An Amino Acid Ester of Menthol Elicits Defense Responses in Plants

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Abstract

Terpenoids, including menthol, exhibit potent abilities as plant defense potentiators in agriculture and horticulture. In the current study, we developed new terpene derivatives that consisted of menthol and various amino acids and that were expected to act as powerful plant defense potentiators. We used 6 amino acids possessing low-reactive sidechains to synthesize an array of amino acid ester of menthol (ment-aa) compounds. *T*ranscript *levels of two defense genes (pathogenesis-related 1* [*PR1*] and *trypsin inhibitor* [*TI*]) *were evaluated in leaves of soybean plants* 24 h after application of aquatic solution of menthol or menthol-aa, and revealed that the menthol menthyl ester of valine (ment-Val) alone elevated the transcript level of defense genes, and it did so only at the low dose of 1 μM, not at higher or lower doses tested. Moreover, it appeared that histone acetylation was involved in this effect. Application of ment-Val enabled soybean plants to sustain the increased transcript levels in their leaves for up to 3 days. Moreover, when ment-Val was additionally applied at day 4, at which time the transcript level had declined to the basal level, the transcript level was re-elevated, indicating the possibility that ment-Val could be repeatedly used to sustain pest control. M*ent-Val was found to be chemically stable and effective* for defense of several crop species. Collectively, these data show that terpenoid conjugates are useful for pest control instead of or in addition to pesticides.

Introduction

The substances known as plant *specialized metabolites* (PSMs, ~200,000 metabolites in nature), including terpenoids, phenols, alkaloids, fatty acids, etc., play a wide spectrum of roles in plant growth/development and environmental adaptation (Arimura and Maffei 2016). PSMs also play important roles in defense against herbivores and pathogens. For instance, limonene, a representative monoterpenoid, functions in the peel of citrus fruit as a defense substance against fungal and bacterial pathogens and a repellent of the citrus pest medfly (Rodríguez et al. 2011). A suite of monoterpenoids, especially including their ketones, exhibit insecticidal activity against the house fly, red flour beetle, and southern corn rootworm (Pamela and Coats. 1994). It has been shown that 6 essential oil components (methyl salicylate, carvacrol, thymol, *trans*-cinnamaldehyde, diallyl trisulfide, and *l*-perillaldehyde) display fumigant toxicity against nymphs and adults of thrips (Lu et al. 2020). Moreover, in addition to these compounds with insecticide-like efficacy, vaporable PSMs (volatile organic compounds [VOCs]), including terpenoids and green leaf volatiles, and benzenoids/phenylpropanoids (phenols), are well known to act as airborne cues in repelling herbivores, in attracting pollinators and herbivore enemies, and in between/within plant communications (Arimura et al. 2009).

Plant VOCs also serve as potentiators for plant defense traits. For instance, volatile monoterpenoids emitted from candy mint (1,8-cineole, menthone and menthol) have been shown to induce transcripts of defense genes such as genes for pathogenesis-related 1 (*PR1*) and trypsin inhibitor (*TI*) in soybean leaves, leading to acquired defense properties against herbivore and fungous pests (Sukegawa et al. 2018). Likewise, (*E*)-b-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene have been identified as possible infochemicals in intraspecific communication between lima

bean plants, as shown in studies in which transcripts of defense-related genes were induced in lima bean leaves exposed to those volatile terpenoids (Arimura et al. 2000, Arimura et al. 2002). Sagebrush exposed to either 1,8-cineol or (*E*)-beta-caryophyllene suffered less insect-induced damage than control sagebrush (Shiojiri et al. 2015).

Moving forward, derivatives of PSMs will be highly useful for biogenic, biochemical and pharmacological objectives, as shown previously in the development of aspirin (from salicin) as an analgesic/painkiller (Stahelin and von Wartburg 1991), naloxone and oxycodone (from thebaine) as an analgesic/painkiller (Morlion et al. 2018), irinotecan (from camptothecin) as an anticancer agent (Tsuruo et al. 1988), docetaxel (from taxol) as an anticancer agent (Pazdur et al. 1993), and etoposide (from podophyllotoxin) as an anticancer agent (Stahelin and von Wartburg 1991), and so on. However, derivatives of VOCs have not been commercially developed for use as pharmacological or health-promoting agents or for use in agriculture or horticulture, except in some cases of their use for academic explorations (Samarasekera et al. 2008, Nesterkina and Kravchenko 2017, Shi et al. 2019).

In the current study, we explored and developed a new terpene derivative (menthol menthyl ester of valine [ment-Val]) that acts as a plant defense potentiator. Based on results of a previous study (Sukegawa et al. 2018), we focused on menthol for use as the basic structure for the derivative. Amino acids are advantageous as components of the derivatives, as they make it easy to generate molecular diversity and obtain structure-activity relationships due to their diverse side chain structures. We present studies of the defense abilities of ment-Val and other structurally similar compounds against herbivores in plants, including soybean representatively as well as other crop species.

Materials And Methods

Synthesis of amino acid esters of menthol (ment-aa)

All commercially available reagents and solvents were used without further purification. Reactions were monitored by thin-layer chromatography (TLC) carried out on silica gel-coated aluminum sheets $60F_{254}$ (Merck KGaA, Darmstadt, Germany). Spots on the TLC sheet were visualized using anisaldehyde sulfuric acid and ninhydrin reagents. Flash chromatography was performed on Wakogel C-200 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). ^{1}H NMR spectra were recorded on an Avance DRX-600 or Avance NEO 400 spectrometer (Bruker, Billerica, MA) at 298 K. Chemical shifts (δ) were reported in parts-per-million (ppm) with respect to tetramethylsilane as internal reference (δ = 0.00 ppm in $CDCl_3$). Signal splitting patterns are described as singlet (s), doublet (d), triplet (t), multiplet (m), and broad signal (br). The assignment of ^{1}H resonance was achieved by a combined employment of 1D and 2D (COSY, HSQC, and HMBC) techniques.

The amino acid esters of menthol were synthesized based on a previous report (Harada et al. 1964). One equivalent mol of amino acid (glycine, L-alanine, L-valine, D-valine, L-leucine, L-isoleucine, or L-phenylalanine; FUJIFILM Wako Pure Chemical Corporation), *l*-menthol (1.5 mol eq., FUJIFILM Wako Pure

Chemical Corporation), and *p*-toluenesulfonic acid monohydrate (1.3 mol eq., FUJIFILM Wako Pure Chemical Corporation) were suspended in toluene and refluxed with a Dean-Stark apparatus for 24–72 hr. The reaction mixture was diluted with toluene, washed with 1 M NaOH aq. and brine. The organic phase was dried (Na_2SO_4) and concentrated in vacuo. The resulting oil was purified by flush column chromatography to obtain the amino acid ester of menthol.

