Isobutyl Isothiocyanate is a Potent Heat Tolerance Enhancer for *Arabidopsis*

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Heat tolerance enhancers are chemicals which prevent the physiological damages of plants by heat. Phenethyl isothiocyanate (ITC), which is known as the heat tolerance enhancer, induced the expression of heat shock protein genes in *Arabidopsis*. Here we measured the heat shock response (HSR)-inducing activities of ITCs to find more active ITCs than phenethyl ITC. We prepared transgenic *Arabidopsis* possessing the β -glucuronidase reporter gene driven by the promoter of a small heat shock protein (*HSP17.6C-CI*) gene which was induced by heat tolerance enhancers. We tested 16 naturally occurring ITCs for their HSR-inducing activities: 5 aromatic ITCs, 7 aliphatic ITCs, and 4 sulfur-containing aliphatic ITCs. The results showed that the short-chain aliphatic ITCs, especially isobutyl ITC, promoted the HSR more strongly than the aromatic and sulfur-containing aliphatic ITCs. Isobutyl ITC induced the HSR in the whole *Arabidopsis* seedlings. The isobutyl ITC-treated plants showed higher heat tolerance than the phenethyl ITC-treated plants. These results suggest that isobutyl ITC is a more potent heat tolerance enhancer than phenethyl ITC.

Keywords : heat shock protein, heat shock response, heat tolerance enhancers

INTRODUCTION

Exposure to extreme and prolonged heat is one of the major causes of damage to crop production. Heat stress allows plants to exacerbate various symptoms such as protein denaturation and degradation, increased fluidity of membrane lipids, the production of reactive oxygen species, and the suppression of photosynthesis (Wahid et al., 2007). Plants ameliorate the stress symptoms by physiological countermeasures, including production of compatible solutes, activation of antioxidative systems, and expression of heat shock proteins (HSPs) (Bita and Gerats, 2013). Among these countermeasures, HSPs are major factors in enhancing heat tolerance in plants, because the expression levels of HSP genes in plants are positively correlated with the degree of heat tolerance (Wahid et al., 2007; Waters, 2013).

Although heat is a potent trigger for the heat shock response (HSR) in organisms, other stresses (i.e., osmotic stress, oxidative stress, irradiations, wounding, and chemicals) also induce HSR (Sørensen et al., 2003; Al-Whaibi, 2011). Natural compounds have been reported to exert inducing effects on the HSR in plants. It has been demonstrated that the application of salicylic acid (SA) (Dat et al., 1998), geldanamycin (GDA) (Yamada et al., 2007), sanguinarine (Hara and Kurita, 2014), and (*E*)-2-hexenal and its related compounds (Yamauchi et al., 2015) have been shown to induce the expression of plant HSP genes and enhance the heat tolerance of plants. In *Arabidopsis*, the *HSP17.6C-CI* (*At1g53540*) gene which belongs to the

small HSP family was highly upregulated by sanguinarine (Hara and Kurita, 2014) and (*E*)-2-hexenal (Yamauchi et al., 2015), suggesting that this gene is a common regulon controlled by chemical HSR inducers in *Arabidopsis*.

Isothiocyanates (ITCs) are sulfur-containing metabolites mainly formed from Brassicaceae plants. They are produced through the hydrolysis of the corresponding precursors, i.e., glucosinolates by myrosinase. Biochemical and genetic studies have demonstrated the reaction mechanism of the glucosinolate-myrosinase system, the tissue and cellular localization of myrosinase and glucosinolate, and the biosynthetic pathways of glucosinolates (Halkier and Gershenzon, 2006; Kissen et al., 2009). It has been considered that the production of ITCs is a part of the defense mechanisms of plants, because ITCs show anti-microorganism, animal-repellent, allelopathic, and herbicidal activities (Halkier and Gershenzon, 2006; Hopkins et al., 2009; Hara et al., 2010). Recently, we found that phenethyl ITC enhanced heat tolerance of *Arabidopsis* (Hara et al., 2013). Since ITCs are contained in food condiments, ITCs are promising as heat tolerance enhancers. However, little is known which ITCs show more HSR-inducing activities than phenethyl ITC.

In this study, we tested 16 kinds of ITCs including phenethyl ITC for their HSR-inducing activities by using the transgenic *Arabidopsis* which has the β -glucuronidase (*GUS*) reporter gene controlled by the *HSP17.6C-CI* gene promoter. We found that the ITCs with short branched side chains showed more potent activation of the *HSP17.6C-CI* gene promoter rather than phenethyl ITC.

