Cover page

Short running title

Response to isothiocyanates in *Arabidopsis*

Authors

Masakazu Hara, Yukie Yatsuzuka, Kyoko Tabata, Toru Kuboi
Faculty of Agriculture, Shizuoka University,
836 Ohya, Suruga-ku, Shizuoka,
422-8529, Japan

Name and address for editorial correspondence

Masakazu Hara
Faculty of Agriculture, Shizuoka University,
836 Ohya, Suruga-ku, Shizuoka,
422-8529, Japan
Telephone number: +81-54-238-5134
Fax number: +81-54-238-5134
E-mail address: masahara@agr.shizuoka.ac.jp

Title page

Title

Exogenously applied isothiocyanates enhance glutathione *S*-transferase expression in *Arabidopsis* but act as herbicides at higher concentrations

Authors

Masakazu Hara, Yukie Yatsuzuka, Kyoko Tabata, Toru Kuboi
Faculty of Agriculture, Shizuoka University,
836 Ohya, Suruga-ku, Shizuoka,
422-8529, Japan

Name and address for editorial correspondence

Masakazu Hara
Faculty of Agriculture, Shizuoka University,
836 Ohya, Suruga-ku, Shizuoka,
422-8529, Japan
Telephone number: +81-54-238-5134
Fax number: +81-54-238-5134
E-mail address: masahara@agr.shizuoka.ac.jp

Summary

Isothiocyanates (ITCs) are sulfur-containing compounds that are generated by the glucosinolate-myrosinase system in plants. Although previous greenhouse studies have demonstrated the phytotoxicity of ITCs, their action modes are still unknown. In this study, we report the physiological responses of *Arabidopsis thaliana* treated with three exogenous ITCs: methyl ITC, allyl ITC, and phenethyl ITC. Administration of a high dose of each ITC inhibited plant growth and induced severe bleaching in the rosette leaves. The bleaching was concomitant with the elevation of electrolyte leakage and the generation of hydrogen peroxide. Although the three ITCs showed bleaching symptoms, phenethyl ITC was the most potent. A low dose of phenethyl ITC, at which the ITC did not promote leaf bleaching, enhanced the accumulation of transcripts of glutathione *S*-transferases (GSTs) in *Arabidopsis*. When 16 GST genes were tested, the levels of transcripts corresponding to 5 of the GST genes were enhanced in response to the phenethyl ITC treatment. In particular, the expression of a Tau class gene (*AtGSTU19*, At1g78380) responded to the phenethyl ITC treatment. Enhancement of the *AtGSTU19* gene expression also occurred in the treatment of both allyl ITC and methyl ITC. These results suggest that the administration of ITCs to *Arabidopsis* at high doses has an herbicidal effect by inducing oxidative burst-like responses, but that administration at lower doses enhances the expression of specific GST genes in *Arabidopsis*.

Keywords: Glucosinolate; Glutathione *S*-transferase; Herbicidal activity; Hydrogen peroxide; Isothiocyanate

Abbreviations: GST, glutathione *S*-transferase; ITC, isothiocyanate; RT-PCR, reverse transcription-polymerase chain reaction.

Introduction

Isothiocyanates (ITCs) are sulfur-containing electrophiles produced by many members of the order Brassicales, especially Brassicaceae plants. ITCs show toxic effects against plants. The growth of velvetleaf seedlings was inhibited by benzyl isothiocyanate (Wolf et al., 1984) and sulforaphene (Brinker and Spencer, 1993). ITCs showed allelopathic effects on wheat (Bialy et al., 1990, Vaughn and Berhow, 1999). The shoot density of many weed species was reduced by the application of ITCs (Yamane et al., 1992; Norsworthy and Meehan IV, 2005; Norsworthy et al., 2006). It is estimated that green manures consisting of ITC-producing plants are promising bioherbicides for use in integrated pest management strategies (Vaughn and Boydston, 1997; Brown and Morra, 1995; Gimsing and Kirkegaard, 2009).

ITCs are generated from glucosinolates by the enzymatic reaction of myrosinases which hydrolyze glucosinolates. Thus, the ITC-producing reaction is called the glucosinolate-myrosinase system (Wittstock and Halkier, 2002; Kliebenstein et al., 2005; Grubb and Abel, 2006; Halkier and Gershenzon, 2006; Yan and Chen, 2007). The enzymatic mechanisms of this system have been studied (Thangstad et al., 1993; Rask et al., 2000; Clay et al., 2009; Bednarek et al., 2009). Recent genetic and biochemical studies using *Arabidopsis* have confirmed the backbone pathway of the biosynthesis of glucosinolates from amino acid precursors (Wittstock and Halkier, 2002; Grubb and Abel, 2006; Halkier and Gershenzon, 2006). The glucosinolate-myrosinase system is likely to contribute to plant defense because ITCs have biocidal activities. ITCs cause toxicity, growth inhibition, or feeding deterrence in relation to plant enemies such as mammals, birds, insects, mollusks, aquatic invertebrates, nematodes, bacteria, and fungi (Halkier and Gershenzon, 2006). Since ITCs react with amino and sulfhydryl groups of peptides, it is likely that ITCs influence the functions of peptides nonspecifically in vivo. In addition, ITCs may generate an intracellular oxidative stress in mammals (Nakamura et al., 2000) and fungi (Sellam et al., 2007). However, the mechanisms of the biocidal activity of ITCs are not fully understood (Halkier and Gershenzon, 2006).

The evaluation of ITCs as phytotoxins has mainly been conducted via greenhouse and/or field studies, but these reports have not described the physiological actions of ITCs on plants. It remains necessary to elucidate the physiological responses of plants treated with ITCs. In this paper, we report the physiological symptoms in *Arabidopsis* treated with three kinds of ITCs: methyl ITC, allyl ITC, and phenethyl ITC. In addition, we show that ITCs enhance the transcript accumulation of specific glutathione *S*-transferase (GST, EC 2.5.1.18) genes in *Arabidopsis*. The physiological significance of the responses is also discussed.

Materials and methods

Plant materials and ITC treatment

Arabidopsis thaliana (L.) Heynh ecotype Columbia (Col-0) plants were grown in 6-cm plastic pots filled with vermiculite in a 16-h day (60 μmol m^{-2} s^{-1})/8-h night cycle at 22°C. The density of planting was 3 plants per pot. The plants were periodically fertilized with Hyponex (NPK = 6.5-6-19, Hyponex Japan, Osaka, Japan). Five-week-old unbolted plants were exposed to methyl ITC, allyl ITC, or phenethyl ITC as follows. Before the ITC treatments, The mouth of each plastic pot was sealed completely by a plastic film (a polyvinylidene chloride sheet, Kureha Chemical Industry, Tokyo, Japan). The plastic film was bonded to the pot with cellophane tape (Scotch; Sumitomo 3M, Tokyo, Japan). The sealed pots were kept for 4 d under the conditions described above. Sealing the pot is necessary because ITCs applied to plants could evaporate and thus be transferred to plants in the neighboring pots. The incubation for 4 d functioned to acclimate the plants to the sealed environment. After the acclimation, a water emulsion of each ITC at a concentration of 1, 10, or 100 mM, which was prepared by sonication with the Branson sonifier 150 (Branson Ultrasonics, CT, USA) in continuous mode 5 for 1 min, was sprayed on the plants (400 μL per pot). As a control, the same volume of water was sprayed. The pots treated with ITCs (or water as a control) were immediately sealed with plastic film and maintained until harvest under the same conditions as described above. The aerial parts of the plants were harvested, and their fresh weight was measured to evaluate growth. The aerial parts were kept at -70°C until use.

Chlorophyll analysis

Chlorophyll assays were carried out according to a spectrophotometric method (Wintermans and de Mots, 1965).

Electrolyte leakage

The percentage of electrolyte leakage was measured by a method described previously (Hara et al., 2003) with slight modifications. Two fully-expanded rosette leaves (the 5^{th} and 6^{th}) which were detached from each plant were placed in a polypropylene centrifuge tube (1.5 mL) containing 1 mL deionized water. After incubation at room temperature for 1.5 h, the solution conductivity (initial value A) was measured using a B-173

conductivity meter (HORIBA, Tokyo, Japan). The tube containing the leaves and deionized water was then heated at 95°C for 20 min and allowed to cool to room temperature. After centrifugation at 1,500 x *g* at room temperature for 3 min, the conductivity of the supernatant was measured (final value B). Electrolyte leakage was calculated as the percentage of initial (A) to final (B) conductivity.