Glycine menthyl ester (ment-Gly): ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.74 (ddd, *J* = 4.1, 10.8, 10.8 Hz, 1H, H-1), 3.43–3.36 (m, 2H, Gly-CH_2), 2.00–1.98 (m, 1H, H-6a), 1.86–1.81 (m, 1H, 2′-CH), 1.74 (br s, 2H, Gly-NH_2), 1.71–1.67 (m, 2H, H-3a, 4a), 1.57–1.42 (m, 1H, H-5), 1.40–1.36 (m, 1H, H-2), 1.10–1.03 (m, 1H, H-3b), 1.01–0.95 (m, 1H, H-6b), 0.91–0.85 (m, 7H, H-4b, 2′-CH_3, 5-CH_3), 0.76 (d, *J* = 7.0 Hz, 3H, 2′-CH_3).

L-Alanine menthyl ester (ment-Ala); ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.63 (ddd, *J* = 3.9, 10.6, 10.6 Hz, 1H, H-1), 3.46–3.39 (m, 1H, Ala-α-CH), 1.92–1.86 (m, 1H, H-6a), 1.84–1.75 (m, 1H, 2′-CH), 1.66–1.55 (br m, 4H, Ala-NH_2, H-3a, 4a), 1.47–1.38 (m, 1H, H-5), 1.35–1.30 (m, 1H, H-2), 1.26–1.21 (m, 3H, Ala-β-CH_3), 1.04–0.96 (m, 1H, H-3b), 0.95–0.86 (m, 1H, H-6b), 0.86–0.77 (m, 7H, H-4b, 2′-CH_3, 5-CH_3), 0.69 (d, *J* = 7.0 Hz, 3H, 2′-CH_3).

L-Valine menthyl ester (ment-Val); ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.77–4.70 (m, 1H, H-1), 3.29–3.24 (m, 1H, Val-α-CH), 2.08–1.95 (m, 2H, Val-β-CH, H-6a), 1.92–1.83 (m, 1H, 2′-CH), 1.73–1.65 (m, 2H, H-3a, 4a), 1.55–1.45 (m, 1H, H-5), 1.45–1.36 (m, 2H, Val-NH_2, H-2), 1.12–1.02 (m, 1H, H-3b), 1.02–0.96 (m, 4H, Val-γ-CH_3, H-6b), 0.94–0.83 (m, 10H, Val-γ-CH_3, H-4b, 2′-CH_3, 5-CH_3), 0.80–0.73 (m, *J* = 7.0 Hz, 3H, 2′-CH_3).

D-Valine menthyl ester (ment-DVal); ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.70 (ddd, *J* = 4.3, 10.9, 10.9 Hz, 1H, H-1), 3.27–3.26 (m, 1H, Val-α-CH), 2.13–1.98 (m, 2H, Val-β-CH, H-6a), 1.97–1.85 (m, 1H, 2′-CH), 1.74–1.65 (m, 2H, H-3a, 4a), 1.61–1.35 (m, 4H, Val-NH_2, H-5, H-2), 1.13–0.94 (m, 5H, H-3b, Val-γ-CH_3, H-6b), 0.94–0.81 (m, 10H, Val-γ-CH_3, H-4b, 2′-CH_3, 5-CH_3), 0.74 (d, *J* = 7.0 Hz, 3H, 2′-CH_3).

L-Leucine menthyl ester (ment-Leu); ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.71 (ddd, *J* = 3.5, 10.6, 10.6 Hz, 1H, H-1), 3.41 (t, *J* = 7.0, 1H, Leu-α-CH), 1.99–1.92 (m, 1H, H-6a), 1.91–1.84 (m, 1H, 2′-CH), 1.83–1.75 (m, 1H, Leu-γ-CH), 1.74–1.61 (m, 4H, Leu-NH_2, H-4a, H-3a), 1.58–1.45 (m, 2H, Leu-β-CHa, H-5), 1.44–1.34 (m, 2H, H-2, Leu-β-CHb), 1.13–1.02 (m, 1H, H-3b), 1.02–0.82 (m, 14H, H-4b, H-6b, Leu-δ-CH_3 × 2, 2′-CH_3, 5-CH_3), 0.77 (d, *J* = 7.0 Hz, 3H, 2′-CH_3).

L-Isoleucine menthyl ester (ment-Ile); ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.73 (ddd, *J* = 4.3, 11.0, 11.0 Hz, 1H, H-1), 3.35–3.32 (m, 1H, Ile-α-CH), 2.00–1.94 (m, 1H, H-6a), 1.91–1.83 (m, 1H, 2′-CH), 1.78–1.72 (m, 1H, Ile-β-CH), 1.71–1.65 (m, 2H, H-3a, 4a), 1.55–1.36 (br m, 5H, Ile-NH_2, H-5, H-2, Ile-γ-CHa), 1.24–1.15 (m, 1H, Ile-γ-CHb), 1.10–1.02 (m, 1H, H-3b), 1.01–0.82 (m, 14H, H-6b, H-4b, 2′-CH_3, 5-CH_3, Ile-β-CH_3 Ile-δ-CH_3), 0.75 (d, *J* = 7.0 Hz, 3H, 2′-CH_3).

L-Phenylalanine menthyl ester (ment-Phe); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.31–7.25 (m, 2H, Phe-Ar-CH), 7.24–7.18 (m, 3H, Phe-Ar-CH), 4.77–4.655 (m, 1H, H-1), 3.72–3.65 (m, 1H, Phe-α-CH), 3.06 (dd, *J* = 4.8, 13.6 Hz, 1H, Phe-β-CHa), 2.83 (dd, *J* = 8.0, 13.6 Hz, 1H, Phe-β-CHb), 1.93–1.87 (m, 1H, H-6a), 1.86–1.77 (m, 1H, 2'-CH), 1.70–1.61 (m, 2H, H-3a, 4a), 1.52–1.41 (m, 3H, Phe-NH_2, H-5), 1.41–1.33 (m, 1H, H-2), 1.09–0.97 (m, 1H, H-3b), 0.96–0.80 (m, 8H, H-4b, H-6b, 2'-CH_3, 5-CH_3), 0.74 (d, *J* = 7.0 Hz, 3H, 2'-CH_3).

