Induction of the heat shock response in Arabidopsis by heat shock protein 70 inhibitor VER-155008

Research paper

Title

Induction of the heat shock response in *Arabidopsis* by heat shock protein 70 inhibitor VER-155008.

Running head

Heat shock response induced by HSP70 inhibitor

Authors

Erina Matsuoka[1], Naoki Kato[2,3], Masakazu Hara[1,2]*

[1]Research Institute of Green Science and Technology,
Shizuoka University,
836 Ohya, Shizuoka, Shizuoka 422-8529, Japan

[2]Graduate School of Science and Technology,
Shizuoka University,
836 Ohya, Shizuoka, Shizuoka 422-8529, Japan

[3]R&D Center
Menicon Co., Ltd.
5-1-10 Takamoridai, Kasugai, Aichi 487-0032, Japan

***Name and address for editorial correspondence**

Masakazu Hara
Research Institute of Green Science and Technology,
Shizuoka University,
836 Ohya, Shizuoka 422-8529, Japan
Tel.: +81-54-238-5134
Fax: +81-54-238-5134
E-mail: hara.masakazu@shizuoka.ac.jp

Keywords: *Arabidopsis thaliana*, geldanamycin, heat shock protein 70 inhibitors, heat shock response, sanguinarine, VER-155008.

Abstract

A heat shock protein 90 (HSP90) inhibitor, geldanamycin, is a chemical inducer of the heat shock response (HSR) in *Arabidopsis*. Geldanamycin is thought to activate the heat shock signal by dissociating the HSP90-heat shock factor (HSF) complex. Recent studies have indicated that plant HSP70 is also associated with HSF, suggesting that inhibition of HSP70 may induce the HSR. However such study has not been carried out. Here, we found that a specific HSP70 inhibitor VER-155008 activated the promoter of a small HSP gene (*At1g53540, HSP17.6C-CI*) of *Arabidopsis* which was shown to be activated by geldanamycin and other HSP90 inhibitors. The production of HSP17.6C-CI, HSP70, and HSP90.1 proteins in *Arabidopsis* was enhanced by the addition of VER-155008. The reduction of chlorophyll contents by heat shock was ameliorated by VER-155008. Chaperone analyses indicated that VER-155008 inhibited the chaperone activities of wheat germ extract and human HSP70/HSP40, respectively. These results suggest that the inhibition of HSP70 by VER-155008 enhanced the heat tolerance of *Arabidopsis* by inducing the HSR in the plant.

Introduction

Extreme heat, which severely decreases crop production, is an important agricultural issue. Heat stress triggers diverse physiological changes in plants. The denaturation of proteins, the acceleration of membrane fluidity, the generation of reactive oxygen species, and the damage of photosynthesis are the major symptoms of plants that are exposed to extreme heat (Wahid *et al*. 2007; Waters 2013). To prevent severe damage due to heat, plants accumulate compatible solutes, activate antioxidative systems, and express heat shock proteins (HSPs) (Bita and Gerats 2013). HSPs reactivate heat-denatured proteins by assisting in their folding and preventing their aggregation.

HSPs are divided into five classes on the basis of molecular weight: HSP60s, HSP70s, HSP90s, HSP100s, and small HSPs. Many reports demonstrated that overexpression of HSP genes enhanced the heat tolerance of plants (Sung and Guy 2003; Wang *et al*. 2016; Masand and Yadav 2016), and mutants of HSP70 genes were hypersensitive to heat (Su and Li 2008; Leng *et al*. 2017), indicating that the expression of HSPs is related to the establishment of heat tolerance in plants.

Heat and various other stresses promote the expression of HSP genes in plants (Sørensen *et al*. 2003; Al-Whaibi 2011; Cottee *et al*. 2013). It has been reported that the heat shock response (HSR) in plants is efficiently induced by some organic compounds, including salicylic acid (Dat *et al*. 1998), benzyl alcohol (Saidi *et al*. 2005), celastrol (Saidi *et al*. 2007), geldanamycin (Yamada *et al*. 2007), isothiocyanates (Hara *et al*. 2013), sanguinarine (Hara and Kurita 2014), (*E*)-2-hexenal (Yamauchi *et al*. 2015), and monoterpenes (Hara *et al*. 2018). Among these compounds, geldanamycin has the best characterized HSR induction mechanism. Geldanamycin is an ansamycin-type heat shock protein 90 (HSP90) inhibitor produced by *Streptomyces hygroscopicus*. In plants without stress, HSP90 is thought to suppress heat shock factors (HSFs, the transcription factors of HSP genes). Geldanamycin specifically inhibits HSP90 activity and can activate HSFs by releasing them from the HSP90-HSF complex. Accordingly, geldanamycin induced the HSR in plants not treated with heat (Yamada *et al*. 2007). Other HSP90 inhibitors, such as macrocyclic types (e.g., radicicol and monocillin I), benzamide types (e.g., PF-04929113 and SNX-2112), purine types (e.g., BIIB021 and PU-H71), resorcinol types (e.g., AT13387, AUY922, and Ganetespib), all induced the HSR of *Arabidopsis* (McLellan *et al*. 2007; Yoshida *et al*. 2011; Murano *et al*. 2017), suggesting that the inhibition of HSP90 triggers HSR induction in plants.

Recently, it has been documented that in unstressed plants HSFs are sequestered by not only HSP90 but also HSP70 (Jacob *et al*. 2017). This supports the concept that the inhibition of HSP70 may induce the HSR in plants. However, little is known about whether HSP70 inhibitors can induce the HSR in organisms. Here, we report the HSR-inducing activities of an HSP70 inhibitor, VER-155008, in *Arabidopsis* seedlings. VER-155008 inhibited the chaperone activities of not only human HSP70/HSP40 but also of wheat germ extract (WGE). The heat tolerance of *Arabidopsis* was enhanced by the administration of VER-155008. We also discuss the mechanism underlying the

HSR-inducing activity of the chaperone inhibitors in plants.