Hydrogen peroxide detection

The accumulation of hydrogen peroxide in the aerial parts of the plants was detected by a diaminobenzidine staining method as described elsewhere (Thordal-Christensen et al. 1997) with modifications. One plant was immersed in the diaminobenzidine solution (1 mg/mL, 5 mL) under light at room temperature for 4 h. The plant was then placed in a polypropylene centrifuge tube (1.5 mL) containing 1 mL of 95% ethanol, and destained by heating at 75°C for 10 min. After the destaining was repeated twice, the plant was rehydrated in deionized water and photographs were then taken.

Measurement of transcripts accumulation

The levels of transcripts 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). One microgram of RNA was subjected to a semiquantitative RT-PCR system (QuantumRNA 18S Internal Standards Kit, Ambion, TX, USA). In this system, both the target RNA and 18 S rRNA (an internal standard) were amplified together. Reverse transcription was performed at 45°C for 30 min. PCR proceeded through 26 cycles of 94°C for 30 s, corresponding annealing temperatures for 30 s, and 72°C for 90 s. The annealing temperatures are denoted in a supplementary table (Table S1). After determining the band intensity using NIH-Image software, the relative mRNA contents were deduced from the intensities of the target PCR products and rRNA PCR products according to the instruction manual for the QuantumRNA 18S Internal Standards Kit. In this work, 20 genes were analyzed. The corresponding gene codes, primer sequences, annealing temperatures, and sizes of the RT-PCR products for the constitutively spliced transcripts are shown in Table S1. The RT-PCR product for 18 S rRNA was 315 bp.

Results

Effects of ITCs on growth of *Arabidopsis*

First, we added ITCs to 5-week-old *Arabidopsis* plants by spraying them as water emulsions. The concentrations of ITCs in the sprayed emulsions are shown in Fig. S1. Phenethyl ITC was the most stable in the sprayed emulsion among the three ITCs tested (Fig. S2). Although the concentrations of ITCs in the sprayed emulsions were lower than those of ITCs that were combined with water, we describe our results below using the latter concentrations. If the ITC emulsion was sprayed as described in Materials and methods, the rate was approximately 130 μl of the ITC emulsion per 1 g fresh weight of a rosette leaf. Figure 1 shows the effects of the three kinds of ITCs, i.e., methyl ITC, allyl ITC, and phenethyl ITC, on the growth of *Arabidopsis*. Although no statistically significant difference was found, ITCs showed a tendency to inhibit growth. Methyl ITC showed growth inhibition at the concentration of 100 mM after 3 d of application. However, 1 mM and 10 mM methyl ITC did not influence growth. Although allyl ITC inhibited *Arabidopsis* growth at 10 mM and 100 mM, 1 mM allyl ITC did not reduce growth. The effect of phenethyl ITC was similar to that of allyl ITC. Visible symptoms are shown in Fig. 2. High-dose applications (100 mM) of the three ITCs promoted bleaching in the rosette leaves. In the case of 10 mM applications, phenethyl ITC showed complete bleaching as observed with the 100 mM application, but allyl ITC showed partial bleaching. The addition of 10 mM methyl ITC did not cause a visible change. The application of each ITC at 1 mM did not induce any change except for a slight curling of the rosette leaves that occurred during the phenethyl ITC treatment. Next, the chlorophyll contents were measured to quantify the degree of bleaching (Fig. 3). The chlorophyll content decreased after 3 d of the methyl ITC application at 100 mM. In the allyl ITC and phenethyl ITC treatments, applications at 10 mM and 100 mM reduced the chlorophyll contents. When the results were compared between Fig. 2 and Fig. 3, alteration of the chlorophyll content resulted in a visible change in the plant. These results show that ITC application inhibits growth and promotes chlorophyll loss in *Arabidopsis*. Although allyl ITC and phenethyl ITC show strong phytotoxicity, the effects of phenethyl ITC were slightly stronger than those of allyl ITC.

Enhancement of solute leakage and hydrogen peroxide accumulation by ITCs

Bleaching is known to be a typical symptom of an oxidative burst in plants. In the case of ozone-induced oxidative damage, chlorophyll loss is concomitant with solute leakage and hydrogen peroxide accumulation (Bray et al., 2000). Thus, we investigated whether ITCs enhance solute leakage and hydrogen peroxide accumulation. Applications of methyl ITC (100 mM),

allyl ITC (10 mM and 100 mM), and phenethyl ITC (10 mM and 100 mM) promoted electrolyte leakage from the leaf tissue of *Arabidopsis* (Fig. 4). Phenethyl ITC was the most potent promoter of electrolyte leakage, as it induced electrolyte leakage within 1 h at concentrations of 10 mM and 100 mM. Diaminobenzidine staining indicated that the three ITCs enhanced hydrogen peroxide accumulation in leaves (Fig. 5). In this experiment, the application of phenethyl ITC (10 mM and 100 mM) for 1 h promoted a significant accumulation of hydrogen peroxide. Taken together, these results suggest that ITCs promote oxidative burst-like responses including chlorophyll loss, solute leakage, and hydrogen peroxide accumulation. Among the 3 ITCs tested, phenethyl ITC was the most potent promoter of the responses.

Alteration of stress-related gene expression by ITCs

Since exogenous ITCs have damaging effects on *Arabidopsis*, we analyzed the transcript accumulation of stress-related genes during ITC treatments. We chose 5 kinds of genes: *invertase/pectin methylesterase inhibitor family protein* (At3g47380), *chitinase class IV* (At3g54420), *peroxidase* (At5g64120), *phenylalanine ammonia-lyase 1* (At2g37040), and *GST AtGSTF6* (At1g02930). The *invertase/pectin methylesterase inhibitor family protein*, *chitinase class IV*, and *peroxidase* were characterized as wounding-responsive genes by Cheong et al. (2002). *Phenylalanine ammonia-lyase 1* and *AtGSTF6* are oxidative stress-related genes (Zeier et al., 2004). In this analysis, phenethyl ITC was used because it had the most potent phytotoxic effects on *Arabidopsis* (Figs. 1 to 5). When phenethyl ITC was applied to *Arabidopsis* at concentrations of 10 mM and 100 mM, RNA could not be extracted because the plant tissues were severely damaged. Thus, we used the RNA of plants treated with 1 mM phenethyl ITC. Figure 6 indicates that phenethyl ITC slightly enhanced the expression of 2 out of the 5 genes, namely *the invertase/pectin methylesterase inhibitor family protein* (Fig. 6A) and *AtGSTF6* (Fig. 6E). Because the expression of the *AtGSTF6* gene was more enhanced than that of the *invertase/pectin methylesterase inhibitor family protein* gene, the *AtGSTF6* gene is likely to be a susceptible gene in response to phenethyl ITC.

It is known that *Arabidopsis* possesses 47 GST genes (Wagner et al., 2002). Recently, Dixon et al. (2009) reported that 51 *Arabidopsis* GSTs were found to be transcribed. We therefore tested whether other kinds of GST genes are also activated by phenethyl ITC. Sixteen genes of GSTs, namely *AtGSTF2*, *AtGSTF3*, *AtGSTF6*, *AtGSTF7*, *AtGSTF8*, *AtGSTF9*, *AtGSTF10*, *AtGSTT1*, *AtGSTZ1*, *AtGSTU5*, *AtGSTU11*, *AtGSTU13*, *AtGSTU19*, *AtGSTU20*, *AtGSTU26*, and *AtGSTU27*, were chosen because they were reported to be

highly expressed in *Arabidopsis* (Wagner et al., 2002). Phenethyl ITC showed tendencies to enhance the expression of 4 genes: *AtGSTF6*, *AtGSTF7*, *AtGSTZ1*, and *AtGSTU19* (Figs. 7A–D). *AtGSTU19* showed a clear response to phenethyl ITC at 1 h after treatment, and the degree of expression then gradually decreased (Fig. 7D, closed circles). *AtGSTU19* did not respond to the water treatment (Fig. 7D, open circles). *AtGSTF6*, *AtGSTF7*, and *AtGSTZ1* responded to phenethyl ITC, but also responded to water. On the other hand, *AtGSTF2*, *AtGSTF3*, *AtGSTF8*, *AtGSTF9*, *AtGSTF10*, *AtGSTT1*, *AtGSTU5*, *AtGSTU11*, *AtGSTU13*, *AtGSTU20*, *AtGSTU26*, and *AtGSTU27* showed either no response or only a slight response to phenethyl ITC. As an example, the result of *AtGSTF2* expression is shown in Fig 7E. This result suggests that enhancement of the *AtGSTU19* expression depends on the phenethyl ITC application. Figure 8 indicates that not only phenethyl ITC, but also methyl ITC and allyl ITC, showed a tendency to elevate the level of the *AtGSTU19* transcript. However, phenethyl ITC is the most effective inducer of *AtGSTU19* expression, and a concentration of 0.1 mM is sufficient to enhance the transcript accumulation of *AtGSTU19*.

