Monoterpenes induce the heat shock response in Arabidopsis

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Abstract: Monoterpenes are common constituents of essential oils produced by plants. Although it has been reported that monoterpenes enhanced the heat tolerance of plants, the mechanism has not been elucidated. Here, we tested whether 13 monoterpenes promoted the heat shock response (HSR) in *Arabidopsis*. To assess the HSR-inducing activity of monoterpenes, we produced transgenic *Arabidopsis*, which has the β-glucuronidase gene driven by the promoter of a small heat shock protein (*HSP17.6C-CI*) gene. Results indicated that two monocyclic and four bicyclic monoterpenes showed HSR-inducing activities using the reporter gene system. In particular, (–)-perillaldehyde, which is a monocyclic monoterpene, demonstrated the most potent HSR-inducing activity. (–)-Perillaldehyde significantly inhibited the reduction of chlorophyll content by heat shock in *Arabidopsis* seedlings. Our previous study indicated that chemical HSR inducers such as geldanamycin and sanguinarine inhibited the activity of plant chaperones in vitro. (–)-Perillaldehyde also inhibited chaperone activity, indicating that it might promote the expression of heat shock protein genes by inhibiting chaperones in the plant cell.

Keywords: heat shock protein; heat shock response; heat tolerance enhancers; monoterpenes; (–)-perillaldehyde.

1 Introduction

Extreme heat stress adversely affects plant growth and crop productivity [1]. Heat causes physiological symptoms such as protein denaturation, an increase in the fluidity of membrane lipids, photosynthetic damage, and production of reactive oxygen species (ROS) [2, 3]. To ameliorate these symptoms, plants promote various stress responses, including the accumulation of compatible solutes, enhancement of a scavenging system against ROS, and the expression of heat shock proteins (HSPs) [4, 5]. HSPs are common molecular chaperones that mediate the folding of heat-denatured proteins, and thereby prevent protein aggregation. Because the level of heat tolerance is positively correlated with the magnitude of HSP expression, HSPs are thought to be involved in enhancing the heat tolerance of plants [2, 6].

Although heat is a general cue to induce the heat shock response (HSR), chemical stimuli can also enhance the HSR in plants. It has been reported that diverse natural products, such as salicylic acid [7], benzyl alcohol [8], celastrol [9], geldanamycin (GDA) [10], phenethyl isothiocyanate [11], sanguinarine [12], and (*E*)-2-hexenal and its related compounds [13], induced plant HSR. The fact that natural HSR inducers are widely varied in their structures facilitates the testing of whether other chemical groups enhance HSR in plants.

Monoterpenes are volatile compounds that are produced by many plant species. The diverse C10-isoprenoid structures are biosynthesized from geranyl diphosphate and/or neryl diphosphate by monoterpene synthases of plants [14]. Many studies have demonstrated that monoterpenes are toxic to insects, fungi, bacteria, and animals, allowing plants to avoid natural enemies [15]. Monoterpenes also have physiological functions for plants, such as inhibition of seed germination [16], herbicidal activity [17], promotion of the senescence that is concomitant with apoptosis-like cell death [18], and enhancement of heat tolerance [19, 20]. Regarding the heat tolerance-enhancing effects, it is thought that monoterpenes might ameliorate the heat stress of plants by scavenging ROS, which are produced by leaves under high temperatures [20, 21]. On the other hand, no research has focused on the relationship between HSP gene expression and the heat tolerance of monoterpene-treated plants.

Recently, we produced the HSR assay system using transgenic *Arabidopsis*, which contains the β-glucuronidase (GUS) gene driven by the promoter of a small HSP gene of *Arabidopsis* (*At1g53540*, *HSP17.6C-CI*) [22]. The *small HSP* gene was remarkably activated by natural HSR inducers, such as GDA and sanguinarine [12]. In this article, 13 monoterpenes (i.e. 5 monocyclic, 5 bicyclic, and 3 acyclic monoterpenes) were subjected

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to the assay system to investigate which monoterpene could induce HSR in *Arabidopsis*. Our findings indicated that (–)-perillaldehyde showed the highest HSR-inducing activity. Moreover, (–)-perillaldehyde inhibited the chaperone activity of wheat germ, suggesting that (–)-perillaldehyde might induce the HSR by suppressing chaperone activity in *Arabidopsis*. We also discuss mechanisms for enhancing the heat tolerance of plants by monoterpenes.