Materials and methods

Chemicals

VER-155008 and pifithrin-μ were purchased from Cayman Chemical (Ann Arbor, MI, USA) and R&D Systems (Minneapolis, MN, USA), respectively. YM-08, 116-9e, and sanguinarine were obtained from Sigma (Tokyo, Japan). Geldanamycin was from LC Laboratories (Woburn, MA, 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 purchased from Wako (Tokyo, Japan).

Reporter gene assay

The gene construct, which was made by inserting the 1-kb promoter region of the *HSP17.6C-CI* (*At1g53540*) gene into the 5' end of the β-glucuronidase (GUS) gene, was introduced to *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0). The whole-plant reaction system (Weigel & Glazebrook 2002) was applied, with slight modifications, to determine the GUS activity (Murano *et al*. 2017).

The transgenic plants were sown on a 1/5 MS agar medium containing 1% sucrose in 6-well plates (TPP #92406, Trasadingen, Switzerland) under sterile conditions. The plants were grown in a chamber (NK Systems, Tokyo, Japan), conditioned at 22 ºC with a 16-h day (60 μmol m^{-2} s^{-1})/8-h night cycle. The seedlings at 6 days after germination (DAG) were treated with the solutions [5% dimethyl sulfoxide (DMSO) in water (v/v) containing VER-155008 (0.005-1000 μM), YM-08 (0.005-500 μM), pifithrin-μ (0.005-500 μM), 116-9e (0.005-500 μM), geldanamycin (50 μM), and sanguinarine (5 μM)] in 1.5-mL microtubes (two seedlings per tube). The control treatment was 5% DMSO. After the tubes were incubated at 22 ºC for 6 h under illumination as described above, the seedlings were rinsed with water and then treated with the GUS assay solution [200 μL, 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] in a 96-well microplate (Iwaki,

Funabashi, Japan) for 16 h at 22 ºC in the dark. The incubation condition was set in the previous report (Murano *et al*. 2017). The reaction was terminated by adding 100 μL of 1-M Na_2CO_3. The enhanced fluorescence of the product (4-MU) was measured at excitation (365 nm) and at emission (455 nm) (Varioskan Flash, Thermo Fisher Scientific, Yokohama, Japan). The concentration of 4-MU was determined by the calibration curve of authentic 4-MU.

GUS tissue staining

Seedlings of transgenic *Arabidopsis* were subjected to GUS staining (Weigel and Glazebrook 2002). The 7 DAG seedlings were treated with VER-155008 (50 μM), YM-08 (50 μM), pifithrin-μ (50 μM), and 116-9e (50 μM) in 5% (v/v) DMSO aqueous solution for 6 h. Heat shock (37 ºC for 1 h and then 22 ºC for 5 h) was applied. The control was 5% DMSO aqueous solution. Seedlings were destained with acetone. GUS activity was detected with X-Gluc.

Immunoblot analysis

Test solution [50-μM VER-155008 in 5% DMSO (v/v)] was prepared in 5 ml test tubes. *Arabidopsis* seedlings (6 DAG) were immersed in the solution for 1, 6, 24, and 48 h under illumination (60 μmol m^{-2} s^{-1}) at 22 ºC. Control was 5% DMSO. Heat shock was applied at 37 ºC for 1 h, and then the heat-shocked samples were incubated at 22 ºC under illumination. The sampling periods were 1, 6, 24, and 48 h after the start of heat shock. In all cases, zero-time samples were taken. The seedlings (25 mg fresh weight) were homogenized with 250 μL of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The homogenate was centrifuged at 10,000 *g* for 10 min at 4ºC, and then the supernatant was heated at 95ºC for 5 min. The proteins (10 μg each) separated by SDS-PAGE were blotted with primary antibodies, i.e., anti-GUS (Sigma), anti-HSP17.6C-CI (Agrisera, Vännäs, Sweden), anti-HSP70 (Agrisera), and anti-HSP90.1 (Agrisera) antibodies (dilution 1/5000). A polyvinylidene difluoride membrane filter (Immun-Blot; Bio-Rad, Tokyo, Japan) and Mini Trans-Blot Cell (Bio-Rad) were used. Horseradish peroxidase conjugated anti-rabbit IgG (Bio-Rad)

was the secondary antibody (dilution 1/5000). Chemiluminescence generated by the chemiluminescent Clarity Western ECL (Bio-Rad) was detected with the LAS-4000 imaging system (Fujifilm, Tokyo, Japan).

Heat tolerance

Heat tolerance was assessed according to the previous method (Murano *et al*. 2017). Briefly, seedlings of wild-type *Arabidopsis* were grown on a filter paper disk placed on 1/5 MS medium containing 0.8% agar and 1% sucrose in 6-well plates at 22 ^{o}C under a 16 h/day photoperiod of 60 μmol m^{-2} s^{-1}. At 6 DAG, the filter paper disk was transferred to new medium containing VER-155008 (0, 0.5, 5, 50, and 200 μM) and DMSO [5% (v/v)] in 6-well plates. After the plates were incubated as described above for 24 h, each plate which was sealed individually in a polyethylene zipper bag was incubated in water at 46 ^{o}C for 1 h. The control was no treatment. The plates were placed for 3 days under the above conditions, and then the fresh weight and chlorophyll contents were determined (Lichtenthaler and Buschmann 2001).

Inhibition of plant and human chaperones

WGE (Promega, Tokyo, Japan) was used for the plant chaperone assay (Murano *et al*. 2017). Recombinant luciferase (QuantiLum; Promega, Tokyo, Japan), which was diluted to 0.3 μg ml^{-1} with CCLR buffer (Promega), was heat denatured at 40 ^{o}C for 5 min. The WGE solution (45 μl, double-diluted with distilled water) was combined to various concentrations of VER-155008 (0.5 μl in DMSO) and then incubated for 30 min at 25 ^{o}C. The renaturation reaction was initiated by the addition of the denatured luciferase (5 μl). At this step, the VER-155008 concentrations ranged from 0.01 to 200 μM. The LUC assay system (Promega) was applied to detect the luciferase activities (LAS-4000, Fujifilm, Tokyo, Japan). The renaturation activities were determined from the initial velocities in the reaction for 20 min at 25 ^{o}C. The activity without VER-155008 was standardized to 100%.

