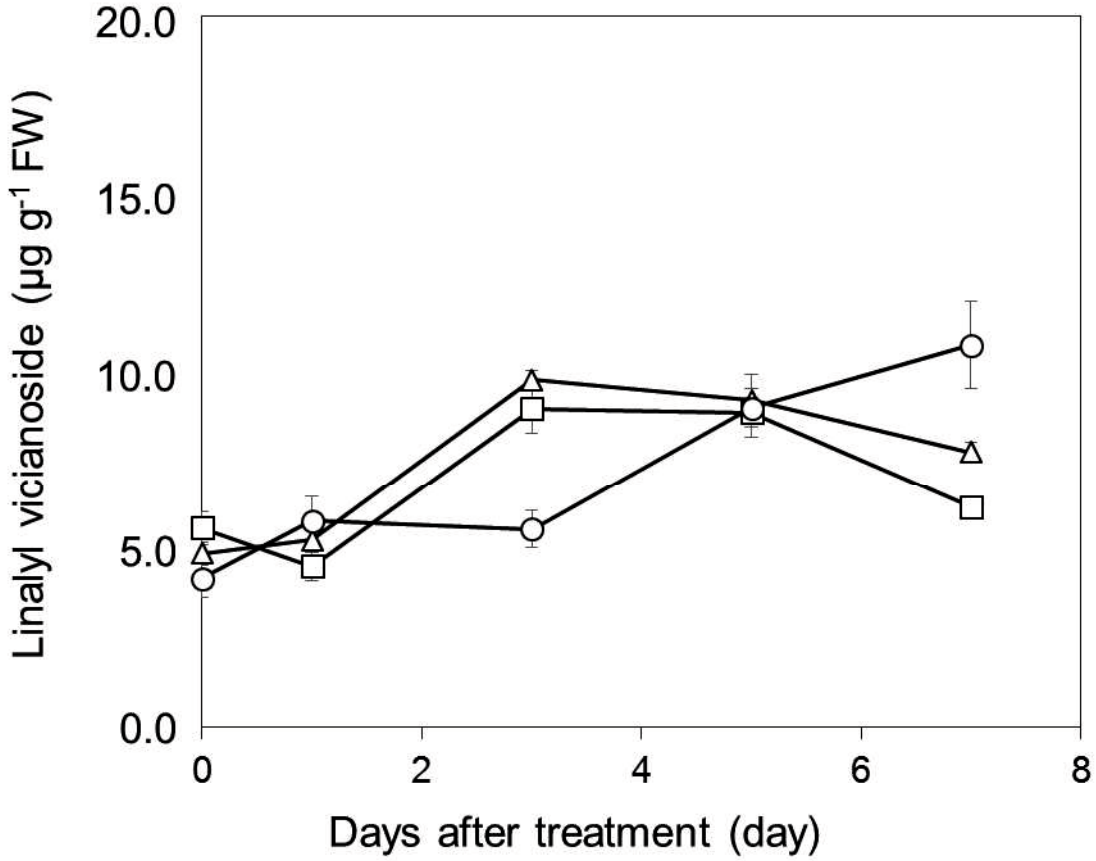


Fig. 2-S4. Effect of methyl jasmonate on the amount of linalyl vicianoside in soybean leaves. The soybean plants at the V3 stage were treated with 0 (circle), 50 (square), or 200 (diamond) μM of methyl jasmonate, and the trifoliate leaves were harvested at a specified time for the glycoside analysis. The means ± standard errors (n = 3) are shown. Significant differences were not identified with two-way analysis of variance analyses ($P < 0.05$, Tukey's test).