2 Results

2.1 Effects of monoterpenes on HSR

To measure HSR levels, we used the transgenic *Arabidopsis* possessing the *HSP17.6C-CIProGUS* construct (see Supplementary Material, Figure 1). In this construct, the GUS gene was controlled by the promoter region (1 kb) of a small HSP gene of *Arabidopsis* (*HSP17.6C-CI*, *At1g53540*). The *HSP17.6C-CI* gene was chosen for the HSR assay because the gene expression was responsive to the addition of natural HSR inducers such as GDA and sanguinarine. Prior to experiments with monoterpenes, GDA (50 μM) and heat shock (37 °C for 1 h) were applied to the transgenic *Arabidopsis* (see Supplementary Material, Figure 2). As expected, both treatments apparently induced HSR, although heat shock was more potent.

We used 13 monoterpenes: 5 monocyclic monoterpenes [(+)-limonene, LIM; (–)-perillaldehyde, PER; (–)-carvone, CVN; (–)-carveol, CVL; (–)-menthol, MEN]; 5 bicyclic monoterpenes [(+)-camphor, CAM; (–)-borneol, BOR; (+)-limonene oxide, LOX; (+)-1,8-cineol, CIN; and α-pinene, PIN]; and 3 acyclic monoterpenes [(+)-citronellol, CTN; geraniol, GER; citral, CTA] (Table 1). The chemical structures of these monoterpenes are shown in Figure 3 of the Supplementary Material. The HSR assay indicated that two monocyclic monoterpenes (LIM and PER) and four bicyclic monoterpenes promoted the HSR (Figure 1). Among them, PER showed the highest HSR-inducing activities. PER (1 mM) displayed approximately four times higher activity than GDA (50 μM), which was used as a positive control. The activity of LIM (1 mM) was about half that of PER (1 mM). The activities of four bicyclic monoterpenes were lower than those of GDA.

Although LIM and PER showed remarkable HSR-inducing activities at a concentration of 1 mM, less activity was observed at 0.1 and 10 mM, suggesting that the maximum activity occurred at concentrations between 0.1 and 10 mM. Thus, we measured the activities of LIM and PER in a detailed series of concentrations between 0.1 and 10 mM (Figure 1). Both LIM and PER showed sharp responses at 1 mM, indicating that these monoterpenes act in the narrow range of concentration of approximately 1 mM in this experimental system.

Table 1: Monoterpenes used in this study and their HSR-inducing activities.

Names	Abbreviations	Activities[a]
Monocyclic monoterpenes		
(+)-Limonene	LIM	++
(–)-Perillaldehyde	PER	++++
(–)-Carvone	CVN	–
(–)-Carveol	CVL	–
(–)-Menthol	MEN	–
Bicyclic monoterpenes		
(+)-Camphor	CAM	+
(–)-Borneol	BOR	+
(+)-Limonene oxide	LOX	–
(+)-1,8-Cineol	CIN	+
α-Pinene	PIN	+
Acyclic monoterpenes		
(+)-Citronellol	CTN	–
Geraniol	GER	–
Citral	CTA	–

[a]Relative 4-MU productions were shown in Figure 1. The highest average in each monoterpene reached between 0 and 0.3 (–), between 0.3 and 1.0 (+), between 1.0 and 2.0 (++), between 2.0 and 3.0 (+++), and between 3.0 and 4.0 (++++), respectively.

The transgenic *Arabidopsis* seedlings treated with PER were subjected to GUS staining. Administration of PER at 1 mM resulted in apparent staining in all parts of the seedlings (Figure 2). However, seedlings treated with 0, 0.1 and 10 mM PER were not stained. GUS staining was obtained by heat, whereas the intensity of the stain promoted by heat was more intensive than that promoted by PER at 1 mM. When wild-type *Arabidopsis* seedlings were treated with PER, the expression of *HSP17.6C-CI* and *HSP70* genes were upregulated within 1 h after the treatment (Supplementary Material, Figure 4).

2.2 Inhibition of chaperone by PER

It has been reported that some HSR inducers, such as monocillin I, GDA, and sanguinarine, inhibited the chaperone activities of plants [22, 23]. These inducers were thought to promote HSR through activating heat shock factors (HSFs) by inhibiting the chaperones. We therefore examined the chaperone-inhibiting activities of PER, LIM, and GER (Figure 3). A wheat germ extract was used as the active chaperone mixture. PER significantly inhibited the chaperone activity at concentrations of 100 and 1000 μM.