Discussion

Greenhouse and field studies have reported that ITCs show phytotoxicity against many kinds of plants. In the present study, we found that ITCs promote oxidative burst-like responses in *Arabidopsis* when applied at high concentrations. It is likely that this action by ITCs results in the phytotoxic effects. Among the 3 ITCs tested, phenethyl ITC showed the most potent effects and methyl ITC the mildest. Such differences of effectiveness may be derived from variations in the side chains of ITCs. Borek et al. (1998) reported the relationships between the molecular structures of the isothiocyanates and their toxicities to black vine weevil eggs. Borek et al predicted that ITCs with higher numbers of carbon atoms or those bearing aromatic moieties could show high toxicities to the insect.

The present investigation indicated that the *AtGSTU19* gene responded to the phenethyl ITC treatment. The *AtGSTU19* gene, which was formerly called GST8, belongs to a Tau class GST family. The *AtGSTU19* gene was reported to be a major GST gene expressed by the administration of herbicide safeners (DeRidder et al., 2002; Smith et al., 2004; DeRidder and Goldsbrough, 2006; Brazier-Hicks et al., 2008) and salicylic acid (Sappl et al., 2004). The *AtGSTU19* gene also responded to oxidative stress, exogenous plant hormones, and wounding (Bianchi et al., 2002). Generally, GST is believed to play roles in the detoxification of xenobiotics and endogenous compounds (Dixon et al., 1998; Edwards et al., 2000).

Because recombinant AtGSTU19 protein showed an activity to conjugate between glutathione and benzyl ITC (DeRidder et al., 2002; Wagner et al., 2002; Dixon et al., 2009), the ITC-responsive *AtGSTU19* expression may be a detoxifying system of ITCs generated in *Arabidopsis*. In addition, phenethyl ITC enhanced the expression of *AtGSTZ1*, which is known as a hydroperoxide-specific GST (Wagner et al., 2002). Taken together, these results suggest that the ITCs that are generated from the glucosinolate-producing tissues may enhance detoxifying activities in plants.

Recently, new glucosinolate metabolic pathways containing glutathione conjugation steps have been proposed (Clay et al., 2009; Bednarek et al., 2009). It is postulated that the pathways are related to antifungal defense in *Arabidopsis*. The conjugation steps likely occur after the activation of glucosinolates by *PEN2* myrosinase. Our results suggest that GST may contribute to the glutathione conjugation.

It is still an open question whether wounded *Arabidopsis* can produce sufficient amounts of ITCs to promote the oxidative burst-like responses and the activation of GST genes. The S-cells, which are sulfur-rich cells localized in the floral stalk of *Arabidopsis*, contain approximately 100 mM glucosinolates (Koroleva et al., 2000, Halkier and Gershenzon, 2006). If the S-cells are wounded, ITCs can be emitted at a concentration similar to 100 mM at the wounded site. Such a concentration of ITCs is sufficient to induce oxidative burst-like responses and GST gene activation. Thus, it is likely that the responses to ITCs may occur in the wounded *Arabidopsis*. ITCs possess the -N=C=S group as a common structure. Because the central carbon atom is highly electrophilic, ITCs act as electrophiles. Recently, it has been reported that naturally occurring electrophiles, such as 12-oxo-phytodienoate (Stintzi et al., 2001), malondialdehyde (Weber et al., 2004), and methyl vinyl ketone (Alméras et al., 2003), induced the gene expression of GSTs including *AtGSTU19*. Since ITCs enhanced *AtGSTU19* expression, they may share the core signal transduction with the electrophilic mediators described above. ITCs may be mediators that control defense-related signaling in the glucosinolate-producing plants.

It has been well documented in mammalian experimental systems that ITCs have cancer-preventive activities. Chemoprevention by ITCs is believed to occur through an induction of phase 2 detoxification enzymes, including GST (Zhang and Talalay, 1994; Zhang et al., 2005; Juge et al., 2007). When the *Brassica* crop pathogen *Alternaria brassicola* was exposed to ITCs, the pathogen produced a specific GST which preferred ITCs as substrates (Sellam et al., 2006). Here we demonstrated an enhancement of GST gene expression by ITCs, suggesting that the response of GST genes to ITCs may be an ubiquitous response that occurs in some kingdoms of organisms. At present, it is not known how ITCs enhance the expression of

the GST genes. Elucidating the mechanisms of the responses to ITCs in plants will help us understand the physiological roles of the glucosinolate-myrosinase system.

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Legends to figures

Figure 1. Effects of methyl ITC, allyl ITC, and phenethyl ITC on *Arabidopsis* growth. The change in fresh weight is shown as the relative growth of the plant. The fresh weight value at zero time (average: 37 ± 8 mg) is standardized. MITC, methyl ITC; AITC, allyl ITC; PEITC, phenethyl ITC. Open circles, 0 mM; open triangles, 1 mM; closed circles, 10 mM; closed triangles, 100 mM. Values and bars represent means ± S.D. of 3 plants. The Student's t-test was performed. No statistically significant difference was found at the $P < 0.05$ level. The chemical structure of ITC is inserted in each graph.

Figure 2. Visible changes in *Arabidopsis* plants exposed to ITCs. Methyl ITC (MITC), allyl ITC (AITC), and phenethyl ITC (PEITC) were sprayed. Photographs were taken 3 d after the treatments. White bars indicate 1 cm.

Figure 3. Effects of methyl ITC, allyl ITC, and phenethyl ITC on chlorophyll contents in *Arabidopsis*. MITC, methyl ITC; AITC, allyl ITC; PEITC, phenethyl ITC. Open circles, 0 mM; open triangles, 1 mM; closed circles,

10 mM; closed triangles, 100 mM. Values and bars represent means ± S.D. of 3 plants. The Student's t-test was performed. * significant difference from the value at 0 time in each treatment at the $P < 0.05$ level.

Figure 4. Time-courses of electrolyte leakage in *Arabidopsis* treated with methyl ITC, allyl ITC, and phenethyl ITC. One hundred percent electrolyte leakage indicates a complete leakage of electrolyte from the rosette leaf. MITC, methyl ITC; AITC, allyl ITC; PEITC, phenethyl ITC. Open circles, 0 mM; open triangles, 1 mM; closed circles, 10 mM; closed triangles, 100 mM. Values and bars represent means ± S.D. of 3 plants. The Student's t-test was performed. * significant difference from the value at 0 time in each treatment at the $P < 0.05$ level.

Figure 5. Diaminobenzidine staining to detect hydrogen peroxide. Methyl ITC (MITC), allyl ITC (AITC), and phenethyl ITC (PEITC) were applied to *Arabidopsis* at concentrations of 1 mM, 10 mM, and 100 mM. Brown pigment indicates the presence of hydrogen peroxide. The staining procedure is described in Materials and methods. The bar indicates 1 cm.

Figure 6. Effects of phenethyl ITC on the expression of stress-related genes. Transcript accumulation of each gene was measured by using the semiquantitative RT-PCR system described in Materials and methods. Expression level of 0 time control in each gene is standardized. A, *invertase/pectin methylesterase inhibitor family protein*; B, *chitinase class IV*; C, *peroxidase*; D, *phenylalanine ammonia-lyase 1*; E, *AtGSTF6*. Open and closed columns were control (water) and phenethyl ITC treatment (1 mM), respectively. Values and bars represent means ± S.D. of 3 plants. We did not judge significant difference, because the values in this experiment were produced by the semiquantitative method.

Figure 7. Effects of phenethyl ITC on glutathione *S*-transferase gene expression. Transcript accumulation of each gene was measured by using the semiquantitative RT-PCR system described in Materials and methods. Expression level of 0 time control in each gene is standardized. A, *AtGSTF6* (At1g02930); B, *AtGSTF7* (At1g02920); C, *AtGSTZ1* (At2g02390); D, *AtGSTU19* (At1g78380); and E, *AtGSTF2* (At4g02520). Open and closed circles represent control (water) and phenethyl ITC treatment (1 mM), respectively. Values and bars represent means ± S.D. of 3 plants. We did not judge significant difference, because the values in this experiment were produced by the semiquantitative method.