Inhibition of human HSP70 was monitored by using an HSP70/HSP40 Glow-Fold Protein Refolding Kit (R&D Systems). The procedure followed the manufacturer's

instructions. Each test solution (VER-155008, geldanamycin, and sanguinarine in DMSO, 0.5 μl) was added to the HSP70/HSP40 reaction mix (4.5 μl) containing luciferase. Here, the concentrations of the test compounds ranged from 0.1 to 200 μM. The samples were incubated for 30 min at 25 ºC and then for 7 min at 45 ºC. After the samples were cooled to 25 ºC, the luciferase activity was determined by using the LUC assay system. The renaturation activities were determined as described above.

Statistical analysis

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

Results

HSR of Arabidopsis *induced by VER-155008*

To assess the HSR-inducing activities of HSP70 inhibitors, we used the transgenic *Arabidopsis* in which the *β-glucuronidase* (*GUS*) gene was controlled by the promoter of a small HSP gene (*HSP17.6C-CI*, *At1g53540*) of *Arabidopsis.* The construct is shown in Figure S1. It has been reported that the expression of the *HSP17.6C-CI* gene in *Arabidopsis* was upregulated by chemical HSR inducers such as geldanamycin, sanguinarine, PU-H71, and perillaldehyde (Matsuoka *et al*. 2016; Murano *et al*. 2017; Hara *et al*. 2018). We used four commercially available HSP70 inhibitors (VER-155008, YM-08, pifithrin-μ, and 116-9e) that belong to different structural groups (Fig. S2).

The promoter analysis indicated that VER-155008 induced the HSR at concentrations from 50 to 1000 μM (Fig. 1). Activity peaked with the addition of 500 μM VER-155008. This peak value occurred between the activities of geldanamycin (50 μM) and sanguinarine (5 μM), which were used as positive controls. However, YM-08, pifithrin-μ, and 116-9e did not induce the HSR in this experimental system. Blue GUS staining occurred at cotyledons, true leaves, and roots of the transgenic seedlings when VER-155008 was applied at 50 μM (Fig. 2). The staining did not appear by the treatments with the other HSP70 inhibitors. Heat potently induced GUS expression in

the seedlings but not in the controls.

The levels of GUS protein in the transgenic seedlings and three HSPs (HSP17.6C-CI, HSP70, and HSP90.1) in the wild-type seedlings were monitored for 48 h after the application of VER-155008 (Fig. 3). VER-155008 enhanced GUS protein production in the transgenic seedlings. The accumulation of GUS protein was initiated at 6 h after the application and peaked at 24 h. When VER-155008 was administered to the wild-type seedlings, the levels of HSP17.6C-CI, HSP70, and HSP90.1 proteins were slightly upregulated during the incubation periods. Heat remarkably induced the accumulation of corresponding proteins within 1 h after treatment. The controls did not influence the protein level in any cases.

Effect of VER-155008 on heat tolerance of Arabidopsis

Since VER-155008 promoted the accumulation of HSPs in *Arabidopsis*, we investigated the heat tolerance of the VER-155008-treated seedlings (Fig. 4). At 6 DAG, seedlings were treated with different concentrations of VER-155008. After 24 h, the seedlings were subjected to 46 ^{o}C heat for 1 h. The seedlings were weighed and the chlorophyll contents were determined at 10 DAG. The fresh weight and chlorophyll contents of the seedlings with no VER-155008 treatment were remarkably reduced after the heat shock (Fig. 4B, C). However, VER-155008 treatment partially inhibited the reduction of chlorophyll contents due to heat at the concentrations of 50 and 200 μM (Fig. 4C), whereas VER-155008 did not affect the reduction in fresh weight and relative water content by heat (Fig. 4B, Fig. S3). Photographs indicate that the seedlings treated with VER-155008 were healthier than the control seedlings (Fig. S4).

Inhibition of chaperones by VER-155008

In this study, VER-155008 was used as the HSP70 inhibitor. However, there is no report that VER-155008 indeed inhibits chaperone activity in the plant system. Thus we measured the chaperone-inhibition activity of VER-155008 by using the wheat germ extract (WGE) system as well as the human HSP70/HSP40 system (Fig. 5). The result was that VER-155008 apparently inhibited the chaperone activities of the WGE system

(Fig. 5A, IC_{50} = 104.3±35.0 μM) and of the human HSP70/HSP40 system (Fig. 5B, IC_{50} = 83.6±31.5 μM). In addition, we tested geldanamycin and sanguinarine, which were used as positive controls in the promoter-GUS assay (Fig. 1) for inhibition of the human HSP70/HSP40 system. It was reported that geldanamycin and sanguinarine inhibited plant chaperones by using the WGE system (Matsuoka *et al*. 2016). Intriguingly, sanguinarine efficiently inhibited the human HSP70/HSP40 system (Fig. 5D, IC_{50} = 23.0±7.8 μM), whereas geldanamycin did not (Fig. 5C, IC_{50} > 200 μM), suggesting that sanguinarine may inhibit plant chaperones at least partially due to interference with the HSP70 system.

Discussion

Geldanamycin has been used to induce the HSR in *Arabidopsis*. The induction mechanism was postulated as follows (Yamada *et al*. 2007). In nonstressed cells, HSFs are kept inactive by binding to HSP90. Geldanamycin facilitates the release of HSFs from the HSF-HSP90 complex by inhibiting HSP90 and activates the HSFs. Thus the HSP genes are efficiently expressed. Since many natural and synthetic HSP90 inhibitors induced the HSR in *Arabidopsis* (McLellan *et al*. 2007; Yoshida *et al*. 2011; Murano *et al*. 2017), the inhibition of HSP90 is likely one of the cues for inducing the HSR in plants.

Besides that, it has been reported that HSFs were associated with HSP70 in tomato (Hahn *et al*. 2011) and *Arabidopsis* (Ohama *et al*. 2016), suggesting that in nonstressed plants HSP70 inactivates HSFs by holding them (Jacob *et al*. 2017). If so, the inhibition of HSP70 may facilitate the disassociation of the interaction between HSP70 and HSFs and the activation of the HSFs. This study first demonstrated that an HSP70 inhibitor, VER-155008, induced the HSR by using the plant system.