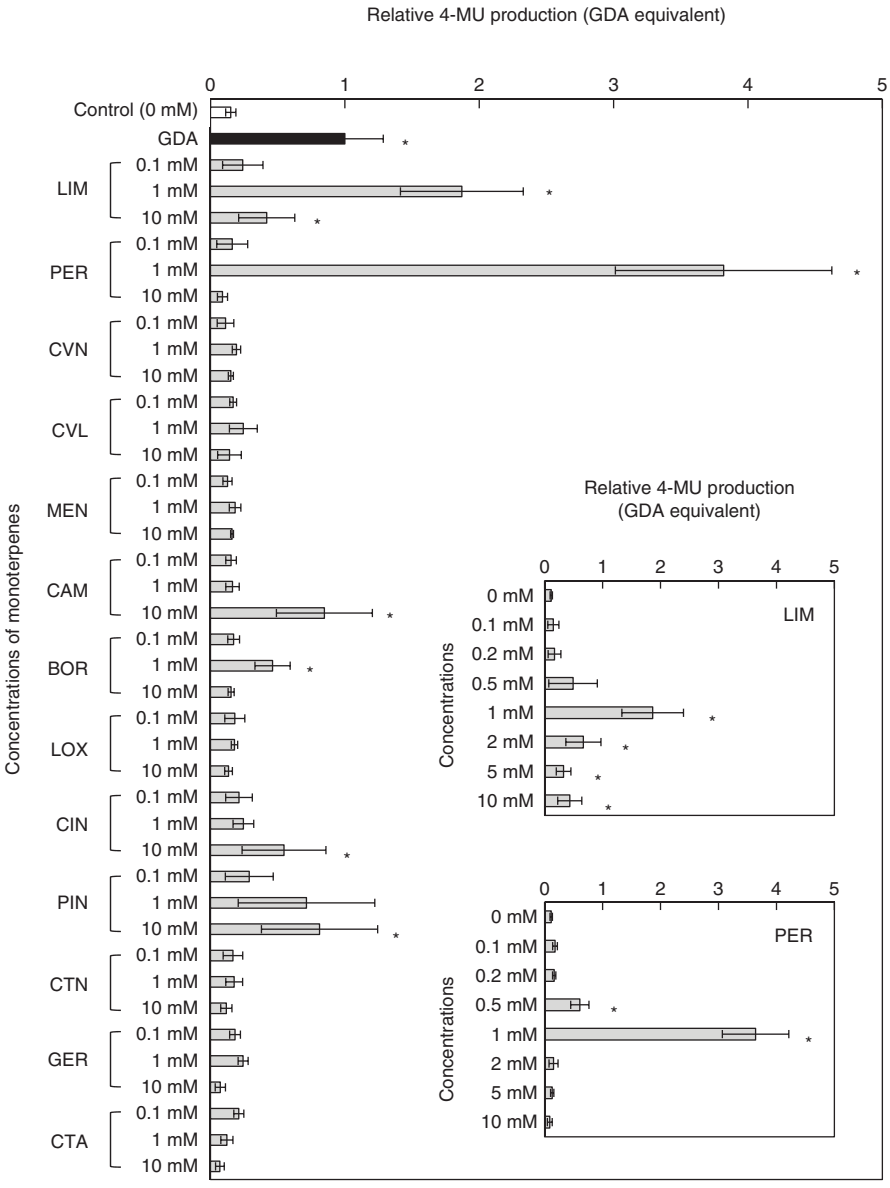

Figure 1: Induction of HSRs by monoterpenes in *Arabidopsis*. GUS activities were measured in HSP17.6C-CIProGUS plants. Results of control (white bar), GDA (black bar), and 13 monoterpenes (gray bars) are shown. Two inserts indicate dose effects of LIM and PER on the GUS expression in plants. The incubation period was 16 h. The GUS activities are presented in terms of 4-MU production. Values and bars represent means and SD (three individual experiments), respectively. Asterisks indicate significant differences ($p < 0.05$) as determined by Student's t-test in comparisons of control (0 mM) and other treatments.

Although LIM and GER did not inhibit chaperone activities, LIM tended to slightly decrease the activity at 1000 μM.