Figure 8. Effects of methyl ITC, allyl ITC, and phenethyl ITC on *AtGSTU19* expression. Transcript accumulation of *AtGSTU19* was measured by using the semiquantitative RT-PCR system described in Materials and methods. Expression level of 0 time control in each ITC is standardized. MITC, methyl ITC; AITC, allyl ITC; PEITC, phenethyl ITC. White, light gray, dark gray, and black columns represent control (water), 0.01 mM ITC treatment, 0.1 mM ITC treatment, and 1 mM ITC treatment, respectively. Values and bars represent means ± S.D. of 3 plants. We did not judge significant difference, because the values in this experiment were produced by the semiquantitative method.

Legends to figures in supplementary materials

Figure S1. Concentrations of ITCs in water emulsion sprayed by a hand-pump aerosol spray bottle. A water emulsion of ITC was sprayed into a 100-mL glass flask using the spray bottle. Immediately, ITC was extracted with chloroform and analyzed by gas chromatograph (GC 2010, Shimadzu, Kyoto, Japan) equipped with a capillary column (Shimadzu CBP1, 25 m, 0.32 mm I.D.). The concentrations of ITCs that were just combined with water were 100 mM (A), 10 mM (B), and 1 mM (C). MITC, methyl ITC; AITC, allyl ITC; PEITC, phenethyl ITC. Values and bars represent means and differences of double measurements.

Figure S2. Stability of ITC in water emulsion sprayed by a hand-pump aerosol spray bottle. A sprayed ITC emulsion was incubated in the same conditions under which *Arabidopsis* grew [a 16-h day (60 μmol m^{-2} s^{-1})/8-h night cycle at 22°C] for 24 h and 72 h. After incubation, ITC was extracted from the incubated emulsion with chloroform and analyzed by gas chromatograph (GC 2010, Shimadzu, Kyoto, Japan) equipped with a capillary column (Shimadzu CBP1, 25 m, 0.32 mm I.D.). The concentration of each ITC that was just combined with water was 100 mM. Values without incubation are standardized to 100%. MITC, methyl ITC; AITC, allyl ITC; PEITC, phenethyl ITC. Values and bars represent means and differences of double measurements.

Table S1. Information about RT-PCR experiments

gene	code	sense primer	antisense primer	AT (°C)	size (bp)
Invertase/pectin methylesterase inhibitor family protein	At3g47380	5'-CTATTCCTCTTATCCACGGC-3'	5'-ATCCGTGCCCTTACTGAGTT-3'	55	512
chitinase class IV	At3g54420	5'-CTCAACAACATCAAAATG-3'	5'-GACATGAGCAAAGAACGCTG-3'	55	414
peroxidase	At5g64120	5'-TTAAACACACACTTCATC-3'	5'-AATGACTGTGTCACGAGCGG-3'	55	465
phenylalanine ammonia-lyase 1	At2g37040	5'-ACGAGATTGGCGATAGCAGC-3'	5'-TAGGCTGCTCTTGCTGCTTCCA-3'	60	689
AtGSTF2	At4g02520	5'-TAGTAACCCAAATCAATGGC-3'	5'-ACTGTGACTGAAGCAAAACC-3'	50	696
AtGSTF3	At2g02930	5'-CTCGGTCTTCAAAGAGTTTC-3'	5'-CTGTGACTGAAGCAAAACCAC-3'	50	716
AtGSTF6	At1g02930	5'-CTCTCTACTTCAATAAATCTCCACC-3'	5'-TGGGCAATTAGGTCATCGCC-3'	50	750
AtGSTF7	At1g02920	5'-CAAAGTCTTAACAAATGGCAGG-3'	5'-TTAGGGCAATGAGGTCATCGCC-3'	50	704
AtGSTF8	At2g47730	5'-TGGAAAAATGGGAGCAATTC-3'	5'-AAGTGAAGAAAGAGAGAGGG-3'	50	822
AtGSTF9	At2g30860	5'-TCACTCAACAAAGCTTAACC-3'	5'-CACCAAGAAGATGAACACATC-3'	50	553
AtGSTF10	At2g30870	5'-TCGCGTCCTGCTATAGTGAG-3'	5'-CCCAAGTCAACATCTCCTTG-3'	50	732
AtGSTT1	At5g41210	5'-CAGTGATCCACAAAATCAGACG-3'	5'-AGTGACTGACCCCTGAAACCGG-3'	50	817
AtGSTZ1	At2g02390	5'-CTGCGAAGAACAACAAATTCC-3'	5'-AGTAGCTTATGGGTTCACAG-3'	50	718
AtGSTU5	At2g29450	5'-GAAAAAGAGAGAGAGACCCC-3'	5'-CCTCACTTCTCAACCACACAAC-3'	50	800
AtGSTU11	At1g69930	5'-GCTGTGAGTTTGATCAATCTC-3'	5'-AAGAACTTTTTGACTACGCC-3'	50	853
AtGSTU13	At1g27130	5'-CAGAAGAAGTCATGGCTCAG-3'	5'-ACGCACAAAGACCAAAAACC-3'	50	832
AtGSTU19	At1g78380	5'-TTTGTGAGCTTTAGCGATCG-3'	5'-GAGCATCTTAAGTCCGAACC-3'	50	725
AtGSTU20	At1g78370	5'-GTGAACTTCAGAGATCCTATAGC-3'	5'-AGCAACAAGAGAACAACAAG-3'	50	708
AtGSTU26	At1g17190	5'-CGATATATCAGAACTAAACCGG-3'	5'-GCCTTCTTGAAACCCAAACC-3'	50	720
AtGSTU27	At3g43800	5'-AAGGATGTCAGAAGAAGAAG-3'	5'-CACCAACTACTTCCTTAAGC-3'	50	768

Gene codes, primer sequences, annealing temperatures (AT), and sizes of the RT-PCR products for the constitutively spliced transcripts are shown. A cycle number of each PCR was 26.