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MATERIALS AND METHODS

Chemicals

Phenethyl ITC (1), benzyl ITC (3), 4-methoxybenzyl ITC (5), hexyl ITC (11), methallyl ITC (12), ethyl ITC (7), butyl ITC (8), isopropyl ITC (9), and 3-(methylthio) propyl ITC (14) were obtained from Sigma-Aldrich (Tokyo, Japan). Phenyl ITC (2), allyl ITC (6), and isobutyl ITC (10) were purchased from WAKO (Tokyo, Japan). 4-Phenylbutyl ITC (4), sulforaphane (13), alyssin (15), and sulforaphane (16) were from LKT Labs (St. Paul, MN, USA). 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. GDA was purchased from TokyoKasei (Tokyo, Japan).

Production of transgenic Arabidopsis

A plasmid vector for the transformation of *Arabidopsis thaliana* was constructed by exchanging the cauliflower mosaic virus (CaMV) 35S promoter region, including AtADH 5'-UTR of pRI201-AN-GUS (Takara, Shiga, Japan), to the 1-kb upstream region from the translational start site of the *HSP17.6C-CI* (At1g53540) gene. The pRI201-AN-GUS plasmid was digested by *HindIII* and *NdeI* to remove the CaMV 35S promoter and AtADH 5'-UTR regions. The upstream region of the *HSP17.6C-CI* gene, which was amplified by polymerase chain reaction, was inserted via the sticky ends of *HindIII* and *NdeI* to the plasmid using the In-Fusion kit (Takara) according to the manufacturer's instruction. Finally, the *HSP17.6C-CI* gene promoter was located adjacent to the 5'-terminus of the GUS gene of the pRI201-AN-GUS plasmid. We designated the plasmid HSP17.6C-CIProGUS. *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) was transformed with the HSP17.6C-CIProGUS plasmid using the *Agrobacterium*-mediated floral dip method. The homozygotes were obtained and named the HSP17.6C-CIProGUS *Arabidopsis* plant.

GUS reporter assay

The GUS activity assay was performed according to the established method of whole-plant GUS reaction (Weigel and Glazebrook, 2002), with slight modifications. Seeds of the HSP17.6C-CIProGUS *Arabidopsis* were sown on a 1/5 MS medium containing 1% sucrose solidified by 0.8% agar in 6-well plates (TPP, Trasadingen, Switzerland, #92406) under sterile conditions. The plates were kept at 6°C for 2 d, and then transferred to the growth chamber (NK System, Tokyo, Japan), conditioned at 22°C with a 16-h day (60 $\mu\text{mol m}^{-2} \text{s}^{-1}$)/8-h night cycle. At 6 d after germination (DAG), whole seedlings were soaked in 250 μL of test solution in 1.5-mL micro test tubes (2 seedlings per tube). The test solutions were 1% dimethyl sulfoxide (DMSO) in water (v/v) containing GDA (5, 50, and 500 μM) and ITCs (0.02, 0.1, 0.2, 1, and 2 mM). Test tubes were incubated at 22°C for 6 h under illumination (60 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Control solution was 1% DMSO. For heat shock (HS), the 6 DAG seedlings soaked in the 1% DMSO solution of the test tubes were incubated at 22°C for 5 h, and then the test tubes were immersed in a water

bath at 37°C for 1 h. The seedlings that were rinsed with water were transferred to the wells (2 seedlings per well) in a 96-well microplate (IWAKI, Funabasi, Japan) containing 200 μL of the GUS assay solution composed 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. Seedlings were incubated at 22°C for 0, 1, 2, 4, 16, 24, and 48 h in the dark. At the end of incubations, 100 μL of 1 M Na_2CO_3 was added and incubated for 10 min to stop the GUS reaction and enhance the fluorescence of the reaction product (4-MU). The solutions were appropriately diluted with 0.33-M Na_2CO_3 . The fluorescence was determined at an excitation and emission wavelength of 365 and 455 nm, respectively, with Varioskan Flash (Thermo Fisher Scientific, Yokohama, Japan). The 4-MU amounts were calculated from the calibration curve made with authentic 4-MU. When ITCs were tested, the incubation period in the GUS assay solution was 16 h.

GUS staining of *Arabidopsis* seedlings was done as previously described (Weigel and Glazebrook, 2002). The 7 DAG seedlings were treated with isobutyl ITC (0.2 mM) as described above for 6 h. The control was without the ITC. After destaining with acetone, the seedlings were stained with X-Gluc, and then observed with light microscopy.

Heat-tolerance experiment

Seeds of the wild *Arabidopsis* were sown on filter paper (No. 1, Advantec, Tokyo, Japan), which was then plated on the 1/5 MS medium containing 1% sucrose solidified by 0.8% agar in 6-well plates (TPP, #92406) under sterile conditions. The plates were kept at 6°C for 2 d (vernalization), and then transferred to the growth chamber (NK System, Japan) conditioned at 22°C with a 16-h d (60 $\mu\text{mol m}^{-2} \text{s}^{-1}$)/8-h night cycle. At 6 DAG, the 6 seedlings with the filter paper were inoculated onto the test medium in a 60-mm plastic Petri dish (IWAKI, Japan).