2.3 Effect of PER on the heat tolerance of *Arabidopsis*

We investigated whether PER, LIM, and GER affected the heat tolerance of *Arabidopsis* seedlings (Figure 4). The schedule of monoterpene treatments is shown in Figure 4A. The monoterpenes were added to the seedlings at 6 days after germination (DAG). After incubation for 24 h, the seedlings were heated at 46 °C for 1 h. This heat treatment was the lowest lethal condition for the control seedlings. The heat-treated seedlings were harvested at 10 DAG, and the fresh weights and chlorophyll contents were determined. When monoterpenes were supplied at 10 mM, fresh weights slightly decreased, suggesting that the concentration was toxic to the seedlings (Figure 4B, white columns). Fresh weights after the heat shock were minimally affected by the

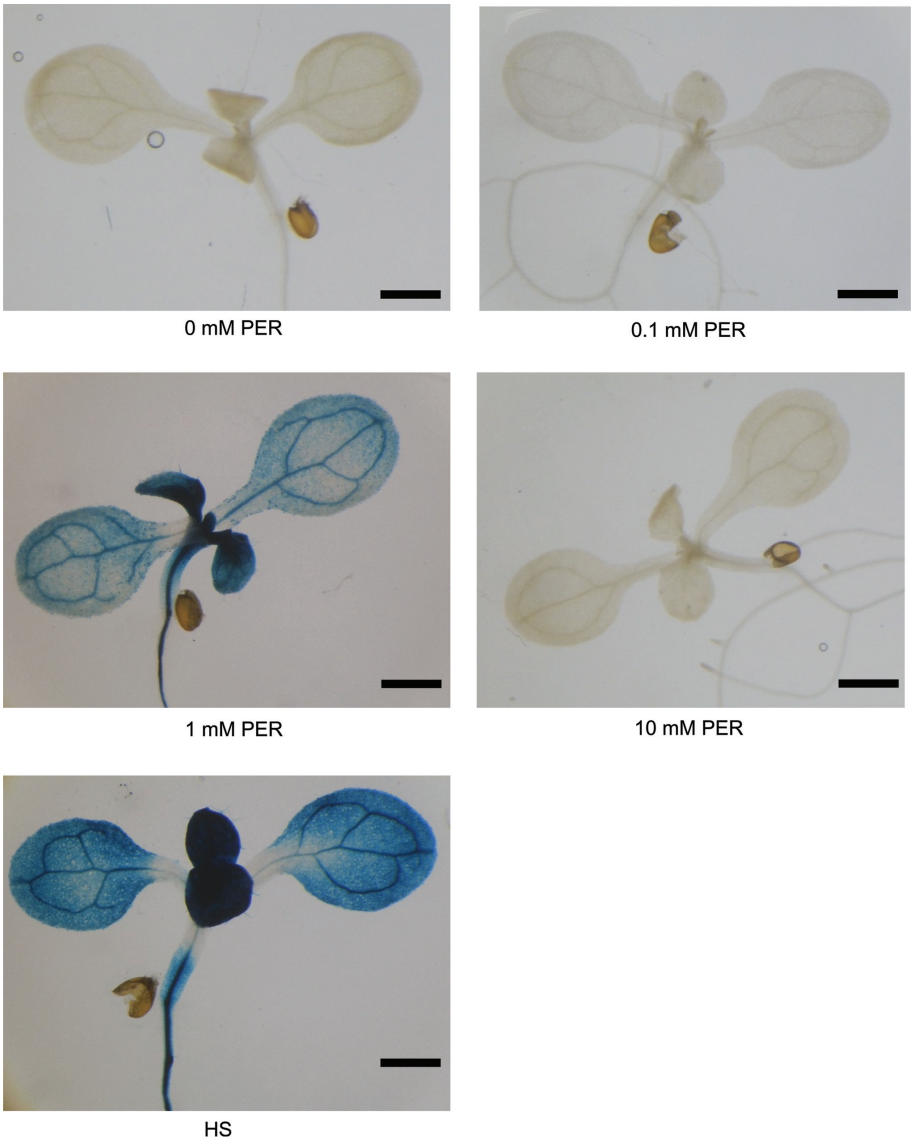

Figure 2: GUS staining of *Arabidopsis* seedlings. Seedlings of HSP17.6C-CIProGUS plants were treated with PER (0, 0.1, 1 and 10 mM for 6 h) and heat (HS, 37 °C for 1 h), respectively. The scale bars are 1 mm.

addition of monoterpenes (Figure 4B, gray columns). Chlorophyll contents were drastically reduced by heat (Figure 4C). The chlorophyll content of seedlings treated with 1 mM PER was significantly higher than that of the nontreated seedlings after heat (Figure 4C, PER, gray columns). LIM and GER did not show such an effect. This indicates that PER, which strongly promoted HSR in *Arabidopsis*, prevented the degradation of chlorophyll in plants due to heat.