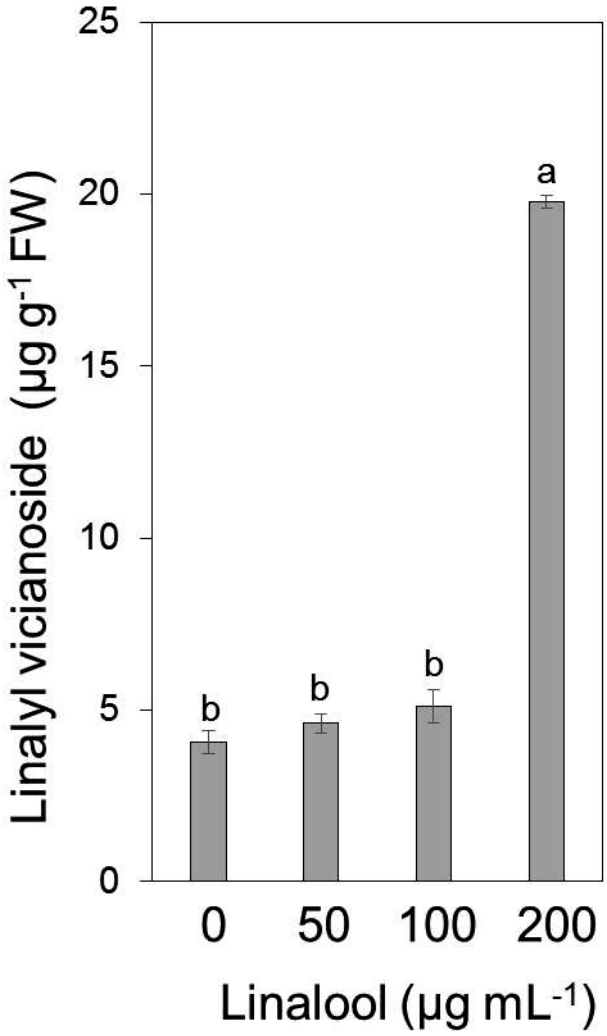


Fig. 2-S5. Effect of vaporized linalool on the amount of linalyl vicianoside in soybean leaves. The soybean plants at the V3 stage were treated with vaporized linalool derived from 1 mL of aqueous solution of linalool at 0, 50, 100, or 200 µg mL^{-1}, and the trifoliate leaves were harvested after 3 days for the glycoside analysis. The means ± standard errors (n = 3) are shown. Significant differences were not identified with two-way analysis of variance analyses (P < 0.05, Tukey's test).