Plants

Soybean plants (*Glycine max* cv. Enrei), *Pisum sativum*, *Brassica rapa* (var. perviridis, cv. Natsurakuten), tobacco (*Nicotiana tabacum* cv. SR1), lettuce (*Lactuca sativa* var. Crispa) and *Zea mays* (cv. Royal Dent), were grown in soil for 14 days (except that tobacco and lettuce were grown for 4 weeks), in a climate-controlled room at 24 ± 1°C with a photoperiod of 16 h (80 μE m^{-2} s^{-1}). *The light period was from 07:00 to 23:00.*

Chemical treatment

A single leaf branch (3 leaves) of a potted plant was evenly sprayed with 3 mL of 10 mM MES buffer (pH 6.0) containing 1% ethanol and 0.1, 1, or 10 μM menthol or ment-Val. Application of 10 mM MES buffer (pH 6.0) alone served as control. The plants were then incubated in a climate-controlled room at 24 ± 1°C with a photoperiod of 16 h (80 μE m^{-2} s^{-1}) for 24 h or 5 days.

For histone acetyltransferase (HAT) inhibitor (*garcinol*) treatment, a single leaf branch (3 leaves) of soybean plants was evenly sprayed with 1 mL of an aqueous solution of *garcinol (*0.1 mM, *Cayman Chemical,* Ann Arbor*, MI, USA*) 24 h before ment-Val treatment.

RNA extraction, cDNA synthesis and quantitative polymerase chain reaction (PCR)

Approximately 100 mg of leaf tissues were homogenized in liquid nitrogen, and total RNA was isolated and purified using Sepasol®-RNA I Super G (Nacalai Tesque, Kyoto, Japan) following the manufacturer's protocol. First-strand cDNA synthesis and qPCR were performed according to the method described previously (Uemura et al. 2020). Primers used in this study are listed in Table S1. Relative transcript abundances were determined after normalization of raw signals with the transcript abundance of a housekeeping gene (actin). We did not use samples or data when sufficient amounts or quality of RNA (> 83 ng $μL^{-1}$) were not obtained from leaves or when abnormal quantification cycle (Cq) values for the actin gene were obtained.

Leaf damage and herbivore growth assays

Eggs of *Spodoptera litura* (Fabricius) were obtained from Sumika Technoservice Co. Ltd. (Takarazuka, Japan). They were incubated in a climate-controlled room at 24 ± 1°C with a photoperiod of 16 h, as reported previously (Uemura et al. 2020).

Five third-instar larvae of *S. litura* (1.8–2.0 mg) were starved overnight and released onto 3 sets of the secondary leaves of a potted soybean plant, in which each solution had been sprayed with each solution before 24 h. The leaves were covered with a mesh bag and kept for 2 h at 24 ± 1°C. The leaves were then scanned, and the total leaf area and the consumed leaf area were determined using ImageJ. Replicate analyses were conducted with 5 independent samples.

Otherwise, a third-instar larva of *S. litura* (1.8-2.0 mg) was incubated on 3 grams of artificial diet (Insecta LFS, Nihon Nosan Kogyo Ltd., Tokyo, Japan) supplemented with 500 µL of menthol or ment-Val in MES (1 µM) or MES alone, in a plastic petri dish (55 mm diameter, 15 mm deep). The fresh diet with each solution was supplied daily. The net body weight that *S. litura* larvae gained was determined during the following 96 h. When a larva died or was lost during the assay, we excluded that sample, and final replicate analyses were conducted with 10 independent samples.

Mite oviposition assays

Tetranychus urticae Koch (Acari: Tetranychidae) were reared as reported previously (Iida et al. 2019). A *T. urticae* adult female (10 days after oviposition) was transferred onto a leaf disc (1.8 cm^2) of soybean on wet cotton in a plastic Petri dish (90 mm diameter). We prepared 1 leaf disc from each of 3 secondary soybean leaves that had been sprayed with each solution before 24 h. The means of the 3 discs were evaluated as a single independent replicate, and final replicate analyses were conducted with 5 independent samples.

Statistics and reproducibility

We performed one-way ANOVA with Holm's sequential Bonferroni post hoc test and post hoc Tukey's HSD using the program (http://astatsa.com/OneWay_Anova_with_TukeyHSD/) for comparing multiple samples. The sample sizes and number of replicates for all of the sets of assays and analyses are indicated in the legends of the corresponding figures.

Results

Mining of ment-aas serving as plant defense potentiators

We focused on 6 amino acids with low-reactive sidechains to synthesize an array of ment-aa compounds *(Fig. 1a). T*ranscript *levels of two defense genes (pathogenesis-related protein 1 [PR1*] and *trypsin inhibitor* [*TI*]) *were evaluated in leaves of soybean plants* 24 h after application of a solution of menthol or its menthyl ester of glycine (ment-Gly), alanine (ment-Ala), valine (ment-Val), leucine (ment-Leu), isoleucine (ment-Ile) or phenylalanine (ment-Phe) (1 µM each) (Fig. 1b). In comparison to control solution (MES buffer), ment-Val alone elevated the transcript levels of the defense genes. Menthol and Val at the same dose were not able to elevate them. Evaluation of the dose-response effect of ment-Val showed that only 1 µM ment-Val was active.

Finally, it should be noted that D-Valine menthyl ester (ment-DVal) was able to induce the transcript levels of the defense genes similarly to the L-Valine menthyl ester. D-Valine was, however, not effective. We thus concluded that both stereoisomers of Val serve as a plant defense potentiator (Fig. S1)

Defense ability of soybean plants treated with menthol or ment-Val

Soybean plants that had been treated with ment-Val solution showed less leaf damage by larvae of the generalist herbivore *S. litura* during 2 h compared to plants that had been treated with the control solution (Fig. 2a). However, menthol did not protect against such leaf damage. Therefore, to test the possibility that ment-Val is detrimental to herbivore performance, we carried out a biological assay in which *S. litura* larvae were incubated on artificial diet infiltrated with 1 μM menthol or ment-Val solution. The results showed that there were no differences among the weights of larvae grown for up to 96 h on the diet infiltrated with control solution, menthol solution, or ment-Val solution, indicating that menthol and ment-Val are not strikingly detrimental to *S. litura* performance (Fig. 2b).