3 Discussion

In this study, we found that PER and LIM remarkably activated the *HSP17.6C-CI* promoter. Bicyclic monoterpenes such as CAM, BOR, CIN, and PIN only weakly enhanced the activity of the *HSP17.6C-CI* promoter. Although PER, which is a monocyclic monoterpene aldehyde, showed strong activity (GDA equivalent was approximately 4), monoterpenes without the aldehyde moiety had lower activity (approximately 2 GDA equivalent for LIM; less than 0.3 GDA equivalents for CVN, CVL, MEN, LOX, CTN, and GER). However, the aldehyde moiety is not the determinant factor for the activity because CTA, which is an acyclic monoterpene aldehyde, showed little activity. It is supposed that the whole structure of PER may be needed to induce the HSR of *Arabidopsis*.

It has been shown that some chemicals with HSR-inducing activities in plants inhibited molecular

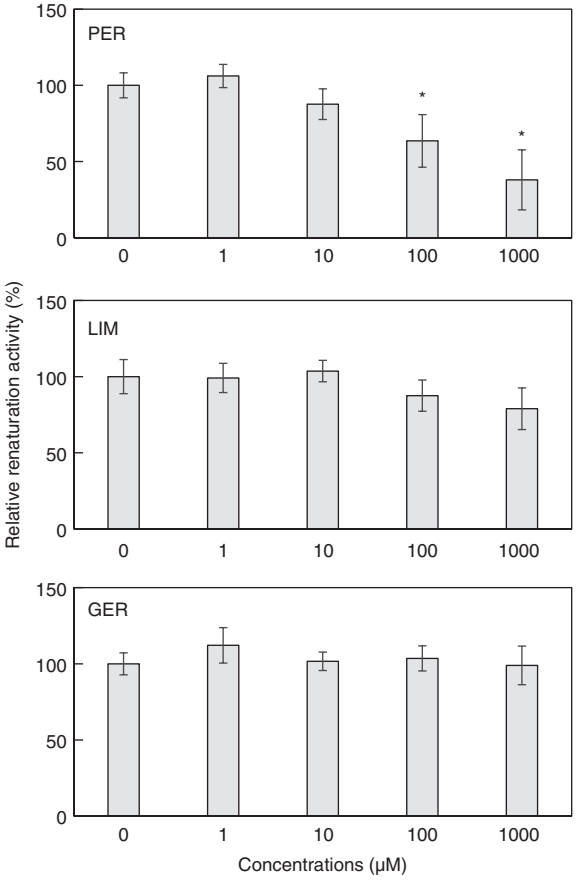

Figure 3: Effects of monoterpenes on chaperone activity. Heat-denatured luciferase was renaturated by wheat germ extract. The mean values of no treatment (0 μM) were standardized to 100% in each compound. The values and bars represent means ± SD (four individual experiments), respectively. Asterisks show significant differences ($p < 0.05$) as determined by Student's t-test in a comparison of no treatment (0 μM) and the treatments.

chaperones. For instance, GDA, monocillin 1, and sanguinarine inhibited chaperone activities, i.e. the reactivation of heat-denatured luciferase by wheat germ lysate [22, 23]. It is possible that chaperone inhibition causes the accumulation of denatured proteins. This could be a trigger to induce HSR [24]. In addition, HSP90-specific inhibition by HSP90 inhibitors such as GDA and monocillin 1 may activate HSR through the HSP90-HSFs system. Active HSP90 interferes with the transcription-regulating activities of HSFs under normal conditions. HSP90 inhibitors activated HSFs by specifically inhibiting the chaperone activity of HSP90. This reaction also resulted in the potent induction of the HSR in *Arabidopsis* [10]. The results in Figure 3 indicate that only PER, which strongly induced the HSR, significantly inhibited the wheat germ chaperone. This suggests that PER may induce HSR by inhibiting the chaperones in *Arabidopsis*. However, the inhibition of the wheat chaperone by PER was weaker than that by GDA because GDA significantly inhibited the chaperones at 1 μM [22].