VER-155008 was designed to specifically bind to the ATP-binding pocket located in the nucleotide-binding domain of human HSP70 (Massey 2010). VER-155008 has been used to inhibit the HSP70 activity of *Arabidopsis* in studies of plant hormones (Merret *et al*. 2015; Zhang *et al*. 2015). It was noted that the amino acid sequences of *Arabidopsis* HSP70s were highly similar to those of human proteins, and the amino acids involved in VER-155008 binding showed perfect conservation between human

and *Arabidopsis* HSP70s (Merret *et al*. 2015). Actually, we showed that VER-155008 inhibited the chaperone activity of the WGE which contained HSP70s (Fig. 5A). The IC_{50} for the WGE system (IC_{50} of 104 μM) was similar to that for the human HSP70/HSP40 system (IC_{50} of 84 μM). These results suggest that VER-155008 can inhibit plant HSP70 under physiological conditions.

On the other hand, neither YM-08, 116-9e, nor pifithrin-μ showed HSR-inducing activities in *Arabidopsis* seedlings. YM-08 is an analog of MKT-077, a rhodocyanine-type HSP70 inhibitor. This type of inhibitor is known to bind to the allosteric site of HSP70. 116-9e is a derivative of dihydropyrimidines, which are also allosteric modulators of HSP70. Pifithrin-μ, also called phenylethylsulfonamide, inhibits HSP70 by interfering with the association between the co-chaperone and HSP70 at the substrate-binding domain. Taken together the past and present findings indicate that, since only VER-155008 induced the HSR in *Arabidopsis*, it is likely that direct competition for the ATP-binding site of HSP70 can promote the efficient induction of the HSR in plants.

A putative mechanism for the HSR-inducing activities of VER-155008 in *Arabidopsis* is shown in Fig. 6. HSP70 holds HSF and suppresses its transcriptional activity (Fig. 6a). VER-155008 inhibits HSP70 and may release the active HSF (Fig. 6b). Thus the transcription of HSP genes was promoted and the heat tolerance of *Arabidopsis* was enhanced (Fig. 6c). Besides that, the chaperone activity of HSP70, i.e., restoring denatured proteins to their native state, can also be inhibited by VER-155008. This means that VER-155008 may transiently accumulate denatured proteins (Fig. 6d). It is likely that the denatured proteins also release the active HSF from HSP70 and HSP90, because these HSP families access the denatured proteins. Sanguinarine is a natural alkaloid that showed HSR-inducing activity in *Arabidopsis*. The alkaloid efficiently inhibited the chaperone activities of both systems, i.e., human HSP70/HSP40 (Fig. 5) and WGE (Matsuoka *et al*. 2016), suggesting that sanguinarine may act by mechanisms similar to those of VER-155008 as postulated in Fig. 6. Geldanamycin induces the HSR by inhibiting HSP90 (Yamada *et al*. 2007). Conclusively, VER-155008, sanguinarine, and geldanamycin may induce the HSR by the chaperone-inhibition mode, whereas the target chaperones are different.

Heat waves are significant environmental threats that apparently reduce primary

production (Teskey *et al*. 2015). Because the induction of HSPs is a common response for surviving under high temperature in many plant species, compounds that promote the HSR can be promising agrochemicals to prevent the reduction of crop production. We call them heat tolerance enhancers. Since inhibitors of HSP70 and HSP90 induced the HSR in plants, HSP70 and HSP90 may be endogenous modulators of heat tolerance. This indicates that new heat tolerance enhancers can be found by searching for compounds that inhibit molecular chaperones of plants.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

This research did not receive any specific funding.

References

Al-Whaibi MH (2011) Plant heat-shock proteins: A mini review. *Journal of King Saud University - Science* **23**, 139-150.

Bita CE, Gerats T (2013) Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. *Frontiers in Plant Science* **4**, 273.

Cottee NS, Wilson IW, Tan DKY, Bange MP (2013) Understanding the molecular events underpinning cultivar differences in the physiological performance and heat tolerance of cotton (*Gossypium hirsutum*). *Functional Plant Biology* **41**, 56-67.

Dat JF, Lopez-Delgado H, Foyer CH, Scott IM (1998) Parallel changes in H_2O_2 and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard

seedlings. *Plant Physiology* **116**, 1351-1357.

Hahn A, Bublak D, Schleiff E, Scharf KD (2011) Crosstalk between Hsp90 and Hsp70 chaperones and heat stress transcription factors in tomato. *The Plant Cell* **23**, 741-755.

Hara M, Harazaki A, Tabata K (2013) Administration of isothiocyanates enhances heat tolerance in *Arabidopsis thaliana*. *Plant Growth Regulation* **69**, 71-77.

Hara M, Kurita I (2014) The natural alkaloid sanguinarine promotes the expression of heat shock protein genes in *Arabidopsis*. *Acta Physiologiae Plantarum* **36**, 3337-3343.

Hara M, Yamauchi N, Sumita Y (2018) Monoterpenes induce the heat shock response in *Arabidopsis*. *Zeitschrift für Naturforschung C* **73**, 177-184.

Jacob P, Hirt H, Bendahmane A (2017) The heat-shock protein/chaperone network and multiple stress resistance. *Plant Biotechnology Journal* **15**, 405-414.

Leng L, Liang Q, Jiang J, Zhang C, Hao Y, Wang X, Su W (2017) A subclass of HSP70s regulate development and abiotic stress responses in *Arabidopsis thaliana*. *Journal of Plant Research* **130**, 349-363.

Lichtenthaler HK, Buschmann C (2001) Chlorophylls and carotenoids - measurement and characterization by UV-VIS. *Current Protocols in Food Analytical Chemistry (CPFA)*, (Supplement 1), F4.3.1-F4.3.8. John Wiley, New York.