Moreover, a lower rate of oviposition of adult female two-spotted spider mites (*T. urticae*) was observed on leaves of soybean plants that had been treated with ment-Val solution, compared to that on plants treated with the control solution (Fig. 2c). As the same held true for soybean plants that had been treated with menthol solution (Fig. 2c), indicating that both menthol and ment-Val were useful for the control of spider mites.

Role of histone acetylation in ment-Val response

Because the innate machineries for epigenetic regulation are based on the transcriptional memory response to VOCs (Sukegawa et al. 2018), we assessed the effect of garcinol*, a* HAT inhibitor, on transcriptional activation of *PR1* and *TI* in response to ment-Val for 1 day. We found that garcinol treatment dramatically suppressed the elevation of the *transcript levels* of these defense genes in the ment-Val-treated leaves (Fig. 3).

Sustainability of defense gene transcript accumulation and epigenetic regulation

Application of ment-Val enabled soybean plants to sustain the increased transcript levels of both *PR1* and *TI* in their leaves for up to 3 days (Fig. 4). Moreover, when ment-Val was applied again at day 4, at which time these transcripts had decreased to the basal levels, they were again increased after an additional 1 day (day 5). However, neither menthol nor MES buffer alone caused such an increase. All these findings indicated that the effect of ment-Val was sustainable by repeated application of the chemical.

Applicability of ment-Val for crops

Finally, in order to assess the effect of ment-Val in several crop species, ment-Val was applied to *Pisum sativum*, *Brassica rapa*, *Nicotiana tabacum*, *Lactuca sativa* and *Zea mays*. ment-Val was able to induce the transcript levels of *PR1* at 1 μM, except that in the case of *B. rapa* 0.1 μM ment-Val was effective (Fig.

5). On the other hand, menthol was not active at all for any of the crops at any of the concentrations used. Taken collectively, these results indicate the specific applicability of ment-Val for several crops as a plant defense potentiator.

Molecular stability of ment-Val

To assess the molecular stability of ment-Val, ment-Val (10 mM) was exposed to various environmental stresses (UV [254 nm], heat (60˚C), acid [10 mM HCl, pH 2], alkalinity [10 mM NaOH, pH12]) for up to 8 h. Analysis of the stressed products by TLC showed no remarkable degradations to menthol or other possible products under any of these stresses (Fig. 6).

Discussion

The Val conjugate of monoterpenoids serves as plant defense potentiator

Functional PSMs conjugated with L-amino acids have been explored academically for multiple pharmacological purposes, as represented by conjugates of menthol and borneol with glycine that exhibit analgesic and anti-inflammatory activities (Nesterkina and Kravchenko 2017), triterpenoids and asiatic acid conjugated with L-amino acids that exhibit antitumor activity (Ukiya et al. 2010, Jing et al. 2015), asiatic acid conjugated with L-amino acids, and glycyrrhizic acid conjugates with Ile (Dengue Virus inhibition) (Baltina et al. 2019). Moreover, besides amino acids, β-pinene (monoterpenoid) derivatives containing amide moieties and acylthiourea moieties that exhibit in vitro antifungal activity have been developed (Shi et al. 2019). To shed light on their potential for pest control for agricultural and horticultural crops, here we present data showing that the new conjugate ment-Val acts as a powerful plant defense potentiator in several crops, including Fabaceae, Solanaceae, Asterales, and Poaceae (Fig. 5).

Intriguingly, both L-Valine and D-Valine menthyl esters act as plant defense potentiators (Figs. 1 and S1). However, menthol conjugated with other amino acids, including Ile and Leu, which are structurally similar to Val, did not have this effect (Fig. 1). It remains unknown why the conjugate with Val is so specific. Unlike glutamate, which can serve as an initiator of systemic signaling in herbivore-damaged *Arabidopsis thaliana* leaves (Toyota et al. 2018), little is known about a special role of Val in plant defense responses. We may be able to accumulate more knowledge about the structure-activity relationships as plant defense potentiator by assessing conjugates with other amino acids, as well as with fatty acids, sugars, etc. that have not yet been tested.

Moreover, it cannot be ignored that we still do not know why only the narrow dose of ment-Val at 1 μM, except in *B. rapa*, in which it worked at 0.1 μM, was specifically effective as a plant defense potentiator. Exceeding the effective concentration of ment-Val may even be detrimental for plants.

Unknown terpenoid sensing system in plants

Little is known about how plants sense and respond to terpenoids. Briefly, some mint essential oil components, including (+)-menthofuran, (+)-pulegone, (+)-neomenthol, (-)-menthol and (-)-menthone, have been shown to stimulate depolarization of the plasma membrane (Maffei et al. 2012), implying that these components have ability to open Ca^{2+} channels, possibly through receptors and ion channels in the plasma membrane. In mice, it has been shown that menthol or borneol conjugates with Gly act through transient receptor potential（TRP） channels, leading to analgesic and anti-inflammatory effects (Nesterkina and Kravchenko 2017). However, nothing is known about whether menthol stimulates ion channels in plants.

Volatiles may also be transmitted inside cells. This is in accord with the finding that (*Z*)-3-hexenol, a green leaf volatile, is converted to (*Z*)-3-hexenylvicianoside in tomato leaf tissues after being incorporated from the atmosphere (Sugimoto et al. 2014). Likewise, caryophyllene (a sesquiterpenoid) has been shown to interact with TOPLESS-like proteins (TPLs) in tobacco (Nagashima et al. 2019). TPLs are known to serve as corepressors bridging transcription factors with chromatin remodeling complexes in the nucleus (Martin-Arevalillo et al. 2017).

Finally, it should be noted that epigenetic regulation may contribute to the effects of ment-Val, probably as well as to the sustainable transition for the effect of ment-Val (Figs. 3 and 4). It was reported that soybean plants exposed to mint volatiles can sustain the activation of defense genes, concomitant with histone acetylation of their upstream promoters, in their leaves (Sukegawa et al. 2018). Experiments using garcinol, *a* histone acetyltransferase (HAT) inhibitor, showed that histone acetylation is relevant to the upregulation of defense genes in ment-Val-treated soybean leaves (Fig. 3). Further studies will be required to understand the details of the regulatory mechanisms involved.

Concluding remarks

New potentiators of plant defense and knowledge on the forefront of sensing systems offer environmentally friendly and healthy strategies for pest management in plant factories (Sukegawa et al. 2018, Ingrao et al. 2019, Arimura 2021). As ment-Val is physically stable (Fig. 6) and has powerful and sustainable effects on several crops, terpenoid conjugates including ment-Val show promise for use for pest control instead of and/or supplemental to pesticides.

