Administration of isothiocyanates enhances heat tolerance in Arabidopsis thaliana

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Administration of isothiocyanates enhances heat tolerance in *Arabidopsis thaliana*

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Abstract

Although it has been documented that plants generate isothiocyanates (ITCs) through the glucosinolate-myrosinase system to defend against biotic stresses, the roles of ITCs in defending against abiotic stresses have scarcely been studied. Here, we report that exogenously applied ITCs enhance the heat tolerance of *Arabidopsis thaliana*. Pre-administration of phenethyl ITC to *Arabidopsis* plants mitigated growth inhibition after heat stress at 55°C for 1 h. Although methyl ITC and allyl ITC also tended to reduce the growth inhibition that the same heat treatment caused, the reduction effects were weaker. The expression levels of heat shock protein 70 genes in *Arabidopsis* were elevated after phenethyl ITC treatment. These results suggest that ITCs may act as heat-tolerance enhancers in plants.

Keywords

Arabidopsis thaliana - heat shock protein - heat tolerance - isothiocyanate

Abbreviations

DAG	Days after germination
GB	Glycinebetaine
HSP	Heat shock protein
ITC	Isothiocyanate
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction
SA	Salicylic acid

Introduction

Global climate changes have potential impacts on crop production worldwide (Hall 2001). High temperature is one of the most serious problems in crop production. An increase in ambient temperature leads to heat stress in plants. Plant growth is inhibited by the heat stress through various symptoms, such as protein denaturation, the inhibition of protein synthesis and degradation, increased fluidity of membrane lipids, and the production of reactive oxygen species (ROS) (Wahid et al. 2007). Photosynthetic processes, such as the oxygen-evolving complex in photosystem II, carbon fixation by Rubisco, and the ATP-generating system, are highly sensitive to heat in plants (Allakhverdiev et al. 2008). Plants manifest physiological responses to heat stress, including the accumulation of compatible solutes, changes in hormone contents and growth regulators, the generation of ROS, the activation of antioxidative systems,

and the expression of heat shock proteins (HSPs) (Iba 2002; Kotak et al. 2007). Although such complicated responses have been recorded in many plants, how plants establish their heat tolerance is not totally understood.

Methodologies of enhancing the heat tolerance of plants have been developed to reduce the negative impact of heat stress on agricultural productivity. Genetic improvements including traditional breeding and genetic transformation have been attempted to produce heat-tolerant plants (Wahid et al. 2007). In addition, considerable attention has been devoted to the induction of heat tolerance using low-molecular-weight compounds. Exogenous applications of salicylic acid (SA) and its derivates enhanced the tolerance to heat in mustard (*Sinapis alba*), bean (*Phaseolus vulgaris*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), cucumber (*Cucumis sativa*), potato (*Solanum tuberosum*), and *Arabidopsis thaliana* (Dat et al. 1998; Senaratna et al. 2000; Horváth et al. 2007). The administration of glycinebetaine (GB) and polyamines also induced heat tolerance in various plant species (Sakamoto and Murata 2002; Wahid et al. 2007; Allakhverdiev et al. 2008). Such chemical treatments are considered to be promising, because the heat tolerance of plants can be enhanced by simple applications to their seeds, leaves, and roots. From the practical point of view, however, the number of chemical inducers of heat tolerance is still limited.

Isothiocyanates (ITCs) are sulfur-containing secondary metabolites mainly produced by Brassicaceae plants. ITCs are generated from corresponding precursors (glucosinolates) by a hydrolyzing reaction of myrosinase (Kliebenstein et al. 2005; Grubb and Abel 2006; Halkier and Gershenzon 2006; Yan and Chen 2007). The reaction mechanism of myrosinase and the biosynthetic pathways of major glucosinolates have been successfully demonstrated by biochemical and genetic studies (see articles cited above). Since ITCs exhibit growth inhibition of herbivores and microorganisms due to their toxicities, it is believed that ITCs are related to chemical defense against biotic enemies (Halkier and Gershenzon, 2006; Clay et al. 2009; Hopkins et al. 2009; Winde and Wittstock, 2011). However, the defensive roles of ITCs against abiotic stresses in plants have been little studied.