Masand S, Yadav SK (2016) Overexpression of MuHSP70 gene from *Macrotyloma uniflorum* confers multiple abiotic stress tolerance in transgenic *Arabidopsis thaliana*. *Molecular Biology Reports* **43**, 53-64.

Massey AJ (2010) ATPases as drug targets: insights from heat shock proteins 70 and 90. *Journal of Medicinal Chemistry* **53**, 7280-7286.

Matsuoka E, Matsubara T, Takahashi I, Murano H, Hara M (2016) The isoquinoline alkaloid sanguinarine which inhibits chaperone activity enhances the production of heat shock proteins in *Arabidopsis*. *Plant Biotechnology* **33**, 409-413.

McLellan CA, Turbyville TJ, Wijeratne EM, Kerschen A, Vierling E, Queitsch C, Whitesell L, Gunatilaka AA (2007) A rhizosphere fungus enhances *Arabidopsis* thermotolerance through production of an HSP90 inhibitor. *Plant Physiology* **145**, 174-182.

Merret R, Nagarajan VK, Carpentier MC, Park S, Favory JJ, Descombin J, Picart C, Charng YY, Green PJ, Deragon JM, Bousquet-Antonelli C (2015) Heat-induced ribosome pausing triggers mRNA co-translational decay in *Arabidopsis thaliana*. *Nucleic Acids Research* **43**, 4121-4132.

Murano H, Matsubara T, Takahashi I, Hara M (2017) A purine-type heat shock protein 90 inhibitor promotes the heat shock response in *Arabidopsis*. *Plant Biotechnology Reports* **11**, 107-113.

Ohama N, Kusakabe K, Mizoi J, Zhao H, Kidokoro S, Koizumi S, Takahashi F, Ishida T, Yanagisawa S, Shinozaki K, Yamaguchi-Shinozaki K (2016) The Transcriptional cascade in the heat stress response of *Arabidopsis* is strictly regulated at the level of transcription factor expression. *The Plant Cell* **28**, 181-201.

Saidi Y, Domini M, Choy F, Zryd JP, Schwitzguebel JP, Goloubinoff P (2007) Activation of the heat shock response in plants by chlorophenols: transgenic *Physcomitrella patens* as a sensitive biosensor for organic pollutants. *Plant, Cell & Environment* **30**, 753-763.

Saidi Y, Finka A, Chakhporanian M, Zrÿd JP, Schaefer DG, Goloubinoff P (2005) Controlled expression of recombinant proteins in *Physcomitrella patens* by a conditional heat-shock promoter: a tool for plant research and biotechnology. *Plant Molecular Biology* **59**, 697-711.

Sørensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role of heat shock proteins. *Ecology Letters* **6**, 1025-1037.

Su PH, Li HM (2008) *Arabidopsis* stromal 70-kD heat shock proteins are essential for plant development and important for thermotolerance of germinating seeds. *Plant Physiology* **146**, 1231-1241.

Sung DY, Guy CL (2003) Physiological and molecular assessment of altered expression of Hsc70-1 in *Arabidopsis*. Evidence for pleiotropic consequences. *Plant Physiology* **132**, 979-987.

Teskey R, Wertin T, Bauweraerts I, Ameye M, McGuire MA, Steppe K (2015) Responses of tree species to heat waves and extreme heat events. *Plant, Cell & Environment* **38**, 1699-1712.

Wahid A, Gelani S, Ashraf M, Foolad MR (2007) Heat tolerance in plants: An overview. *Environmental and Experimental Botany* **61**, 199-223.

Wang X, Yan B, Shi M, Zhou W, Zekria D, Wang H, Kai G (2016) Overexpression of a *Brassica campestris* HSP70 in tobacco confers enhanced tolerance to heat stress. *Protoplasma* **253**, 637-645.

Waters ER (2013) The evolution, function, structure, and expression of the plant sHSPs. *Journal of Experimental Botany* **64**, 391-403.

Weigel D, Glazebrook J (2002) *Arabidopsis: A laboratory manual*. Cold Spring Harbor Laboratory Press, New York

Yamada K, Fukao Y, Hayashi M, Fukazawa M, Suzuki I, Nishimura M (2007) Cytosolic HSP90 regulates the heat shock response that is responsible for heat acclimation in *Arabidopsis thaliana*. *Journal of Biological Chemistry* **282**, 37794-37804.

Yamauchi Y, Kunishima M, Mizutani M, Sugimoto Y (2015) Reactive short-chain leaf volatiles act as powerful inducers of abiotic stress-related gene expression. *Scientific Reports* **26**, 8030.

Yoshida T, Ohama N, Nakajima J, Kidokoro S, Mizoi J, Nakashima K, Maruyama K, Kim JM, Seki M, Todaka D, Osakabe Y, Sakuma Y, Schöffl F, Shinozaki K, Yamaguchi-Shinozaki K (2011) *Arabidopsis* HsfA1 transcription factors function as the main positive regulators in heat shock-responsive gene expression. *Molecular Genetics and Genomics* **286**, 321-332.

Zhang XC, Millet YA, Cheng Z, Bush J, Ausubel FM (2015) Jasmonate signalling in *Arabidopsis* involves SGT1b-HSP70-HSP90 chaperone complexes. *Nature Plants* **1**, 15049.

Figure legends

Fig. 1. GUS reporter assay by using transgenic plants possessing the GUS gene whose expression was controlled by the *HSP17.6C-CI* promoter. Main graph shows the effects of chemicals on GUS activity in transgenic plants. Control (white bar), HSP70 inhibitors (gray bars), geldanamycin (dark gray bar), and sanguinarine (black bar). The structures of the chemicals are shown in Figure S2. The inset indicates the effects of VER-155008 (from 5 to 1000 μM) on GUS activity. Control (white bar) and VER-155008 (gray bars). Asterisks indicate significant differences ($p < 0.05$) as determined by Student's t-test in a comparison between control and treatments.

Fig. 2. GUS staining of transgenic seedlings. Seedlings of *Arabidopsis* HSP17.6C-CIProGUS plants were treated with HSP70 inhibitors (VER-155008, YM-08, pifithrin-μ, and 116-9e) at a concentration of 50 μM for 6 h and with heat (HS, 37 °C for 1 h). Control was no treatment. Scale bars are 0.5 mm.