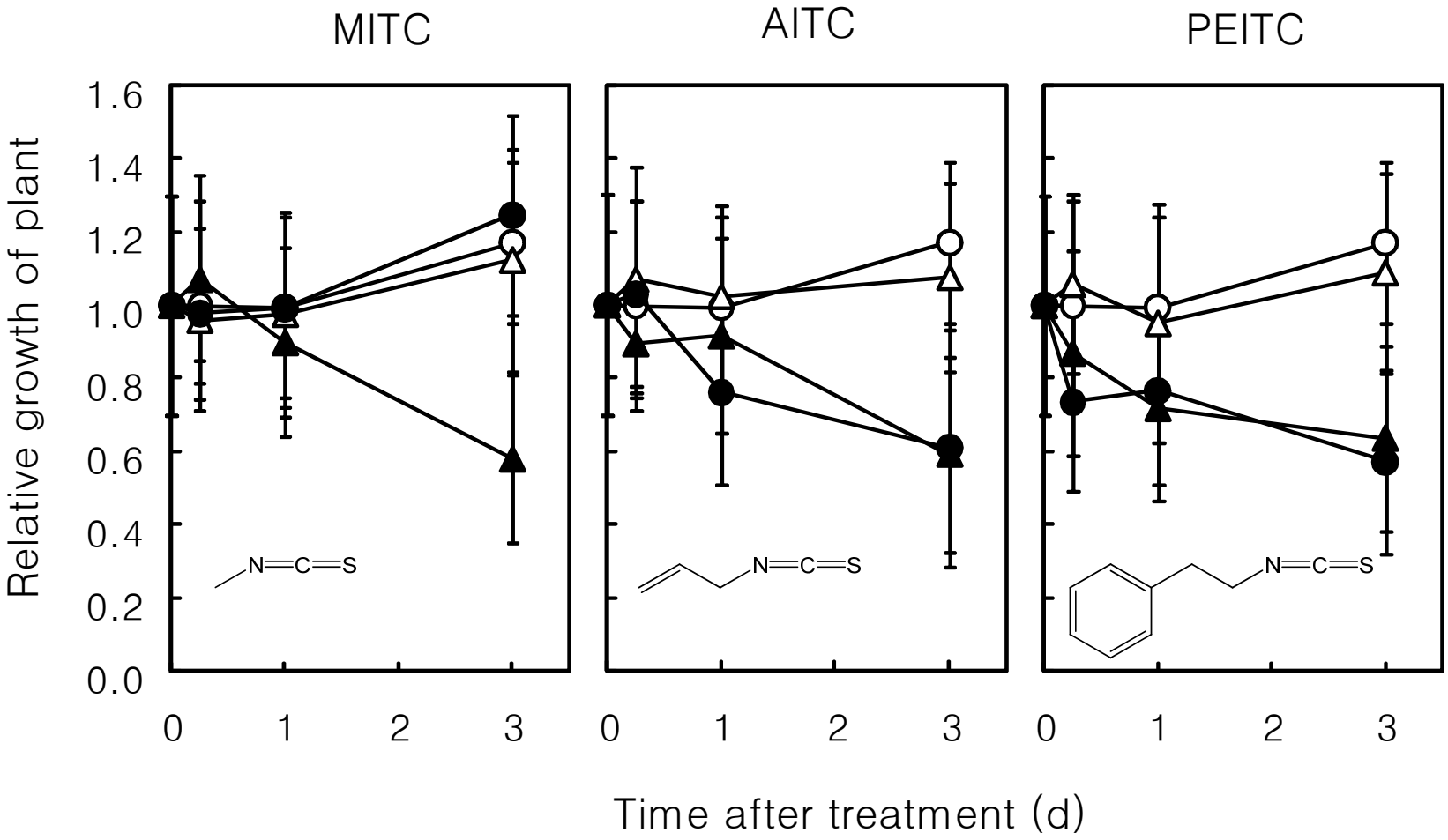
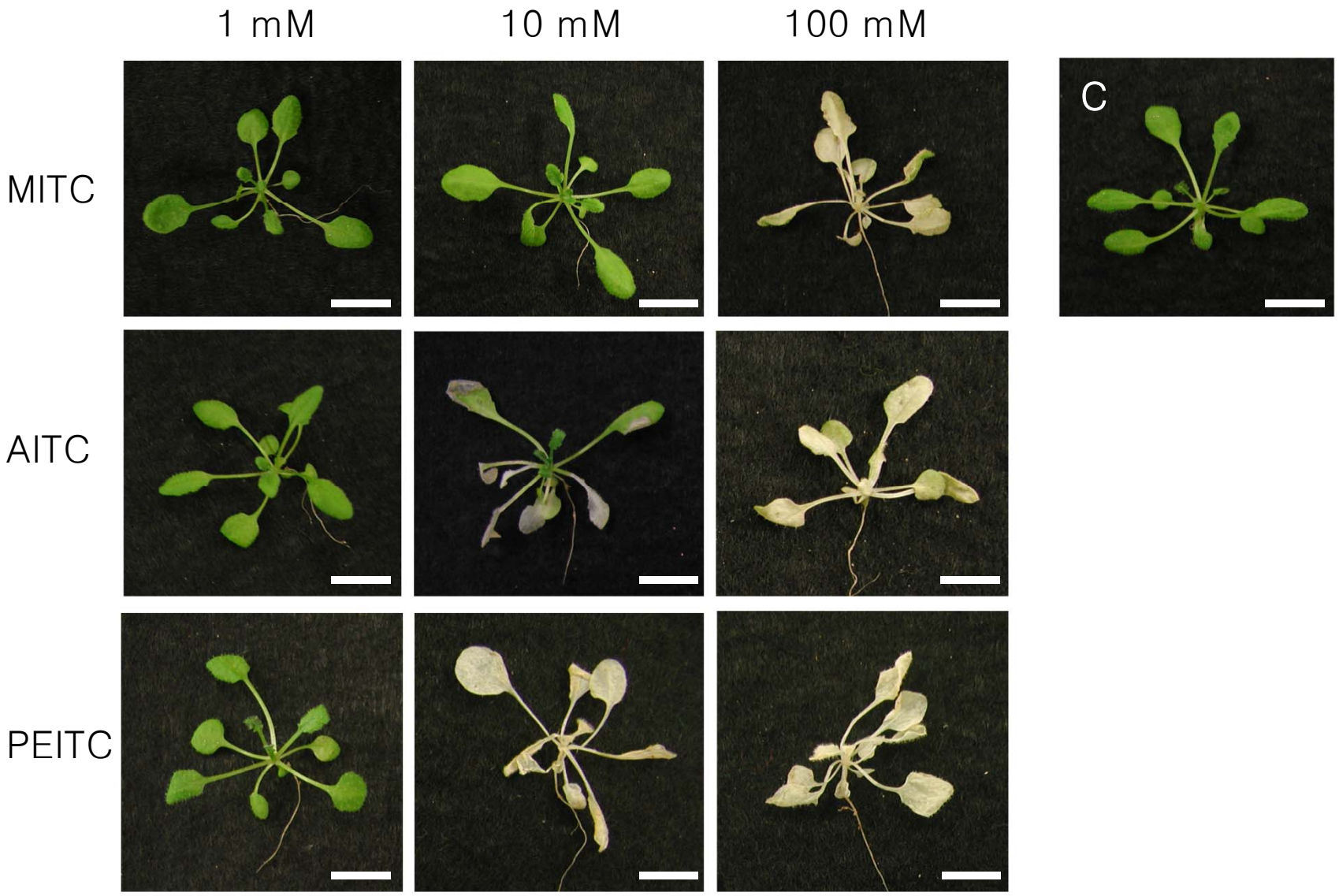


Fig. 1 Hara et al.



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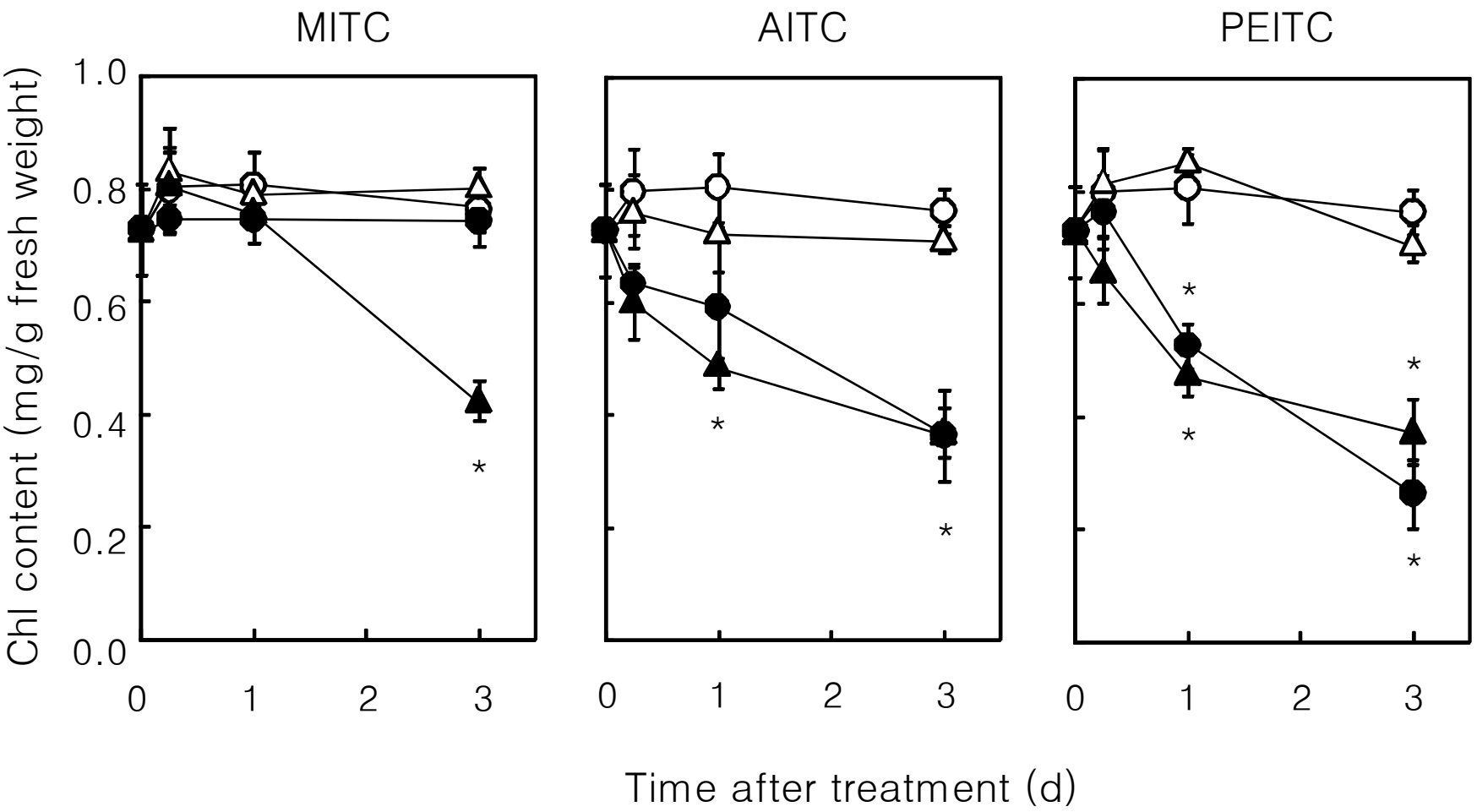