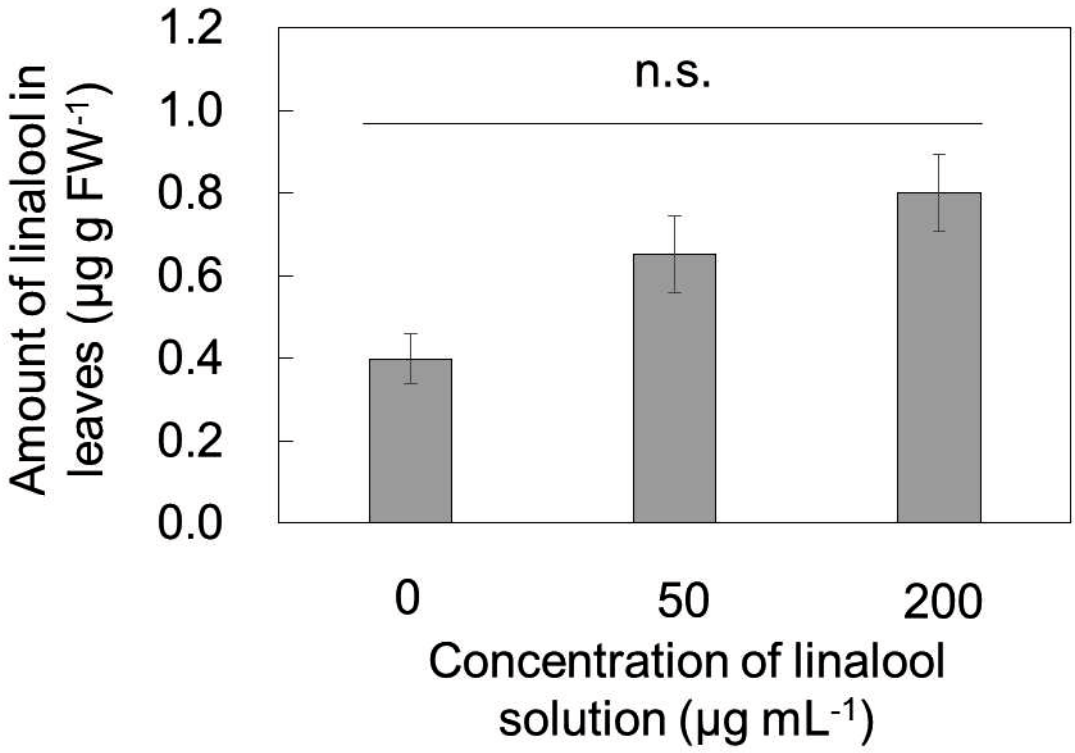


Fig. 2-S6. The amount of linalool (in the free form) in soybean leaves exposed to linalool vapor. Soybean plants were exposed to linalool vapor for three days, and the leaves were harvested for volatile analysis. There were no significant differences among the three treatments (one-way ANOVA, Tukey, *n* = 3).