Fig. 3. Accumulation of GUS and HSPs in *Arabidopsis* seedlings. Transgenic

HSP17.6C-CIProGUS plants (for GUS) and wild-type plants (for HSP17.6C-CI, HSP70, and HSP90.1) were used for immunoblot analysis. The seedlings were treated with VER-155008 (50 μM) and heat (HS, 37 ºC for 1 h). Control means no treatment.

Fig. 4. Effect of VER-155008 on *Arabidopsis* seedlings after heat treatment (46 ºC for 1 h). A: Experimental schedule. VER-155008 was administered 6 days after germination (DAG). Heat (46 ºC for 1 h) was administered at 7 DAG. Fresh weight and chlorophyll contents were measured at 10 DAG. The results are shown in (B) (fresh weight) and (C) (chlorophyll contents). Gray and white bars represent heat-treated and nontreated seedlings, 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.

Fig. 5. Chaperone inhibition by VER-155008, geldanamycin, and sanguinarine. Wheat germ extract (WGE) and human HSP70/HSP40 (hHSP70) were used. Effects of VER-155008 on WGE (A) and hHSP70 (B) are shown. Geldanamycin (C) and sanguinarine (D) were tested by using the hHSP70 system. 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.

Fig. 6. Hypothetical mechanism for the HSR-inducing activities of chaperone inhibitors. HSFs are held by HSP70 and HSP90 under normal conditions (Jacob *et al*. 2017) (a). When VER-155008, sanguinarine, and geldanamycin inhibit HSP70 or HSP90, HSFs are released and activated (b). This promotes the expression of HSP genes (c), and then heat tolerance is enhanced. Moreover, HSP70 and HSP90 can restore denatured proteins (d). VER-155008, sanguinarine, and geldanamycin may increase the accumulation of denatured proteins by inhibiting the HSPs. This may also trigger the release of HSFs from complex (a) because HSP70 and HSP90 can be recruited to the denatured proteins.

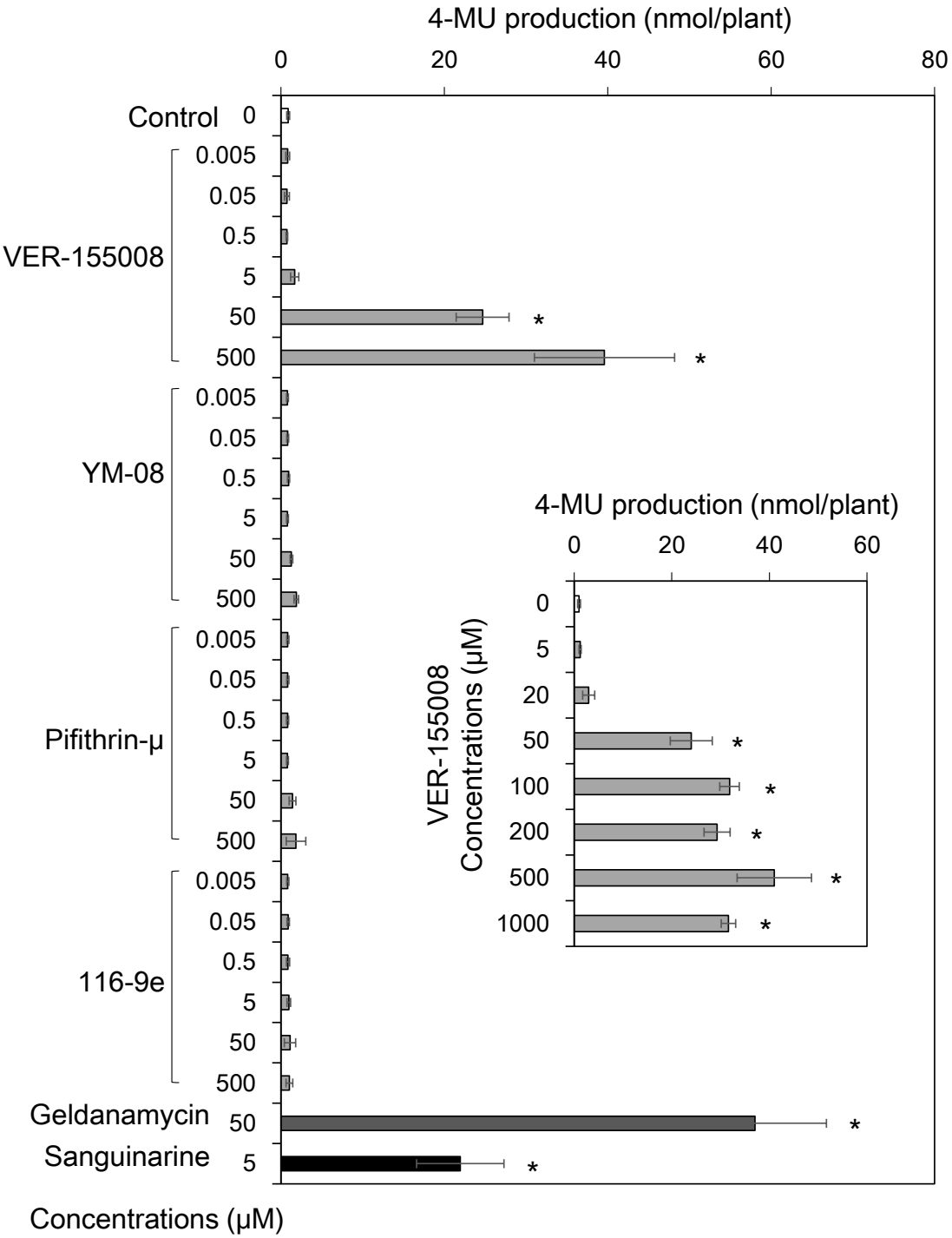


Fig. 1 Matsuoka et al.