Generally, monoterpenes are toxic to microorganisms and serve as feeding deterrents to animals [15, 25]. Monoterpenes also show herbicidal and allelopathic activities [16]. A transcriptomic analysis indicated that the expression of stress- or defense-related genes were upregulated when soil-grown *Arabidopsis* was exposed to myrcene and ocimene [26]. On the other hand, it has been shown that monoterpenes are related to heat tolerance in plants. High temperature promoted the monoterpene emission in *Quercus ilex* [19], *Solanum lycopersicum* [27], and *Musa* species [21]. Exogenously applied monoterpenes increased heat tolerance in *Quercus* species [20, 28]. The physiological mechanisms of heat-tolerance enhancing activities of monoterpenes were predicted to be the stabilization of the membrane and the scavenging of ROS [20, 21]. However, it has not been argued that monoterpenes may enhance the heat tolerance of plants through the induction of HSR. The present results indicate that some monoterpenes, such as LIM, PER, CAM, BOR, CIN, and PIN, can induce the HSR of *Arabidopsis*. In the case of *Q. ilex*, the monoterpene mixtures that were used for the fumigation experiments contained LIM and PIN [20, 19]. It is supposed that LIM and PIN might enhance the heat tolerance of *Quercus* mainly by membrane stabilization and radical scavenging, but partially by HSR induction in the plant.

We found that the uncommon monoterpene PER strongly induced the HSR of *Arabidopsis*. PER is a major component of the essential oil of *Perilla frutescens*, which is used as a traditional Chinese medicine. Although its many bioactivities have been reported, such as antimicrobial, anticancer, antioxidant, anti-inflammatory, and vasodilation, the mechanisms of action are not yet well understood [29]. To the best of our knowledge, this is the first report of the physiological effect of PER on plants. Because many kinds of monoterpenes are produced by various plant species, a comprehensive screening may identify monoterpenes that show more potent HSR-inducing activities. In the future, these natural compounds may be used for managing heat stress in plants.

In our experimental conditions, monoterpenes were supplied as the 1% dimethyl sulfoxide (DMSO) solution. However, it was supposed that monoterpenes in natural conditions could induce HSR in plants. The physiological roles of monoterpenes, as predicted by our results, are shown in Figure 5. Stresses may enhance the emission of monoterpenes, which can induce the HSR not only in monoterpene-emitting plants but also in neighboring plants. The HSR promotes the production of HSP, which