Test ITCs were prepared as DMSO solution at concentrations of 0.25, 2.5, 25, and 250 mM. To make the test medium (8 ml in each Petri dish), the test ITC DMSO solutions (16 μL) were added to the 1/5 MS medium with 0.8% agar and 1% sucrose (7984 μL). The final concentrations of the test medium were 0.5, 5, 50, and 500 μM , respectively. The Petri dishes were sealed with Parafilm to prevent the emission of ITCs from the dish. After incubating the seedlings with the test medium under the same conditions as above for 24 h, the plates were sealed with a polyethylene zipper bag (4-F, 120 mm $\times$ 170 mm, System Polymer, Tokyo, Japan) and soaked in water at 46°C for 1 h. Control plates were placed in the same growth chamber for 1 h. The dishes containing heat-treated and control plants were maintained for 3 d under the described conditions. At 10 DAG, the seedlings were harvested, and the fresh weights were measured. Chlorophyll content was determined spectrophotometrically according to the common method (Wintermans and de Mots, 1965).

Statistical analysis

Data for *P* values were analyzed by Student's *t*-test at a significance level of 0.05.

RESULTS AND DISCUSSION

Reporter gene assay

First, we produced transgenic *Arabidopsis* plants to conduct the reporter assay for HSR in plants. The construct of the transgene is shown in Fig. 1a. We used the promoter of a small HSP gene, i.e., the *HSP17.6C-CI* (*At1g53540*) gene of *Arabidopsis*. A previous study reported that the *HSP17.6C-CI* gene was upregulated by the addition of GDA (Hara and Kurita, 2014). The upstream region (1 kb) of the *HSP17.6C-CI* gene was inserted into the site adjacent to the β -glucuronidase (*GUS*) reporter gene, which was followed by the heat shock protein terminator. The transgenic *Arabidopsis* was designated as the HSP17.6C-CIProGUS plant.

Induction of the HSR by the GDA treatment in the HSP17.6C-CIProGUS plant is shown in Fig. 1b. After treating the HSP17.6C-CIProGUS plant with GDA for 6 h, the plants were inoculated from the GDA solution to the GUS reaction solution containing 4-MUG, which can be converted to fluorescent 4-MU by GUS. It has been established that GUS activity can be detected without extracting the GUS protein from the plants, because both 4-MUG and 4-MU can pass through the plant cell membranes under the reported conditions (Weigel and Glazebrook, 2002). The GDA addition gradually enhanced 4-MU production during the incubation period. GDA concentration of 50 μ M promoted 4-MU production more than concentrations of 5 and 500 μ M. The blank control (0 μ M GDA) enhanced 4-MU production very little. Heat (37°C for 1 h) induced more 4-MU production in the HSP17.6C-CIProGUS plant than the GDA treatment (Fig. 1c). Because the *HSP17.6C-*

CI promoter responded to both the GDA addition and heat, this reporter assay was available to analyze the activity of HSR induction by ITCs.

Induction of HSR in Arabidopsis by ITCs

We measured the HSR-inducing activities of the naturally occurring ITCs listed in Fig. 2 using the reporter assay. We tested 16 ITCs, including 5 aromatic, 7 aliphatic, and 4 sulfur-containing aliphatic ITCs. After the HSP17.6C-CIProGUS plants were treated with the ITCs for 6 h, 4-MU productions by GUS were determined at the 16th h of incubation in the whole-plant reaction. Although all 16 ITCs promoted 4-MU production, promoting effect varied by type of ITC (Fig. 3). Overall, the aliphatic ITCs tended to enhance 4-MU production more strongly than the aromatic and sulfur-containing aliphatic ITCs. In particular, isobutyl ITC (10) and isopropyl ITC (9) potently promoted 4-MU production. The following tests were done using isobutyl ITC (10), because the highest 4-MU production was marked by the administration of isobutyl ITC (10) at 0.2 mM in Fig. 3. GUS staining was clearly observed in almost the whole HSP17.6C-CIProGUS plant treated with isobutyl ITC (10) (Fig. 4), suggesting that most tissues of *Arabidopsis* seedlings could exhibit the HSR to the application of isobutyl ITC (10).

Enhancement of heat tolerance by ITCs

One day after the addition of ITCs to the wild-type *Arabidopsis* seedlings (6 DAG), the plants were treated with heat stress (46°C for 1 h). The heat-stressed seedlings were cultivated for 3 d, and then fresh weight and chlorophyll content were measured (Fig. 5a). Both the fresh weight and chlorophyll content of the seedlings treated with 500- μ M isobutyl ITC (10) were significantly higher than those of the control (0 μ M) seedlings (Fig. 5b, c).