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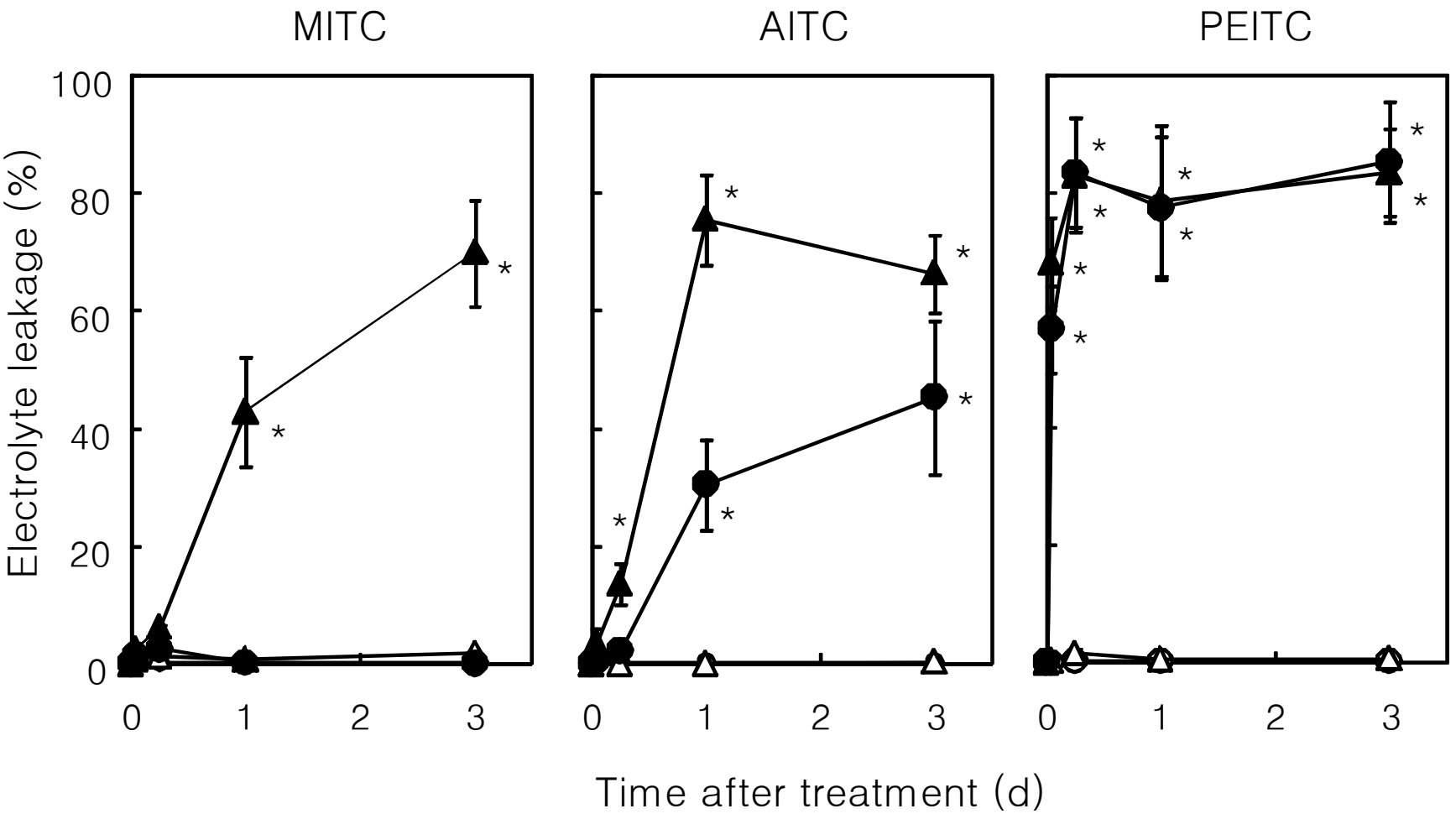

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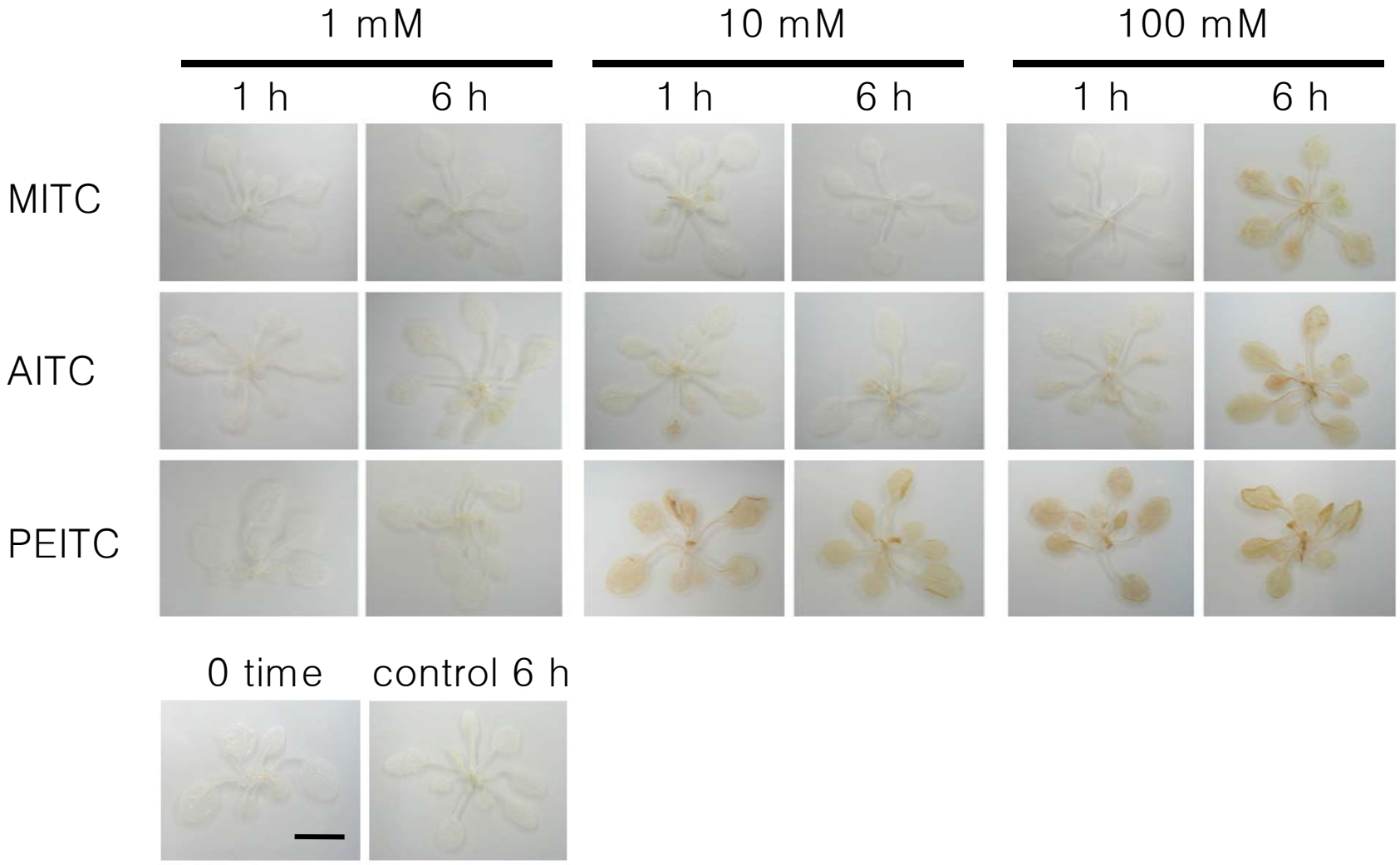