Declarations

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Author contributions

CT, MH, YN, YN, SS, SH, and GA designed experiments and analyzed data. CT, MH, RI, YN, YN, and SS performed experiments. MH and GA wrote the paper.

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Figures

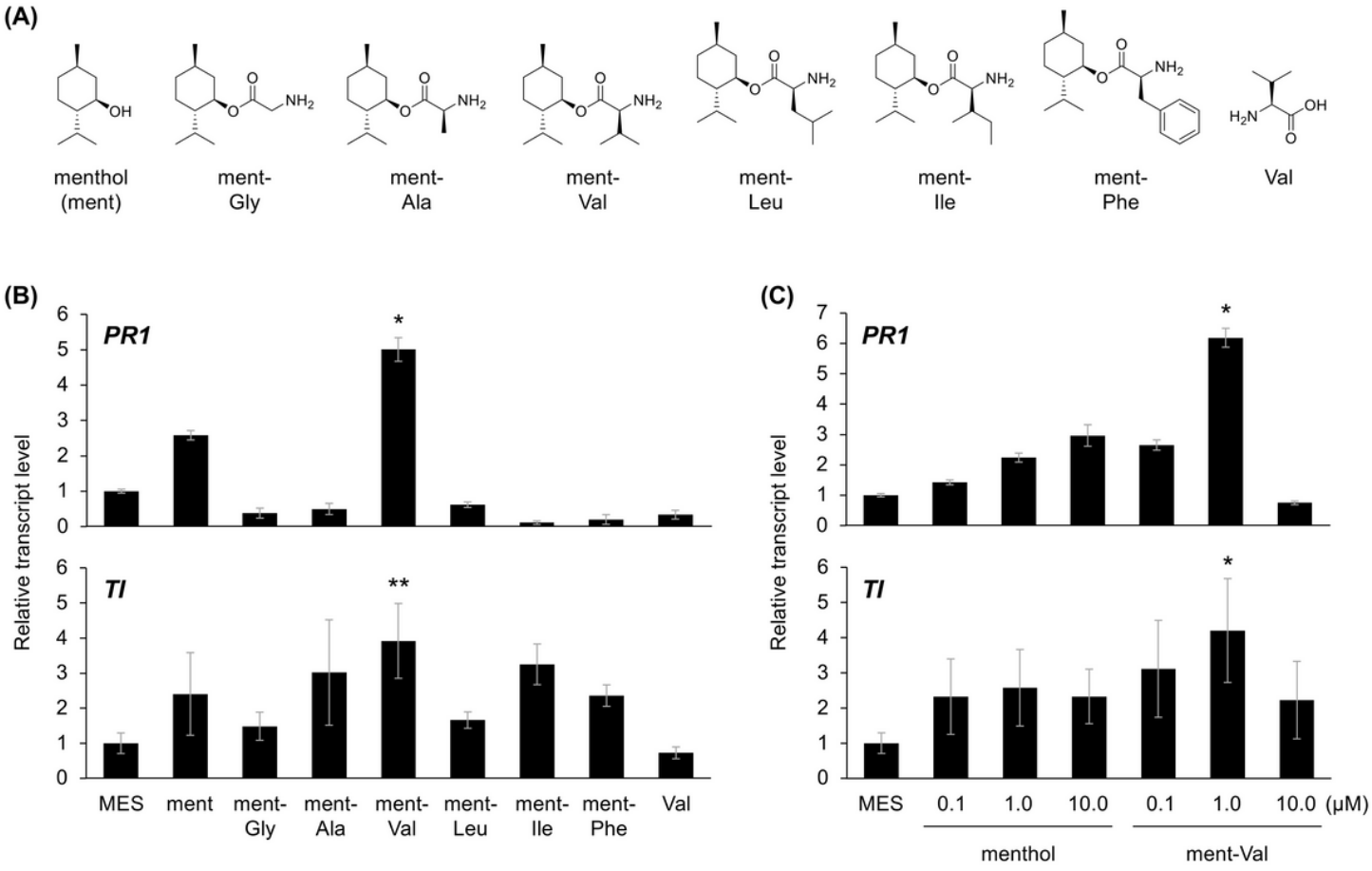

Figure 1

Screening of active amino acid esters of menthol (ment-aa). (A) The chemical structures of menthol (ment), ment-aa and Val used for this study. (B) Relative transcript levels of pathogenesis-related protein 1 (PR1) and trypsin inhibitor (TI) in leaves of soybean plants 24 h after application of MES buffer solution containing ment, valine (Val), menthyl ester of glycine (ment-Gly), alanine (ment-Ala), valine (ment-Val), leucine (ment-Leu), isoleucine (ment-Ile) or phenylalanine (ment-Phe) (1 μM each). Application of MES buffer alone served as control. (C) Likewise, the effect of the dose of menthol or ment-Val (0.1, 1.0 or 10 μM) on the levels of transcripts of genes were assessed. Data represent the mean and standard error (n = 10). Data marked with an asterisk(s) are significantly different from those of MES control, based on an ANOVA with Holm's sequential Bonferroni post-hoc test (**, $0.001 \leq P < 0.01$; *, $0.01 \leq P < 0.05$).