Recently, we reported that ITCs showed herbicidal effects when applied to *Arabidopsis* at high doses (Hara et al. 2010). Before demonstrating the herbicidal effects of ITCs at the field scale, we first attempted to confirm the ITCs' effects on *Arabidopsis* in the greenhouse. During the greenhouse tests, we accidentally found that plants which were pretreated with phenethyl-ITC showed greater heat tolerance than plants pretreated with no phenethyl-ITC under summer high temperatures in Japan. This suggests that the application of ITCs to plants may promote the heat tolerance. In this paper, we investigated the growth of heat-stressed plants to which ITC(s) were pre-administered. We also discuss putative mechanisms of the effects of ITCs in enhancing heat tolerance in plants.

Materials and methods

Plants and ITC treatments

Arabidopsis thaliana (L.) Heynh. (ecotype Columbia) plants were grown in 6-cm plastic pots filled with Peatban (Sakata Seed, Yokohama, Japan). The pots were placed in a growth chamber (NK System, Tokyo, Japan) to control the growth conditions, i.e., a 16-h day (60 μmol m^{-2} s^{-1})/8-h night cycle at 23°C. Three plants were grown per pot. At 20 days after germination (20DAG), each plastic pot in which unbolted plants were growing was put into a plastic bag. The bag was completely sealed with cellophane tape (Scotch; Sumitomo 3M, Tokyo, Japan) and then placed in the same growth chamber as above for 1 day. This treatment was necessary to acclimate the plants to the sealed environment. After the acclimation, a water emulsion of each ITC was sprayed on the plants with a hand-pump aerosol spray bottle (400 μL per pot) at 21DAG. The water emulsion was prepared according to a previous paper (Hara et al. 2010) by sonication with the Branson sonifier 150 (Branson Ultrasonics, Danbury, CT, USA) in continuous mode 5 for 1 min. Phenethyl- and methyl-ITCs were stable in the sprayed water emulsions, because more than 70% of the initial concentration remained for 24 h. However, allyl-ITC was somewhat less stable than phenethyl- and methyl-ITCs (Hara et al. 2010). For the phenethyl-ITC treatment, 0, 1, 2, and 5 mM solutions were prepared (Fig. 1). The 0 mM solution refers to water that was treated with the same sonication as described above. Solutions of methyl- and allyl-ITCs (0, 2, 5, and 10 mM) were sprayed for the test in Fig. 2. The phenethyl alcohol and phenylalanine solutions used in Fig. 2 were applied to plants after they were subjected to sonication. The pots treated with the compounds (or water) were immediately put into the same plastic bags. After incubation for 24 h in the growth chamber as described above, the pots were retrieved from the plastic bags and then placed again in the growth chamber. Twenty-four hours after the bag removal, plants in the pots were exposed to heat stress at 23DAG. The heat stress is described in detail below. After the heat stress, the plants were grown for an additional 19 days (until 42DAG) in the growth chamber as described above. The aerial parts of the plants were harvested, and their fresh weights were measured to evaluate their growth. To investigate gene expression, the aerial parts were kept at -70°C until use.

Heat stress

The pots where the plants grew were placed in the air-conditioned incubator (EYELA, MHS-2000, Tokyo, Japan) without illumination. To avoid the direct influence of the hot wind

which was produced by an electric fan, each pot was wholly covered by a shield whose shape was a cylindrical tube (φ 8 cm x 15 cm) made from paper. The temperatures in the incubator were 23, 35, 45 50, 55, and 65°C, respectively. The incubation period was 1 h. After the heat treatments, the pots were returned to the growth chamber as described above (in the "Plants and ITC treatments" section).

Gene expression analysis

The transcript levels of *HSP70*, *HSP70T2*, *HSP70B*, and *actin* genes in *Arabidopsis* were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from the aerial parts of the plants with the RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). Total RNA (1 μg) was subjected to reverse transcription (45°C for 30 min), and then PCR proceeded under the conditions denoted in Supplemental Fig. S1. After the band intensity was determined using NIH-Image software (http://rsbweb.nih.gov/nih-image/), the relative amounts of the transcripts were calculated by standardizing the band intensities at zero time.