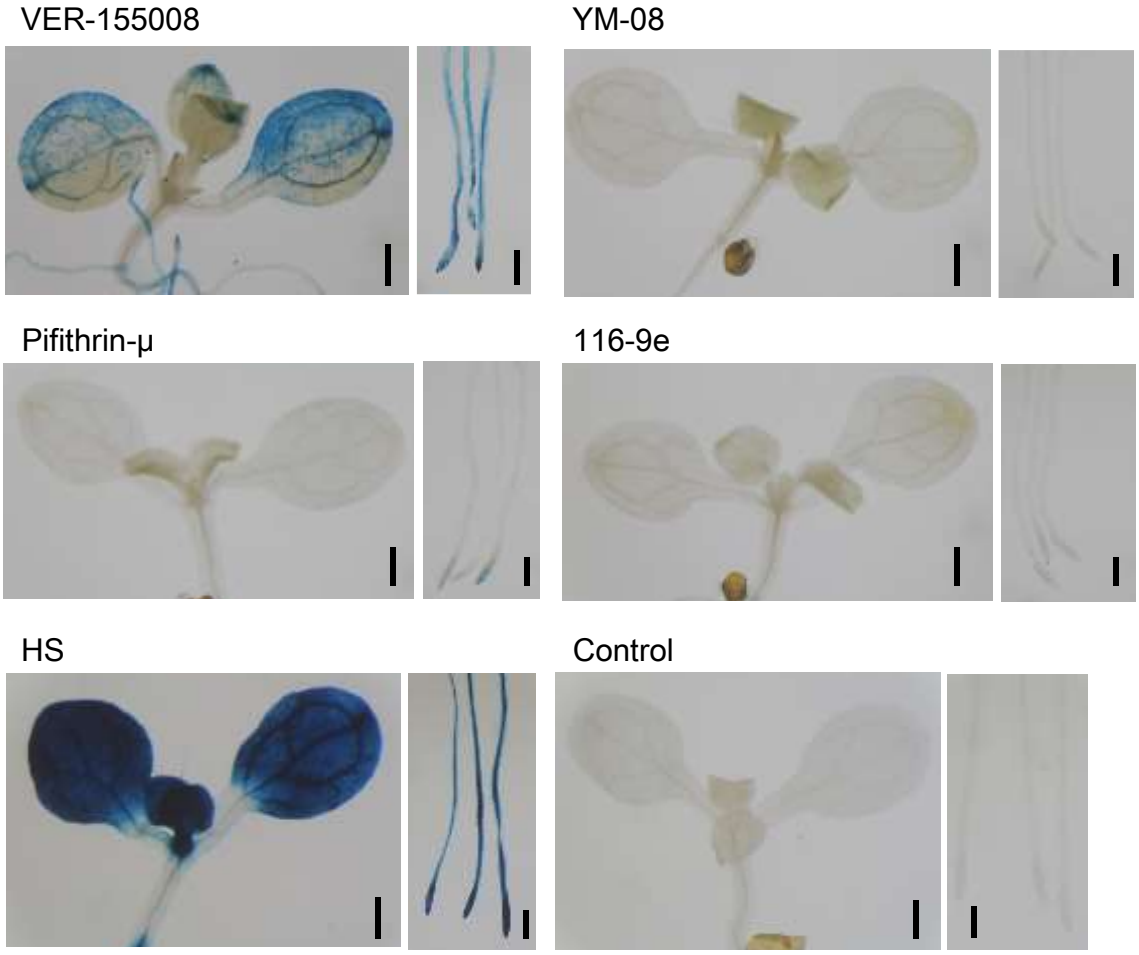


Fig. 2 Matsuoka et al.

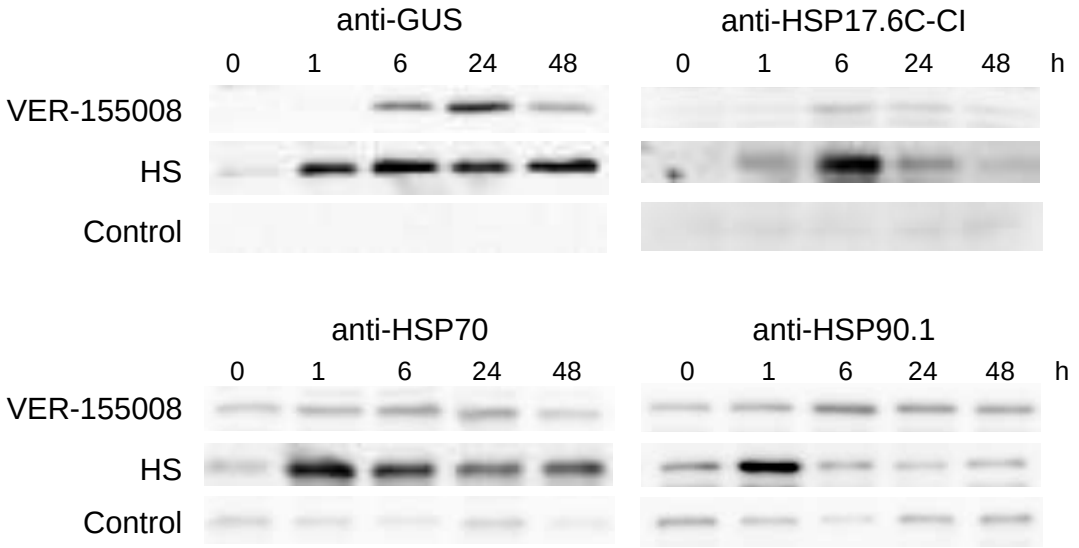


Fig. 3 Matsuoka et al.