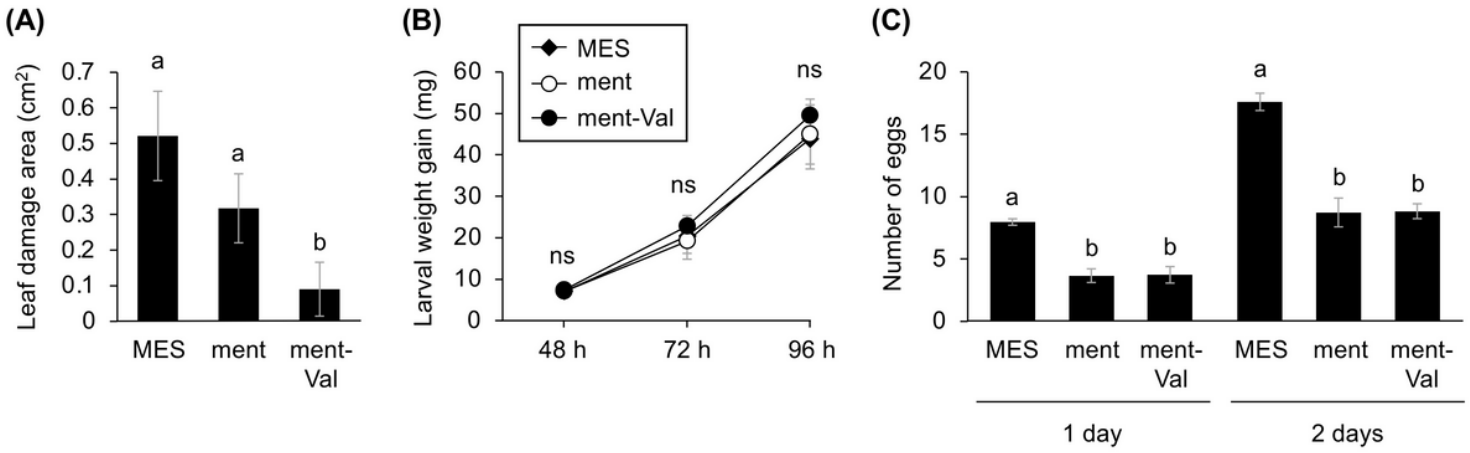

Figure 2

Defensive properties of soybean plants in response to menthol or ment-Val. (A) Spodoptera litura larvae were placed onto the leaf of a potted plant 24 h after application of MES buffer solution containing menthol (ment, 1 μM) or ment-Val (1 μM). Application of MES buffer alone served as control. The area of the leaf damage after 2 h was determined (n = 5). (B) The net body weight of S. litura larvae gained during 48, 72 and 96 h after incubation on artificial diet supplied with each solution (n = 10). (C) An adult female of Tetranychus urticae was placed onto leaf sections prepared from plants 24 h after application of each solution. The number of eggs laid by T. urticae during 24 and 48 h was determined. Data represent the mean and standard error. The means indicated by different small letters are significantly different based on an ANOVA with post hoc Tukey's HSD ($P < 0.05$). ns, not significant.

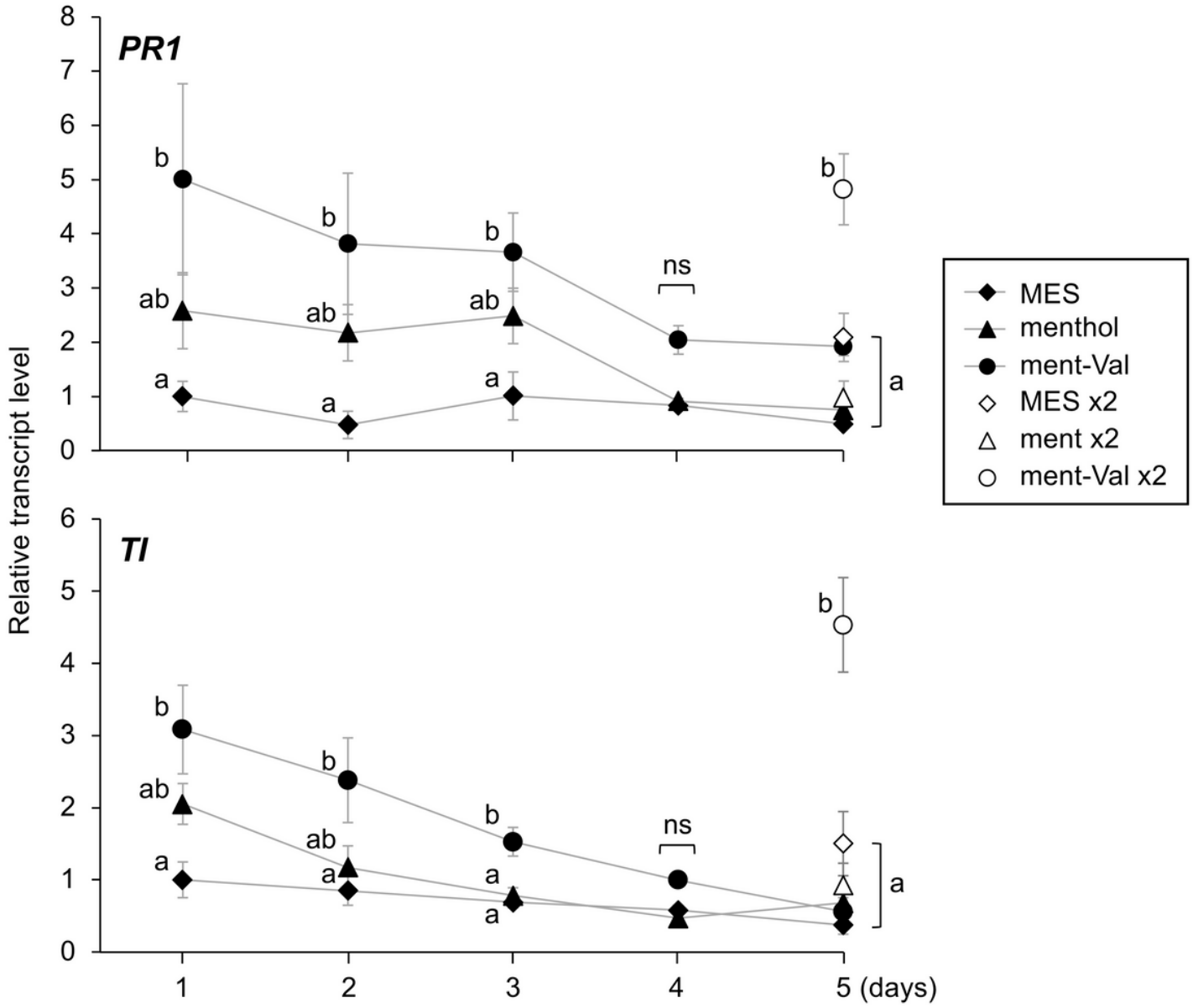

Figure 3

Involvement of histone acetyltransferases (HATs) in ment-Val response. Effects of a HAT inhibitor (garcinol [Gar]) treatment on relative transcript levels of pathogenesis-related protein 1 (PR1) and trypsin inhibitor (TI) were determined in leaves of soybean plants 24 h after application of MES buffer solution containing ment-Val (1.0 μM). Application of MES buffer alone served as control. Data represent the mean and standard error (n = 10). The means indicated by different small letters are significantly different among data of each day, based on an ANOVA with post hoc Tukey's HSD ($P < 0.05$).