Results

We tested the effect of phenethyl-ITC on *Arabidopsis* growth after the heat stresses. Plants at the rosette stage (21DAG) were treated with different concentrations (0, 1, 2, and 5 mM) of phenethyl-ITC (Fig. 1A). Two days after the phenethyl-ITC treatment (23DAG), the plants were exposed to heat stresses (for 1 h at 23, 35, 45, 55, and 65°C). After that, the plants were harvested and weighed at the bolting stage (42DAG). Photographs of the plants, which had been treated with phenethyl-ITC (0, 1, 2, and 5 mM) at 21DAG and then exposed to 55°C for 1 h at 23DAG, were taken at 42DAG (Fig. 1B). The control plants (0 mM phenethyl-ITC) were severely damaged by the heat stress. Some of them could not grow after the heat. However, plants treated with phenethyl-ITC (especially at the concentration of 2 mM) continued to grow after the same heat stress (Fig. 1B). Other concentrations (1 and 5 mM) of phenethyl-ITC were likely to be effective, but their effects were weaker than that of 2 mM phenethyl-ITC (Fig. 1B). Figure 1C shows the fresh weights of plants that were treated with the different concentrations of phenethyl-ITC and then exposed to various temperatures. When the rosette stage *Arabidopsis* plants that had been treated with no ITC (0 mM phenethyl-ITC) were exposed to the heat stresses at 35, 45, 55, and 65°C for 1 h, plant growth tended to slow as the degree of heat stress increased (Fig. 1C, white bars). However, pre-administration of phenethyl-ITC at 1 and 2 mM tended to reduce growth inhibition due to heat stress, especially at 55°C. The phenethyl-ITC treatments (1, 2, and 5 mM) did not alter *Arabidopsis* growth if heat stress was

not administered (23°C). Because all the plants were dead after the 65°C stress, we did not measure plant weight. The time intervals between the phenethyl-ITC treatment (2 mM) and the heat stress (55°C for 1 h) were varied among 1, 2, and 3 days in order to assess the effectiveness of each interval. The results indicated that the 1-day interval was about as effective as the 2-day interval, whereas the 3-day interval was somewhat less effective than either the 1- or 2-day interval (data not shown).

We also investigated whether or not other ITCs, such as methyl-ITC and allyl-ITC, can also enhance the heat tolerance of *Arabidopsis* (Fig. 2). The cultivation conditions were the same as in Fig. 1. The timings of the ITC treatments and the heat stresses were identical to those shown in Fig. 1A. The methyl- and allyl-ITC treatments tended to ameliorate the growth inhibition caused by heat stress at 55°C for 1 h, whereas the fresh weights were not significantly different between the ITC-treated plants and the non-ITC-treated plants (Fig. 2A). The plants treated with 10 mM allyl-ITC were not weighed, because the treatment with 10 mM allyl-ITC immediately killed the plants. Pictures of plants that had been treated with methyl-ITC and allyl-ITC and then exposed to the heat stress are shown (Fig. 2B). The methyl- and allyl-ITC-treated plants grew larger than the control plants.

Two compounds (phenethyl alcohol and phenylalanine) that possess phenethyl moieties were pre-administered to rosette-stage *Arabidopsis* plants at 21DAG. The subsequent exposure to heat stress (55°C for 1 h) at 23DAG showed that phenethyl alcohol and phenylalanine did not affect the growth inhibition caused by the heat stress (Figs. 2A, B). These results suggest that the ITC moiety is more related to the promotion of heat tolerance than the phenethyl moiety.

Generally, it is known that heat stress promotes the gene expression of *HSP*s in most organisms. HSPs are categorized into five classes based on the differences in their molecular weights: HSP100, HSP90, HSP70, HSP60, and small HSPs (Iba 2002; Kotak et al. 2007). Among them, HSP70 has the primary structure that is most conserved through different species (Iba 2002). Transgenic *Arabidopsis*, whose endogenous HSP70 level was lowered, showed less thermotolerance than the wild type (Lee and Schöffl, 1996), suggesting that HSP70 is related to the heat tolerance of *Arabidopsis*. Thus, we checked the expression levels of three kinds of *HSP70* genes, *HSP70* (*At3g12580*), *HSP70T2* (*At2g32120*), and *HSP70B* (*At1g16030*), after the administration of phenethyl-ITC to *Arabidopsis* at the rosette stage. The transcript levels of *HSP70*, *HSP70T2*, *HSP70B*, and *actin* (*At3g18780*, a control gene) were determined by RT-PCR. Typical results of gel electrophoresis are shown in Supplemental Fig. S2. The time courses of the transcript levels of the genes tested in this study are represented in Fig. 3. All three *HSP70* genes were transiently expressed by the addition of phenethyl-ITC. The transcript accumulations of *HSP70*, *HSP70T2*, and *HSP70B* peaked at 1 h after phenethyl-ITC administration, then immediately decreased to almost the same levels as before administration.

During the first 48h after administration, the transcript levels of the *actin* gene were constant.