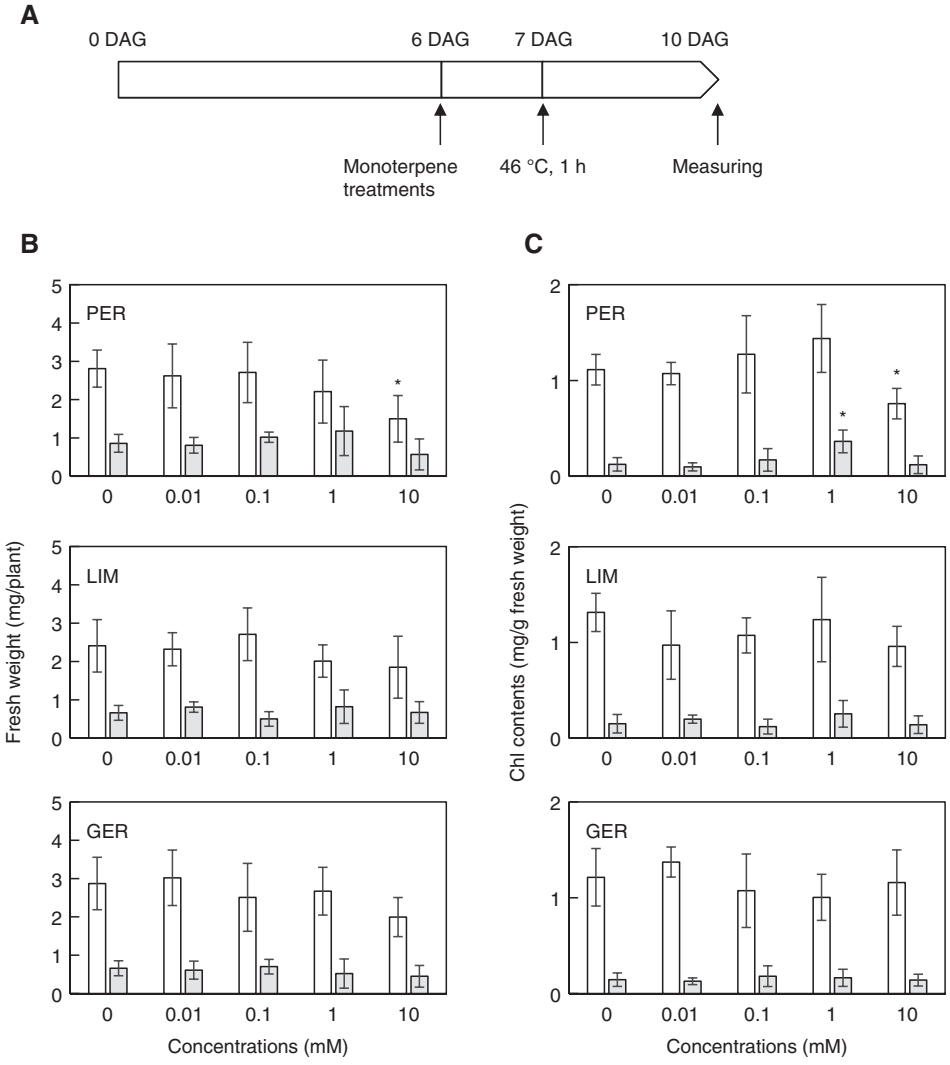

Figure 4: Effects of pretreatments with PER, LIM, and GER on fresh weight and chlorophyll content of *Arabidopsis* seedlings after heat treatment (46 °C for 1 h). This heat treatment was the lowest lethal condition for control seedlings (0 μM). (A) The scheme of the experimental schedule. DAG: days after germination. The fresh weight (B) and chlorophyll contents (C) of the seedlings. White and gray bars represent plants not exposed to heat and exposed to heat, respectively. Values and bars are means and SD (n = 12 in fresh weight, and n = 4 in chlorophyll contents), respectively. Asterisks show significant differences ($p < 0.05$) as determined by Student's t-test in a comparison of no treatment (0 μM) and treatments.

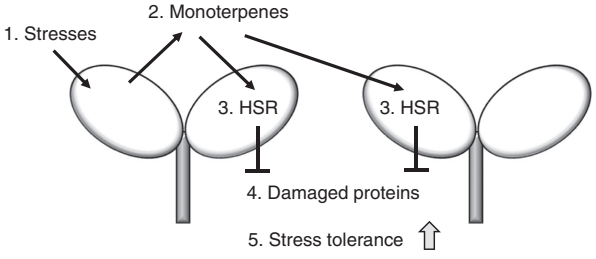

Figure 5: A hypothetical scheme for enhancing stress tolerance in plants using monoterpenes. Stressed plants can emit monoterpenes. The monoterpenes may induce HSR in the stressed plants and neighboring plants. Heat shock proteins produced through the HSR may repair damaged proteins. Thus, stress tolerance can be enhanced.

prevents damage to proteins [30]. As a result, stress tolerance may be enhanced in these plants. Further studies are needed to prove the hypothesis.

4 Experimental

4.1 Chemicals

(+)-Limonene (LIM), (+)-limonene oxide (LOX), (–)-carveol (CVL), (–)-menthol (MEN), (+)-camphor (CAM), (+)-citronellol (CTN), geraniol (GER), citral (CTA),

4-methylumbelliferyl-β-D-glucuronide (4-MUG), 4-methylumbelliferone (4-MU), and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) were obtained from Wako (Tokyo, Japan). (−)-Perillaldehyde (PER), (−)-carvone (CVN), (−)-borneol (BOR), and (+)-1,8-cineol (CIN) were purchased from Tokyo Kasei (Tokyo, Japan). α-Pinene (PIN) was from Sigma-Aldrich (Tokyo, Japan).

4.2 HSR assay

The HSR-inducing activities of monoterpenes were examined by the reporter gene assay using *Arabidopsis*, as previously described [22]. The method was based on the whole-plant GUS assay [31]. Briefly, the CaMV 35S promoter and AtADH 5′-UTR regions were removed from the pRI201-AN-GUS vector (Takara, Shiga, Japan). The region 1 kb upstream from the translational start site of the *HSP17.6C-CI* (*At1g53540*) gene was inserted into the 5′ end of the β-glucuronidase (GUS) gene of the vector. The plasmid, named HSP17.6C-CIProGUS, was introduced into *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) with the *Agrobacterium*-mediated floral dip method. Homozygous seeds of the HSP17.6C-CIProGUS *Arabidopsis* were obtained.