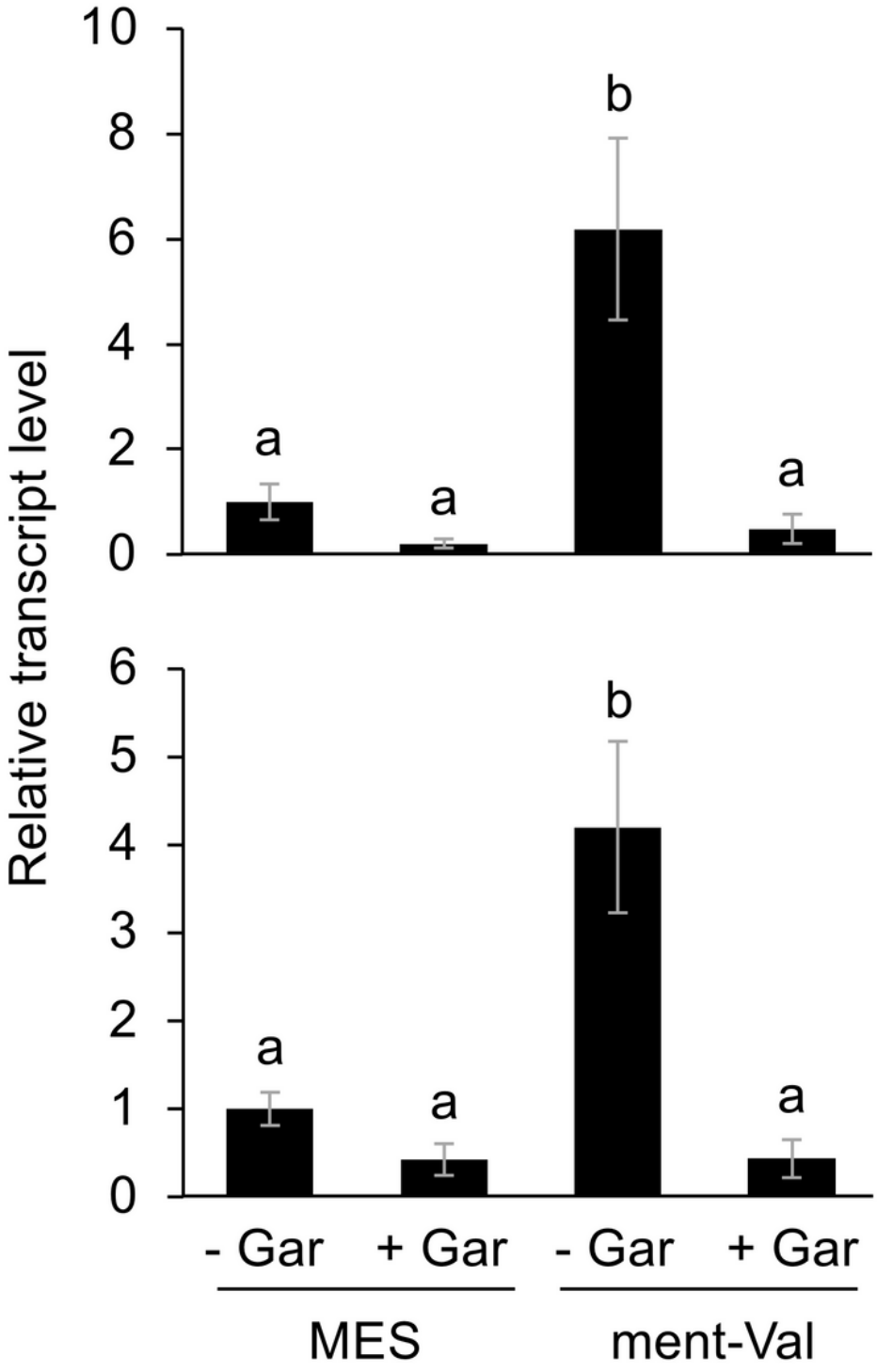

Figure 4

The sustainable effect on defense gene transcript levels. Soybean plants were treated with MES buffer solution containing menthol (1 μM) or ment-Val (1 μM). Application of MES buffer alone served as control. Relative transcript levels of pathogenesis-related protein 1 (PR1) and trypsin inhibitor (TI) were determined in leaves of the soybean plants maintained for up to 5 days. In some plants, the defense genes' relative transcript levels were determined in leaves treated again with the respective solutions at 4

days and incubated for an additional 1 day (MES x2, ment x2, and ment-Val x2). Data represent the mean and standard error (n = 3). The means indicated by different small letters are significantly different among data of each day, based on an ANOVA with post hoc Tukey's HSD ($P < 0.05$). ns, not significant.