Discussion

Although studies of the roles of ITCs have been conducted mainly from the viewpoint of chemical defense in plant-microbe interactions, a small number of reports on the physiological functions of ITCs in the plant itself have been published. Recent research has revealed that ITC may be involved in stomatal closure in *Arabidopsis* (Zhao et al. 2008; Khokon et al. 2011), and exogenous appreciation of ITCs enhanced the expression of *GST* genes in *Arabidopsis* (Hara et al. 2010). Although these results show that ITCs can prime the physiological responses of plants, no report demonstrating that ITCs affect abiotic stress tolerance has been published. In this study, we first report that exogenously applied ITCs can enhance heat tolerance in plants.

Phenethyl-ITC application showed the transient induction of *HSP70s* genes at 1 h after the application. Although the transcript levels of the *HSP70* genes decreased within 24 h to the zero-time levels, the potential of the heat tolerance remained for at least 2 days after the phenethyl-ITC application. This phenomenon may be explained as follows: the accumulation of the HSP70 proteins may continue for 2 days, and/or the exogenous phenethyl-ITC may indirectly affect another mechanism related to the establishment of heat tolerance through the expression of the *HSP70* genes. SA and GB are the heat-tolerance inducers that have been most studied. Exogenous SA induced *HSP* genes (Horváth et al. 2007) to increase the hydrogen peroxide content *in vivo* (Chen et al. 1993). Thus, SA may enhance the heat tolerance in plants by setting the physiological status to be similar to stress-acclimating processes. On the other hand, GB is considered to promote heat tolerance by protecting enzymes and protein complexes from heat-induced denaturation. Although the GB-accumulating transgenic *Arabidopsis* showed more heat tolerance than the wild type, the extent of the *HSP* genes induction was significantly reduced in the transgenics (Alia et al. 1998), suggesting that HSPs may not contribute to enhancing heat tolerance induced by GB. As mentioned above, the administration of phenethyl-ITC to *Arabidopsis* increased the *HSP* genes expression as well as the hydrogen peroxide content. This indicates that the mode of action of phenethyl-ITC is similar to that of SA rather than that of GB.

Benzoic acid is predicted to be the functional moiety of SA that induces heat tolerance in plants (Senaratna et al. 2003). In the present study, we found that phenethyl-, methyl-, and allyl-ITCs appeared to enhance heat tolerance, but phenethyl alcohol and phenylalanine did not. This suggests that the ITC moiety may be the functional group of the ITCs. Because the ITC moiety is structurally unrelated to benzoic acid, the molecular recognition mechanisms by plants may be different between ITCs and SA. SA was proven to bind to catalase, inhibiting its activity

in many plants (Horváth et al. 2007). It has been explained that the catalase inhibition is a trigger to elevate the hydrogen peroxide level. In the animal system, ITCs promote a rapid depletion of glutathione and other thiols in cells by conjugating ITCs to the thiol compounds, thus the ROS (mainly hydrogen peroxide) level increased intensively (Zhang et al. 2005). The depletion of thiol compounds may occur in the plant cells that are exposed to ITCs as well. However, it is still unknown how ITCs elevate the expression of the *HSP* genes. The following studies are needed to clarify the mechanism of the heat-tolerance enhancing effect of ITCs.

Since many kinds of plants used for condiments and foods contain ITCs, and so ITCs are commonly ingested (Fahey et al. 2001). For instance, phenethyl-ITC is present in watercress, cabbage, and horseradish. Allyl-ITC is a major component found in mustard, horseradish, and wasabi. Moreover, methyl-ITC is already applied to control nematode pests in the field (Chitwood 2002). Given these facts, ITCs can be considered to be useful for safely controlling the heat tolerance of plants in farm fields.

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Figure legends

Fig. 1 Effect of pre-administration of phenethyl-ITC on the growth of *Arabidopsis* after heat stress. The schedule of ITC administration (ITC), heat stress (HS), and harvest is shown (**a**). Photographs of the areal parts of plants (**b**). Bar indicates 5 cm. Graph of the fresh weights of the plants exposed to different heat stresses (**c**). White, light gray, dark gray, and black bars represent the administration of 0, 1, 2, and 5 mM of phenethyl-ITC, respectively. Values and bars represent means ± SD (n=5). The fresh weights of plants exposed to 65^{o}C heat stress were not measured (n.m.) because the corresponding plants were dead. The symbols a and b show a significant difference ($p < 0.05$), which was determined by Student's t-test in a comparison between no administration (0 mM) and administration (1, 2, or 5 mM) at each level of heat stress.