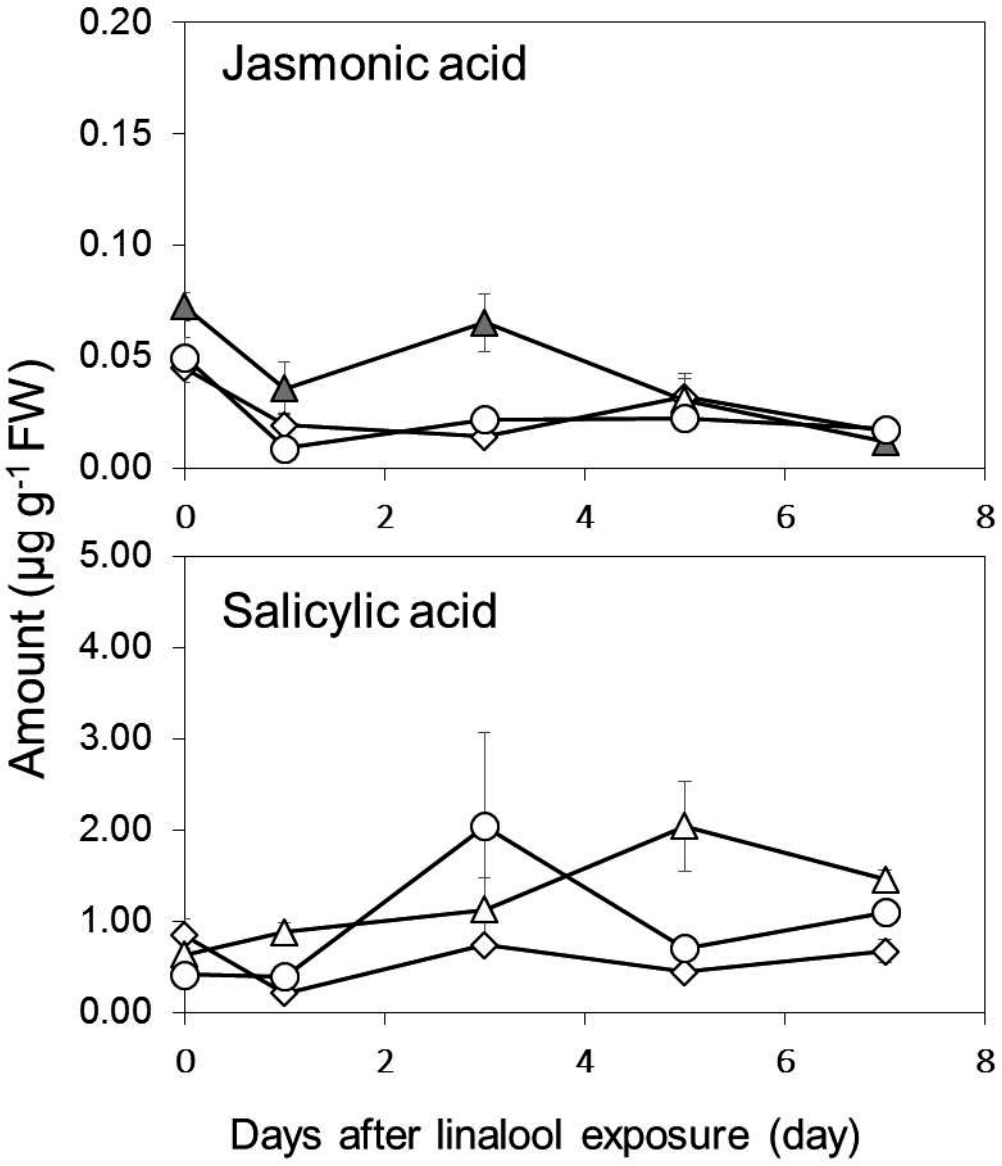


Fig. 2-S7. The effect of linalool fumigation on the amounts of jasmonic acid and salicylic acid in soybean leaves. Soybean plants were exposed to linalool vapor generated from 0 (diamond), 50 (triangle), or 200 (circle) μg mL^{-1} of linalool solution, and the leaves were harvested at the specified times for determination of the amounts of jasmonic acid and salicylic acid. The means ± standard errors (n = 3) are shown. There were no significant differences among the three treatments (one-way ANOVA, Tukey, *n* = 3).

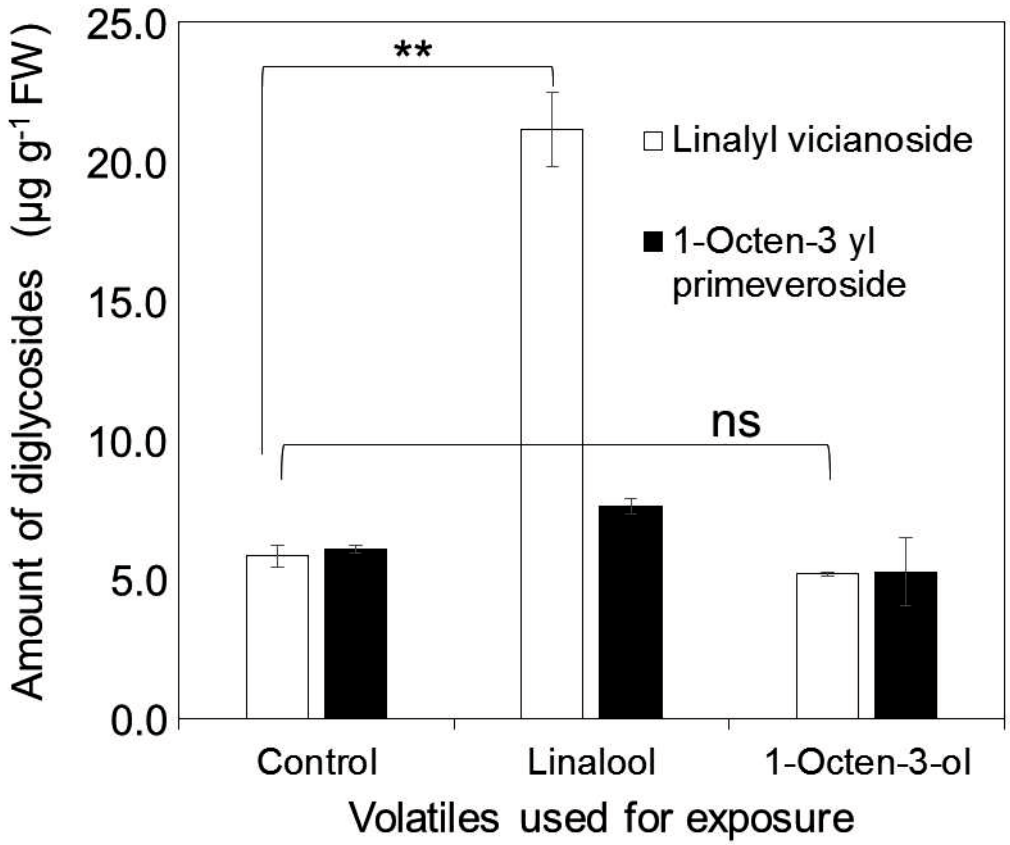


Fig. 2-S8. Effect of exposing soybean plants to linalool or 1-octen-3-ol on the amounts of aroma glycosides. The soybean plants at the V3 stage were treated with vapors of linalool and 1-octen-3-ol generated from respective 200 μg mL^{-1} solutions for three days. The leaves were harvested to determine the amounts of linalyl vicianoside (white bars) and 1-octen-3-yl primeveroside (black bars). The means ± standard errors (n = 3) are shown. Asterisks indicate significant differences between the control and the exposed leaves (P < 0.01), ns. not significant.**