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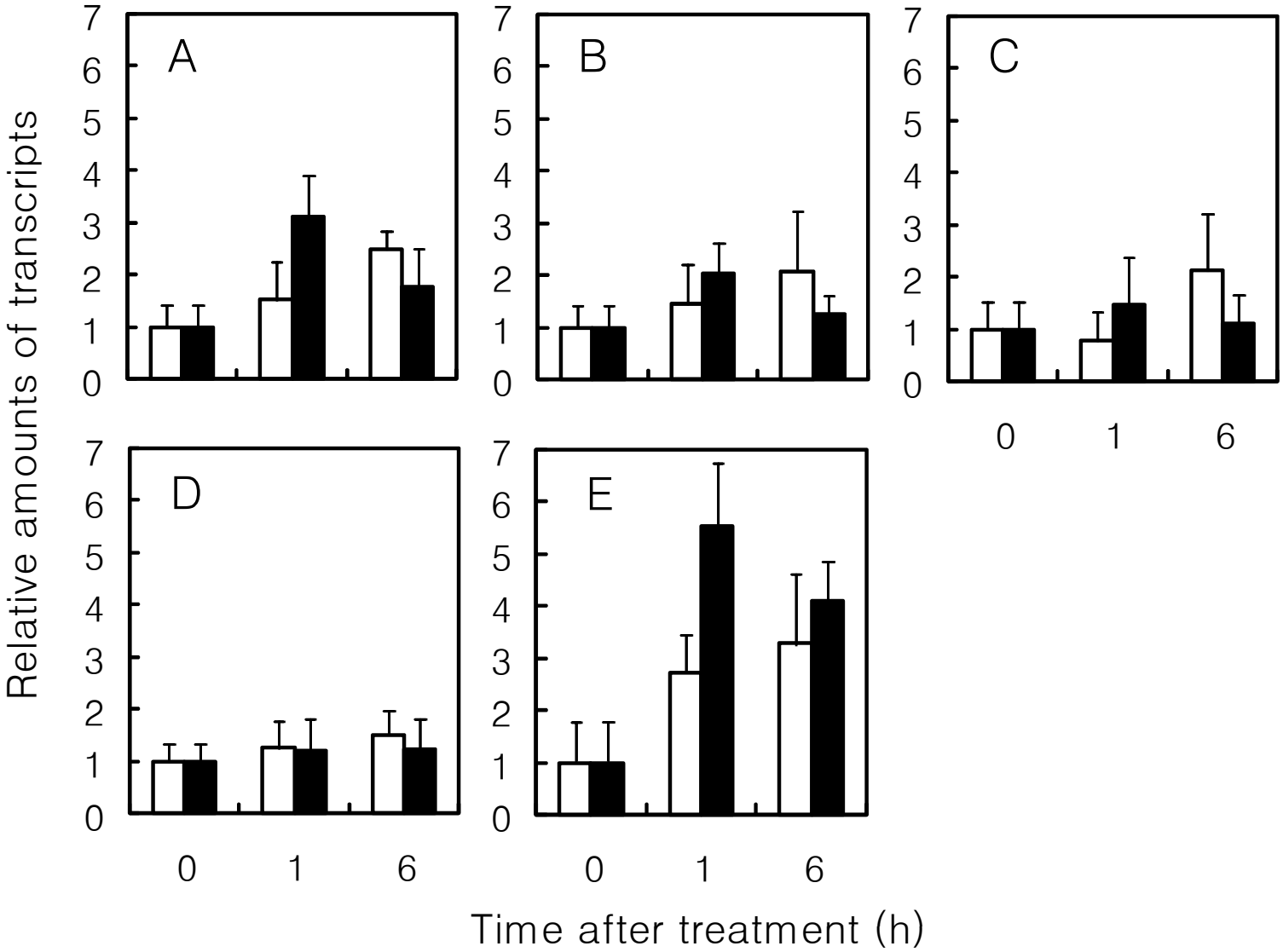

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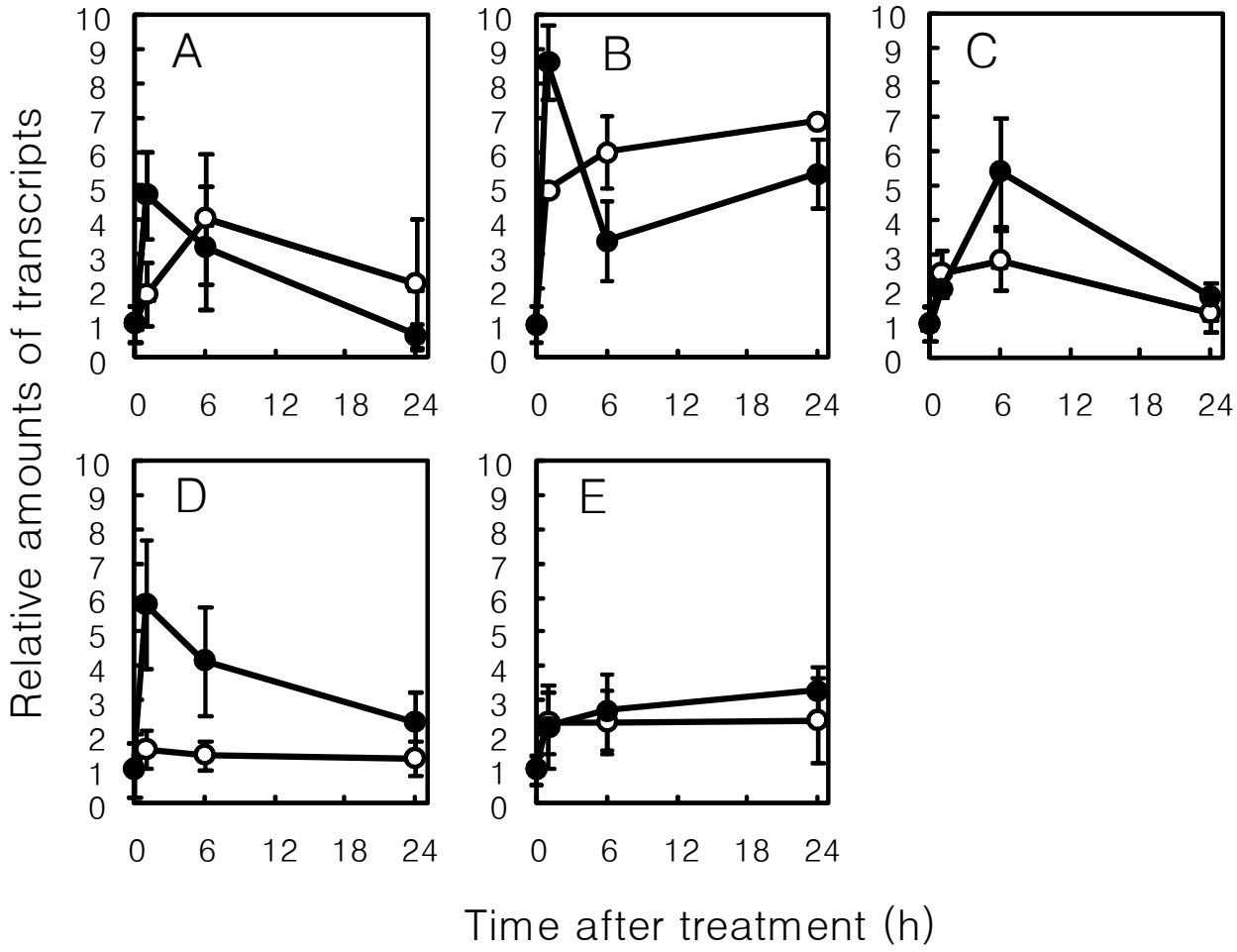