Fig. 2 Effects of the pre-administration of methyl-ITC (MITC), allyl-ITC (AITC), phenethyl alcohol (PA), and phenylalanine (Phe) on the growth of *Arabidopsis* after heat stress. The schedule of ITC administration, heat stress, and harvest was the same as that shown in Fig. 1A. Fresh weights of the areal parts of plants are exhibited (**a**). The concentrations of MITC, AITC, PA, and Phe were 0 mM (white bars), 2 mM (light gray bars), 5 mM (dark gray bars), and 10 mM (black bars, only MITC), respectively. Heat stress was 55^{o}C for 1 h. Values and bars represent means ± SD (n=5). The fresh weights of plants pre-treated with 10 mM AITC were not measured (n.m.) because the corresponding plants were dead. The symbols a and b show a significant difference ($p < 0.05$), which was determined by Student's t-test in a comparison between no administration (0 mM) and administration (2, 5, or 10 mM) at each level of heat stress. Photographs of the areal parts of plants are shown (**b**). Bar indicates 3 cm.

Fig. 3 Effect of phenethyl-ITC on the expression levels of *HSP70* genes. The *HSP70* (*At3g12580*), *HSP70T2* (*At2g32120*), *HSP70B* (*At1g16030*), and *actin* (*At3g18780*) genes were analyzed. Relative amounts of the transcripts were determined by RT-PCR. The control (0 mM phenethyl-ITC) and administration (2 mM phenethyl-ITC) groups are represented by white and black bars, respectively. Values and bars represent means ± SD (n=3). The symbols a and b show a significant difference ($p < 0.01$), which was determined by Student's t-test in a comparison between no administration (0 mM) and administration (2 mM) at each time after treatment.

Legends for supplemental data

Fig. S1 RT-PCR conditions used to determine transcript accumulations of the *HSP70* (*At3g12580*), *HSP70T2* (*At2g32120*), *HSP70B* (*At1g16030*), and *actin* (*At3g18780*) genes.

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Fig. S2 Analyses of the RT-PCR products of the *HSP70* (*At3g12580*), *HSP70T2* (*At2g32120*), *HSP70B* (*At1g16030*), and *actin* (*At3g18780*) genes. The products were separated by agarose gel electrophoresis (1%). The results for 0 h and 1 h are shown. Arrowheads indicate the putative sizes of cDNA derived from matured (spliced) mRNA.

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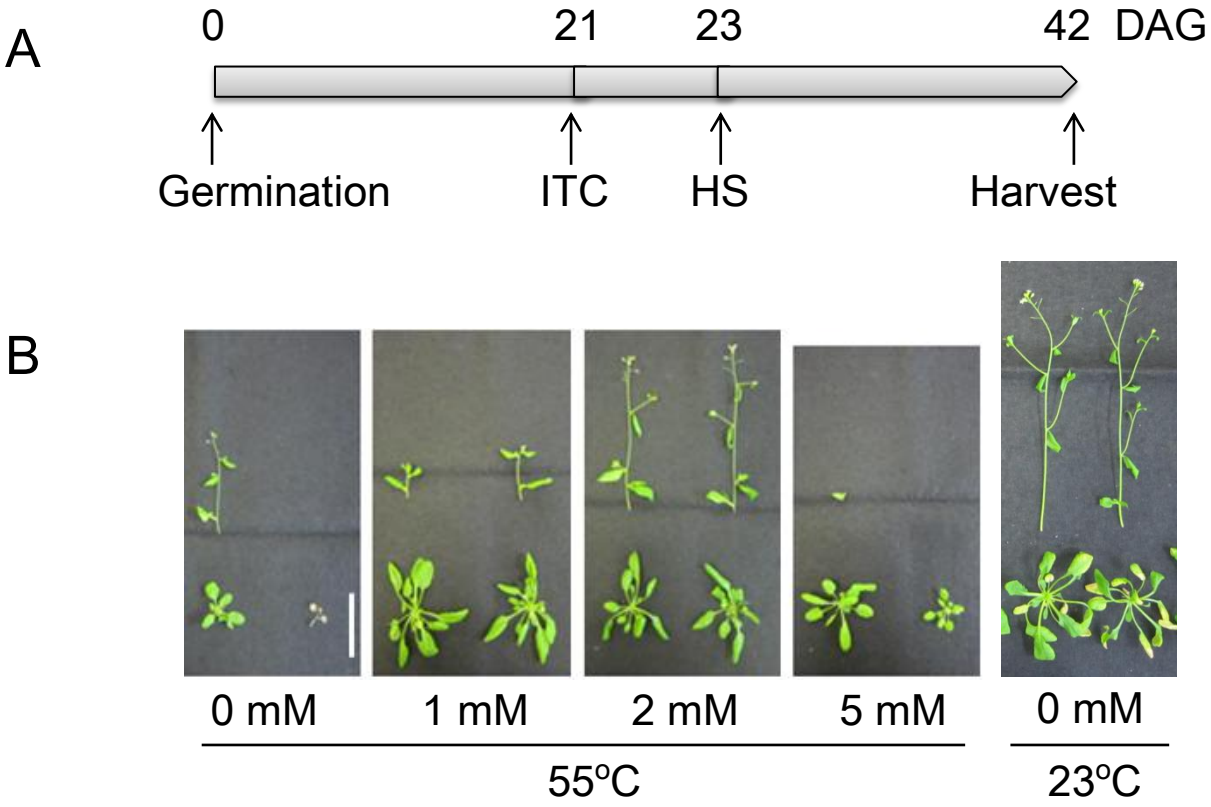