Seedlings were grown on a 1/5 MS medium containing 1% sucrose solidified by 0.8% agar in six-well plates (TPP, Trasadingen, Switzerland, #92406) under sterile conditions. A growth chamber was used (NK System, Tokyo, Japan), which was conditioned at 22 °C with a 16-h day (60 μmol m^{-2} s^{-1})/8-h night cycle. The seedlings at 6 DAG were treated with the test solutions [250 μL, 1% DMSO in water (v/v) containing monoterpenes (0.1, 1 and 10 mM for 13 monoterpenes, 0.1, 0.2, 0.5, 1, 2, 5 and 10 mM for LIM and PER, respectively)] in 1.5 mL test tubes (two seedlings per tube). Control was 1% DMSO solution. The tubes were incubated at 22 °C for 6 h under illumination (60 μmol m^{-2} s^{-1}). After rinsing with water, seedlings were placed in the GUS assay solution [200 μL of 50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA, 0.1% (w/v) Triton X-100, 0.1% (w/v) SDS, and 1 mM 4-MUG], and incubated at 22 °C for 16 h in the dark. The GUS reaction was stopped by the addition of 100 μL of 1 M Na_2CO_3 and incubated for 10 min. The fluorescence of 4-MU was determined using Ex 365 nm and Em 455 nm (Varioskan Flash, Thermo Fisher Scientific, Yokohama, Japan). The 4-MU amounts were calculated from the calibration curve made with authentic 4-MU.

4.3 GUS staining

At 7 DAG, seedlings were treated with PER (at 0.1, 1 and 10 mM for 6 h) and HS (at 37 °C for 1 h) in 1.5 mL test tubes. The control was 1% DMSO solution. The GUS staining was performed according to a protocol described previously [31].

4.4 Chaperone inhibition

The chaperone system from wheat germ [22, 23] was used. Recombinant luciferase (QuantiLum; Promega, Tokyo, Japan) was diluted with the supplied buffer (CCLR; Promega) to a concentration of 0.3 μg mL^{-1}. Denatured luciferase was prepared by heating the diluted sample at 40 °C for 5 min. Wheat germ extract (Promega) was double-diluted with distilled water. The diluted extract (45 μL) was incubated with the DMSO solution of test monoterpenes (0.5 μL) for 30 min at 25 °C. The denatured luciferase (5 μL) was added to start the renaturation of luciferase. The final concentrations of monoterpenes were 0 (control), 1, 10, 100 and 1000 μM. The luciferase activities were monitored by the LUC assay system (Promega) with the chemiluminescence detector (LAS-4000, Fujifilm, Tokyo, Japan). The incubation was for 0 (0 time control), 20, 40 and 60 min at 25 °C. The renaturation activities were calculated from the initial velocities. Values with no monoterpene treatment were standardized to 100%.

4.5 Heat tolerance assay

The assay was performed according to the procedure described in a previous report [32]. Seeds of the wild-type *Arabidopsis* were germinated on a filter paper disk (no. 1, Advantec, Tokyo, Japan), which was placed on 1/5 MS medium containing 0.8% agar and 1% sucrose in six-well plates (TPP, #92406) under sterile conditions. The density of seeds was six per well. Growth conditions were 22 °C with a 16-h day (60 μmol m^{-2} s^{-1})/8-h night cycle. At 6 DAG, seedlings were transferred onto the test medium containing PER, LIM, and GER with the filter paper disk. The concentrations of monoterpenes were 0 (control), 0.01, 0.1, 1 and 10 mM. The plates were incubated for 24 h, as above. Seedlings were inoculated onto the 1/5 MS medium without monoterpenes in the new plates. The plates were sealed with a polyethylene zipper bag (4-F, 120 nm × 170 mm; System Polymer, Tokyo, Japan), and soaked in water at 46 °C for 1 h. Control plates were not heat treated. After incubating for 3 days as above, the fresh weights and chlorophyll contents of the seedlings were determined [33].

4.6 Statistical analysis

Data for p values were analyzed using Student's t-test at a significance level of 0.05.

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