Fig. 1 GUS reporter assay. (a) Construct of the transgene in the HSP17.6C-CIProGUS plasmid. (b) Time course of the 4-MU production by the HSP17.6C-CIProGUS *Arabidopsis* plant treated with GDA. The concentrations were 0 μ M (open circles), 5 μ M (closed circles), 50 μ M (closed triangles), and 500 μ M (open triangles), respectively. (c) Heat shock (37°C for 1 h) was applied to the HSP17.6C-CIProGUS plants. Closed and open circles show heat shock and control, respectively. Values and bars represent means $\pm$ SD (four individual experiments). Asterisks show significant differences ($P < 0.05$) as determined by Student's *t*-test in a comparison between no treatment (0 μ M) and treatments.

Fig. 2 Sixteen ITCs used in this study.

Fig. 3 Promotion of HSR by ITCs in *Arabidopsis*. Numbers represent ITCs corresponding to the numbers in Fig. 2. The 4-MU productions were determined after whole-plant GUS reaction for 16 h. Values are shown as GDA equivalents standardized to the values obtained by the GDA treatment. ITCs were applied at concentrations of 0.02, 0.1, 0.2, 1, and 2 mM, from left to right, respectively, as shown by open triangles. Black and white bars show GDA treatment (G, 0.05 mM) and control (C, 0 mM ITC), respectively. Values and bars represent means $\pm$ SD (three individual experiments). Asterisks show significant high values ($P < 0.05$) as determined by Student's *t*-test in a comparison between GDA and ITC treatments.

However, such effects were not observed when phenethyl ITC (1) was used. The heat condition (46°C for 1 h) was lethal for the phenethyl ITC-treated seedlings. This indicates that isobutyl ITC showed the higher heat tolerance-enhancing effect than phenethyl ITC in the experimental condition.

Use of ITCs with short-branched side chains

The reporter assay indicated that aliphatic ITCs showed stronger HSR-inducing activities than aromatic

and sulfur-containing aliphatic ITCs, whereas most ITCs had more activities than GDA. Since most aliphatic ITCs have smaller molecular weights than aromatic and sulfur-containing aliphatic ITCs, aliphatic ITCs may more easily access the active sites of the plant cell. Among aliphatic ITCs, allyl ITC (6) and methallyl ITC (12) showed weaker activities than other aliphatic ITCs, suggesting that aliphatic ITCs possessing carbon-carbon double bonds may be chemically unstable compared to aliphatic ITCs without

Fig. 4 GUS staining of *Arabidopsis* seedlings. Seedlings were treated with 0.2-mM isobutyl ITC (a, b, and c) and without ITC (d, e, and f). Shoots (a and d), main roots (b and e), and the tips of the main roots (c and f) are shown. The scale bars are 5 mm.

the double bond.

Since ITCs are potent electrophiles which can react with thiol groups of various proteins and glutathione, ITCs may affect protein homeostasis and redox balance in the cell (Murakami, 2013). These effects may be triggers for inducing the HSR. Besides that, it was recently reported that sulforaphane bound to HSP90 and disrupted the HSP90-p50^{Cdc37} complex in pancreatic cancer cells (Li et al., 2012). In *Arabidopsis*, HSP90 is thought to suppress HsfA1, which is a transcription factor for many HSP genes, including *HSP17.6C-CI* (Yoshida et al., 2011), by binding it. It is postulated that when the action of HSP90 was inhibited by HSP90 inhibitors such as GDA, HsfA1 could be released and activated by disrupting the interaction between HSP90 and HsfA1 (Yamada et al., 2007). It

is supposed that ITCs may induce the *HSP17.6C-CI* gene expression by activating HsfA1 via disrupting the HSP90-HsfA1 interaction.

ITCs with short-branched side chains are found in many plant species (Fahey et al., 2001). Isobutyl ITC (10) is a pungent component of horseradish. Isopropyl ITC (9) is contained in Brassicaceae vegetables. This means that these ITCs are safe because they have been taken by humans for a long period. ITCs with short-branched side chains may be promising for the practical enhancers of heat tolerance in plants.

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Fig. 5 Effects of pre-treatments with isobutyl ITC and phenethyl ITC on fresh weights and chlorophyll contents of *Arabidopsis* seedlings after heat (46°C for 1 h). (a) The scheme of the experimental schedule. DAG means days after germination. The fresh weight (b) and the chlorophyll content (c) of seedlings. White and black bars represent plants not exposed to heat and plants exposed to heat, respectively. Values and bars represent means $\pm$ SD ($n = 12$ in fresh weight and $n = 4$ in chlorophyll content). Asterisks show significant differences ($P < 0.05$) as determined by Student's *t*-test in a comparison between no treatment (0 μ M) and the ITC treatments.

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