Fig. 1. Hara et al.

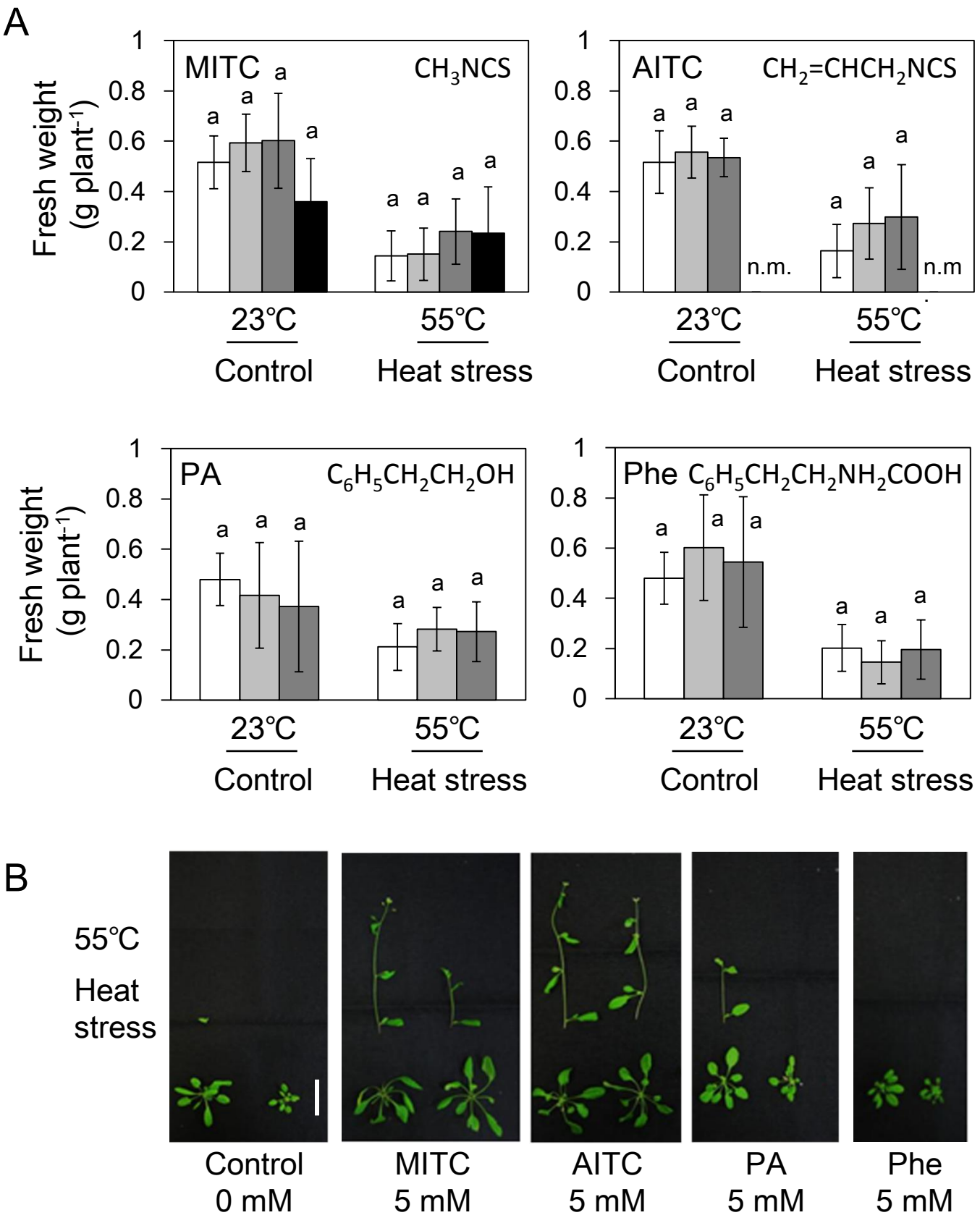

Fig. 2. Hara et al.