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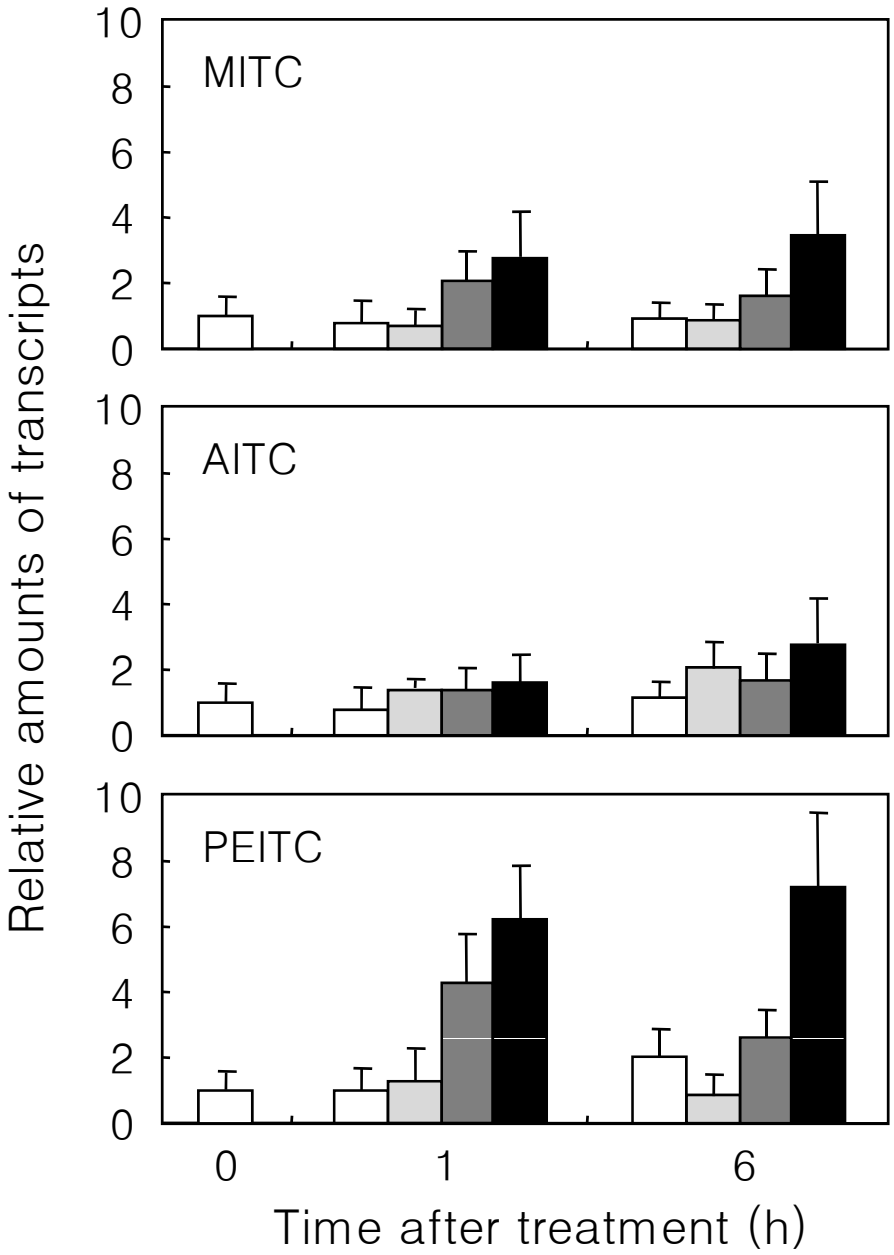

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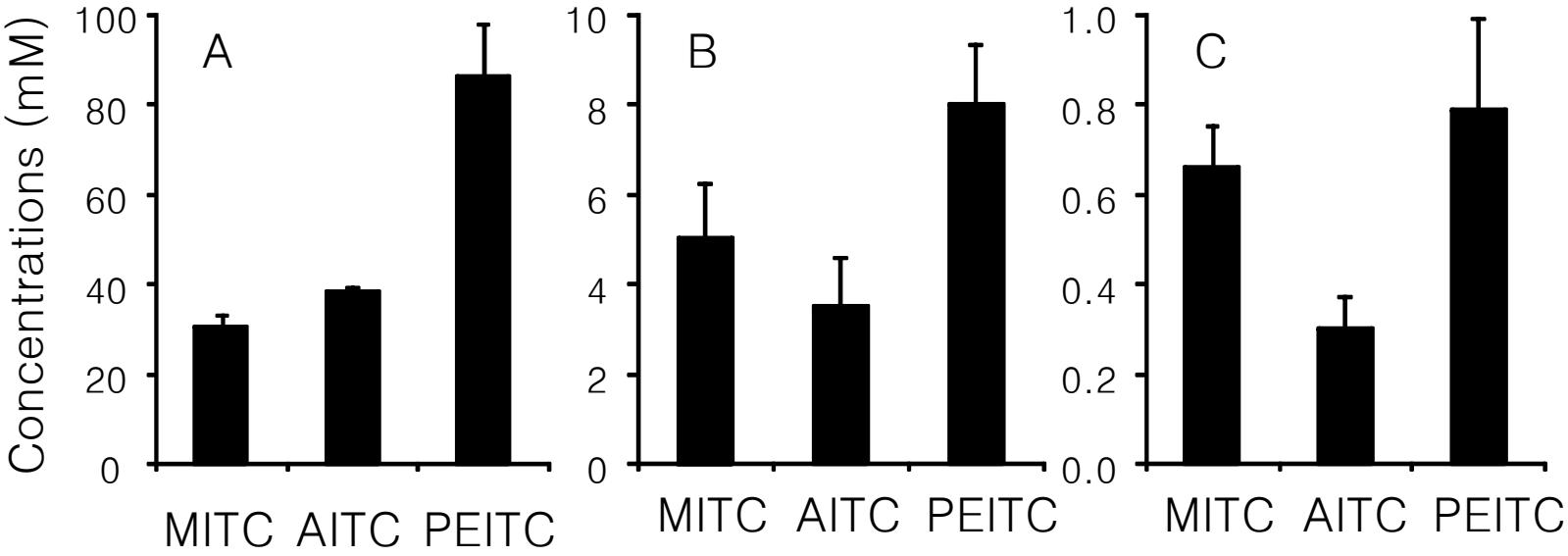
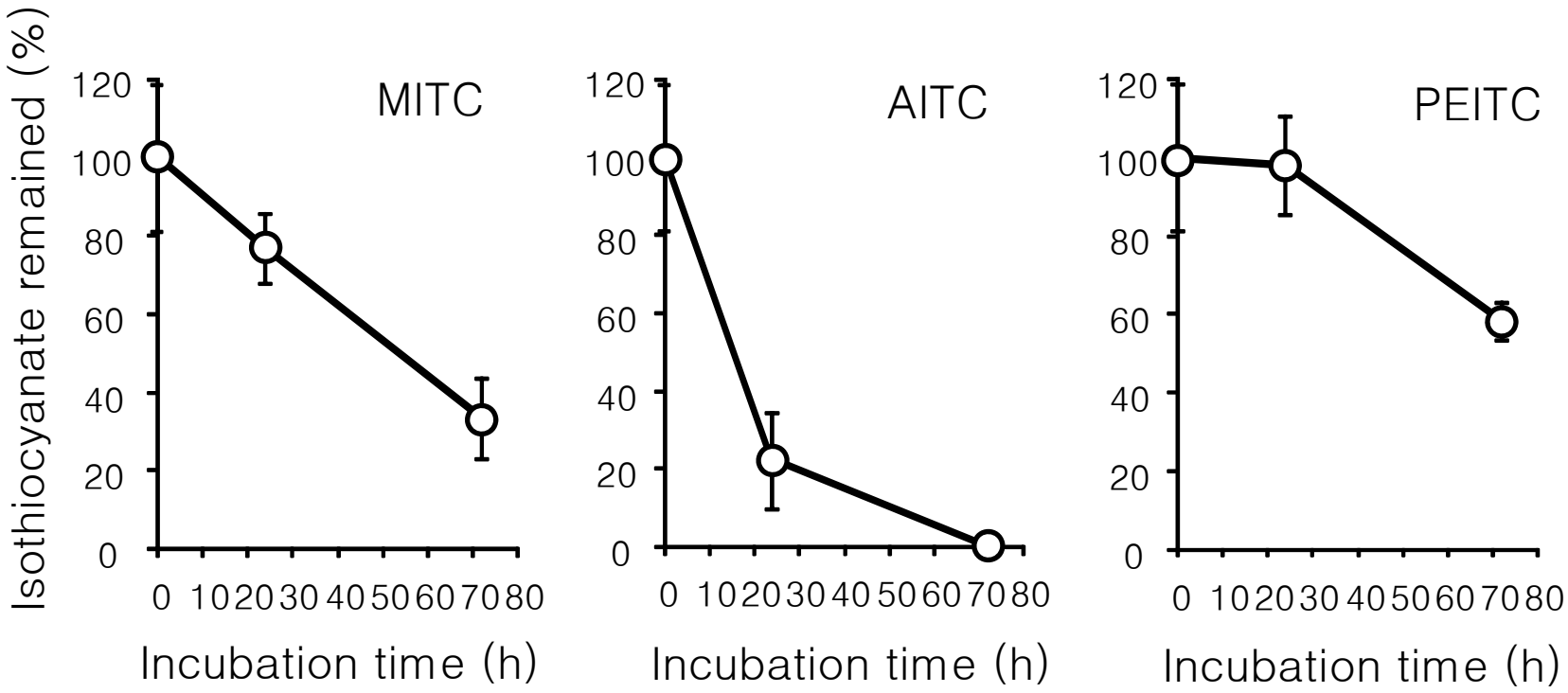


Fig. S1 Hara et al.



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