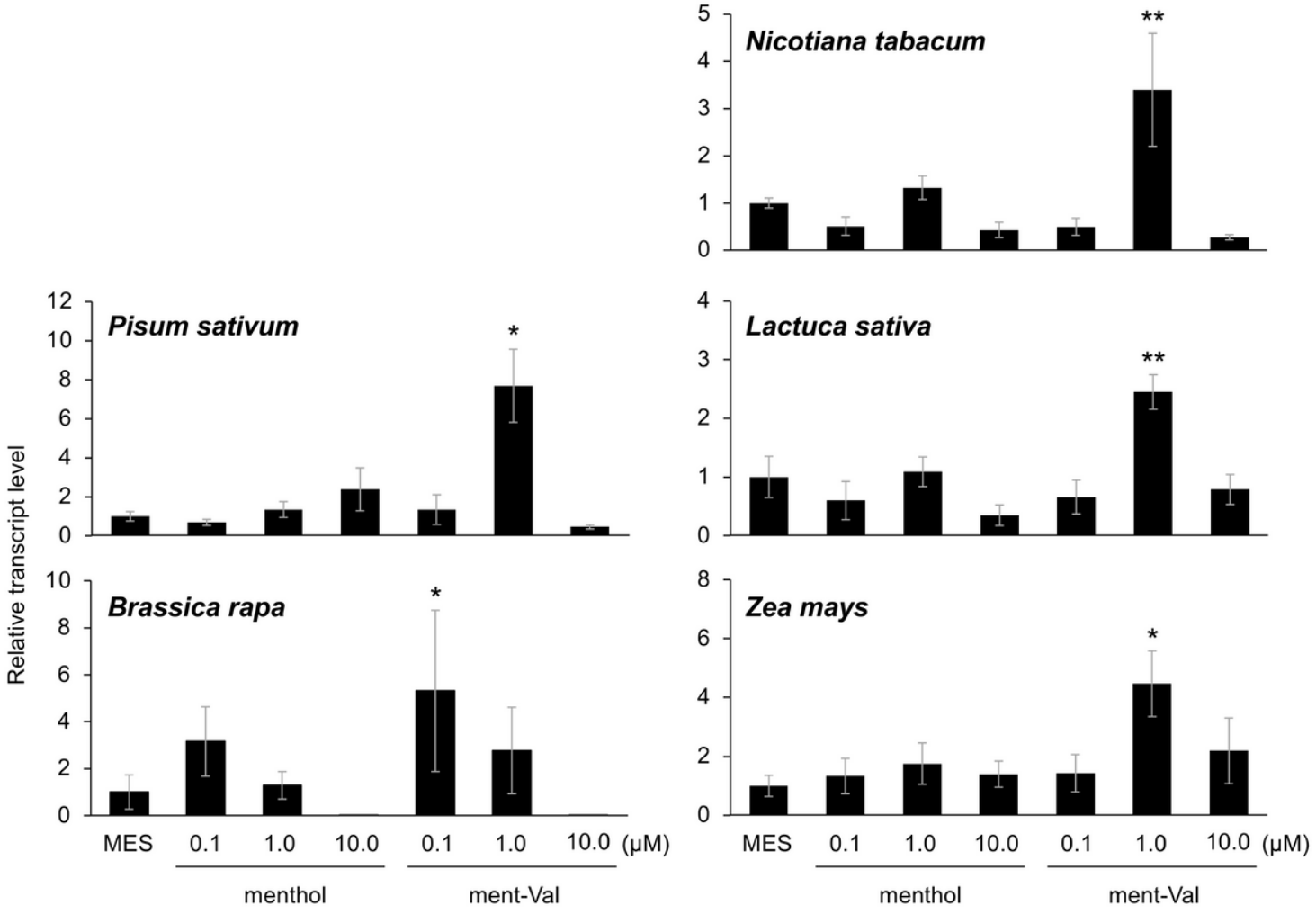

Figure 5

Defensive properties of several plant taxa in response to ment-Val. Relative transcript levels of pathogenesis-related protein 1 (PR1) in leaves of soybean plants 24 h after application of MES buffer solution containing ment-Val (0.1, 1.0 or 10 μM). Application of MES buffer alone served as control. Data represent the mean and standard error (n = 10). Data marked with an double asterisks are significantly different from those of MES control, based on an ANOVA with Holm's sequential Bonferroni post-hoc test (**, $0.001 \leq P < 0.01$; *, $0.01 \leq P < 0.05$).

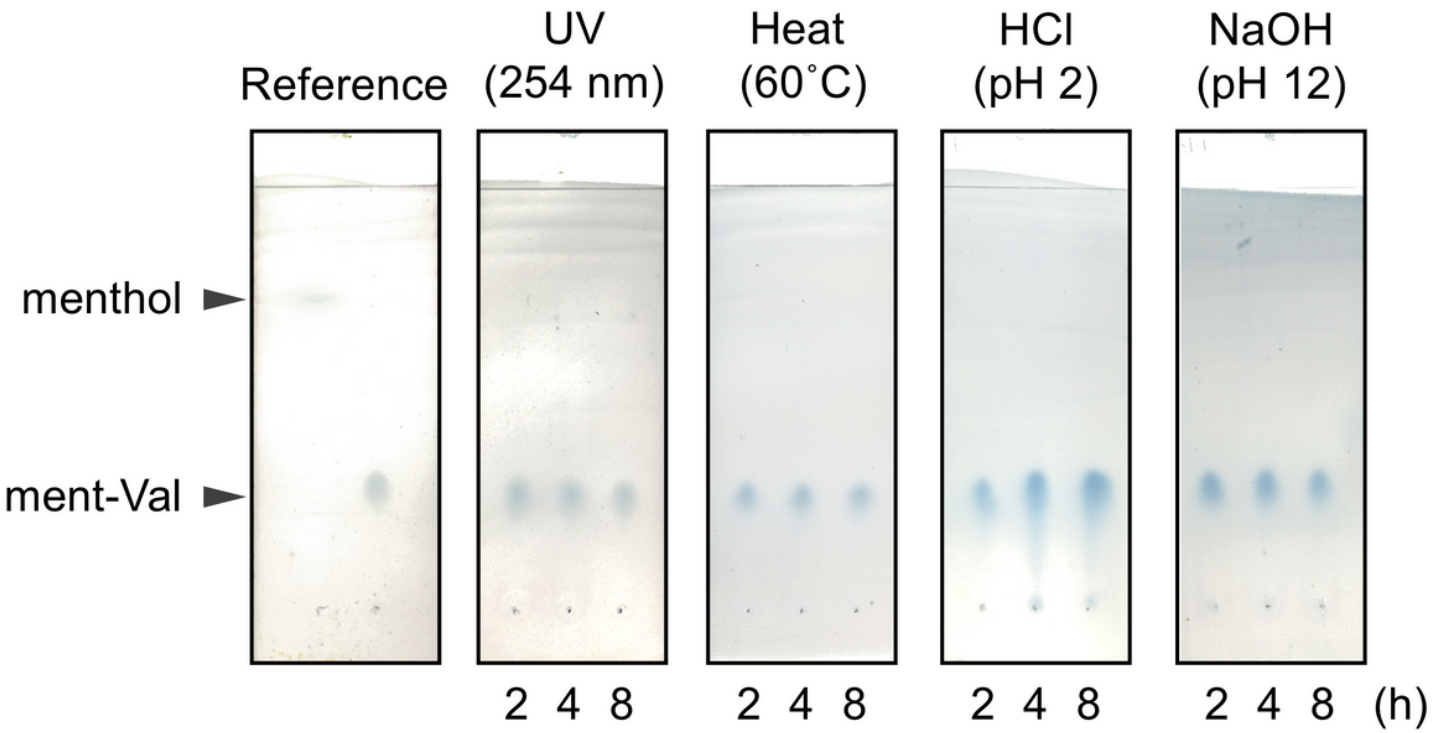

Figure 6

Molecular stability of ment-Val. TLC chromatograms of ment-Val exposed to UV irradiation, heating, acidic, and alkaline conditions. The ment-Val solution (10 mM) was applied at 1 µL per spot. After development with n-hexane/ethyl acetate (2:1, v/v), spots were visualized using p-anisaldehyde/sulphuric acid reagent (Rf values: menthol, 0.72; Ment-Val, 0.29).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Fig.S1.pdf
- TableS1.xlsx