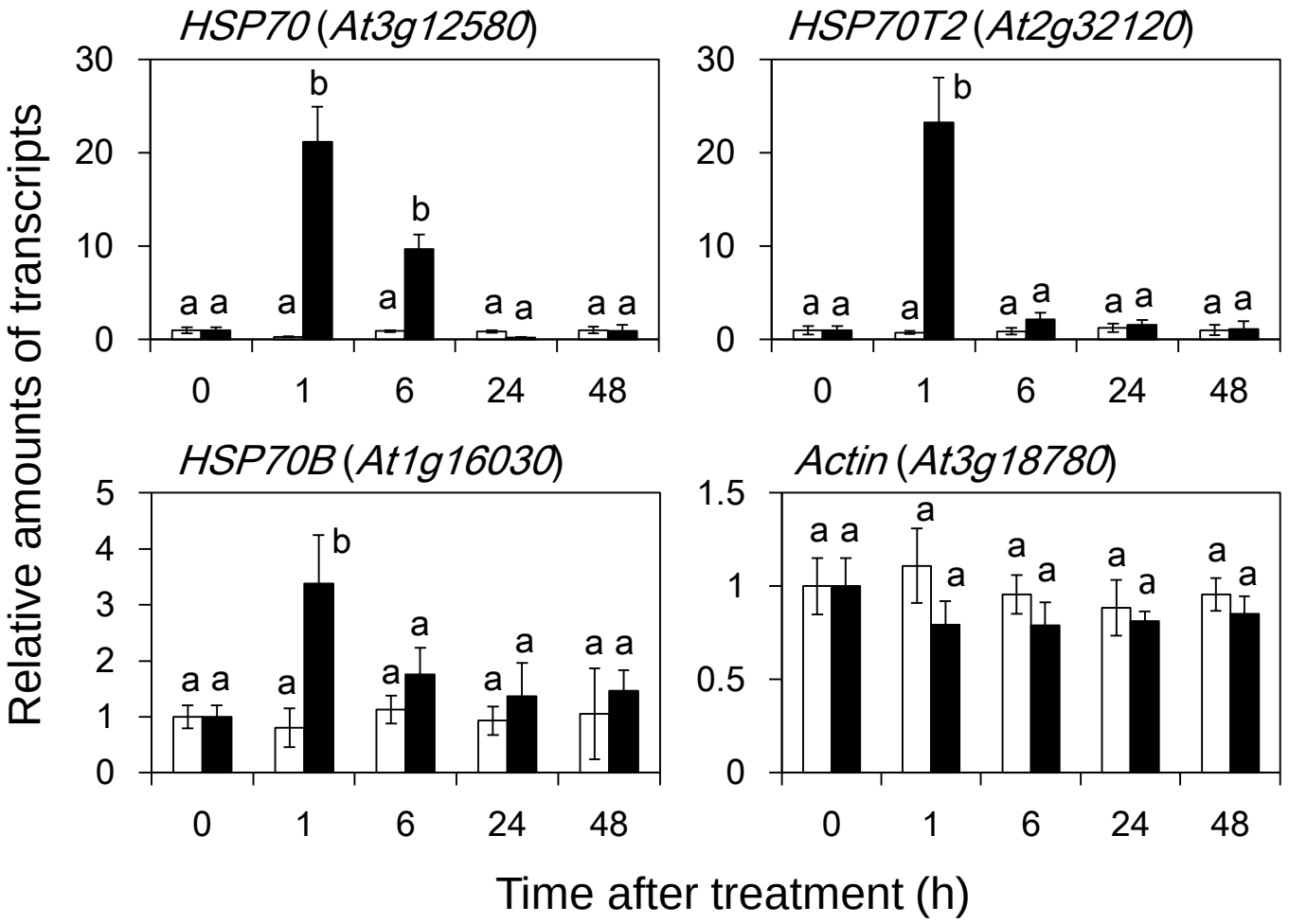

Fig. 3. Hara et al.

HSP70 *At3g12580*	94°C for 30 sec. 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec. Sense primer: 5'-GATCTCTAATAATGGCGGG-3' Antisense primer: 5'-CCAGTTTCAGAGTGACATAG-3' Size: 2,053 bp (spliced) and 2,303 bp (unspliced).
HSP70T2 *At2g32120*	94°C for 30 sec. 32 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec. Sense primer: 5'-CTCTCCTTTCGGTGGTGACA-3' Antisense primer: 5'-TACGAAGCATCCTCGTATC-3' Size: 1,794 bp (spliced) and 2,113 bp (unspliced).
HSP70B *At1g16030*	94°C for 30 sec. 32cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec. Sense primer: 5'-GCAACAATGGCGACGAAATC-3' Antisense primer: 5'-GCCTTCTTGAAACCCAAACC-3' Size: 1,958 bp.
Actin *At3g18780*	94°C for 20 sec. 26 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec. Sense primer: 5'-ACCTTGCTGGACGTGACCTTACTGAT-3' Antisense primer: 5'-GTTGTCTCGTGGATTCCAGCAGCTT-3' Size: 298 bp.

Fig. S1 RT-PCR conditions used to determine transcript accumulations of the *HSP70* (*At3g12580*), *HSP70T2* (*At2g32120*), *HSP70B* (*At1g16030*), and *actin* (*At3g18780*) genes.

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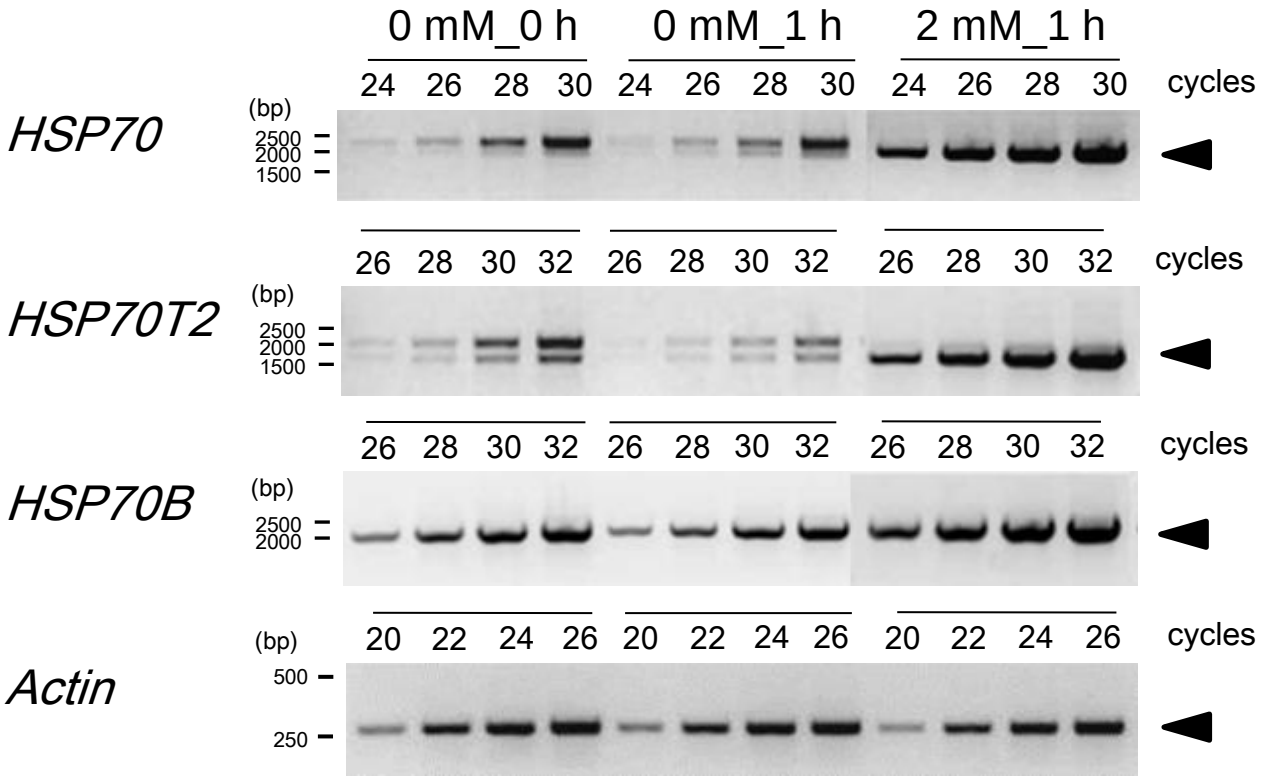

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