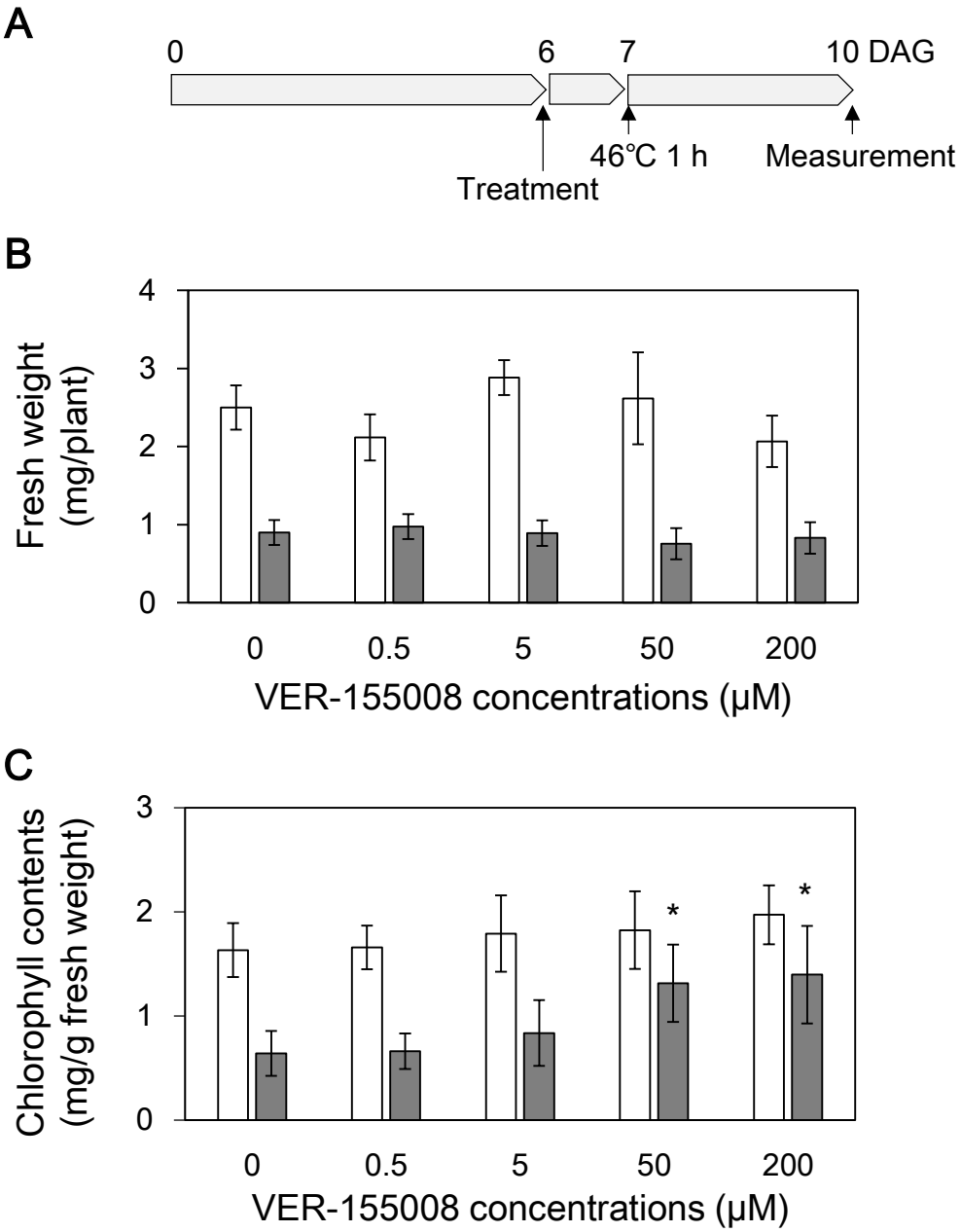


Fig. 4 Matsuoka et al.

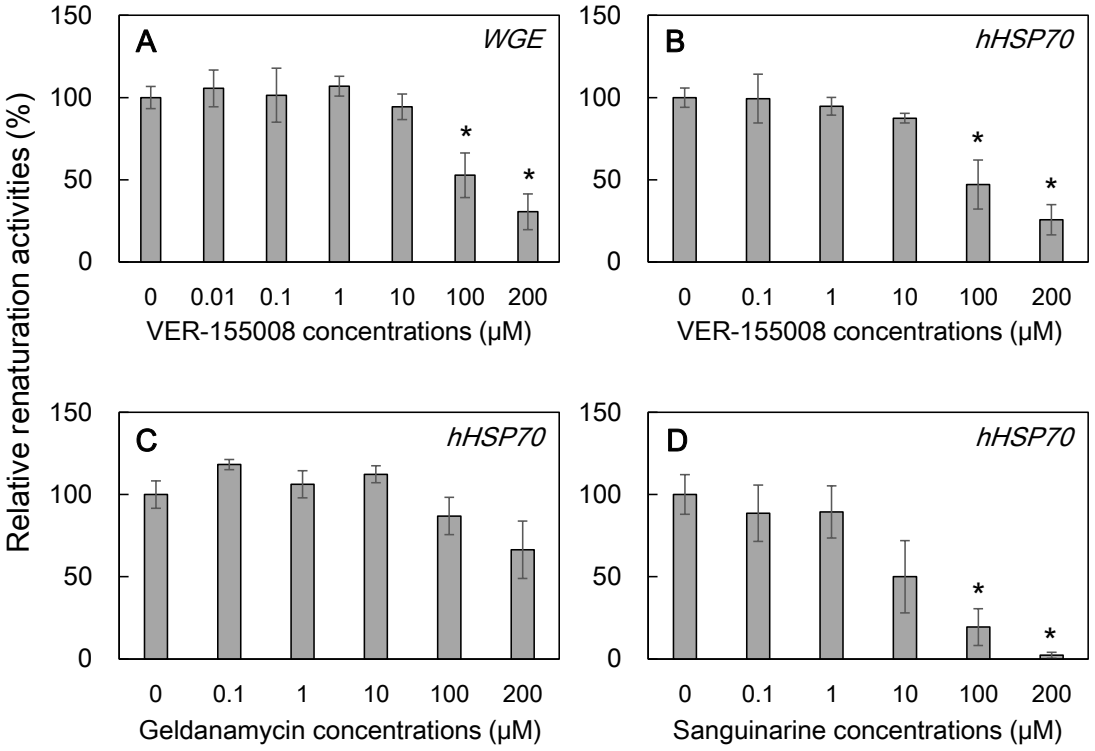


Fig. 5 Matsuoka et al.

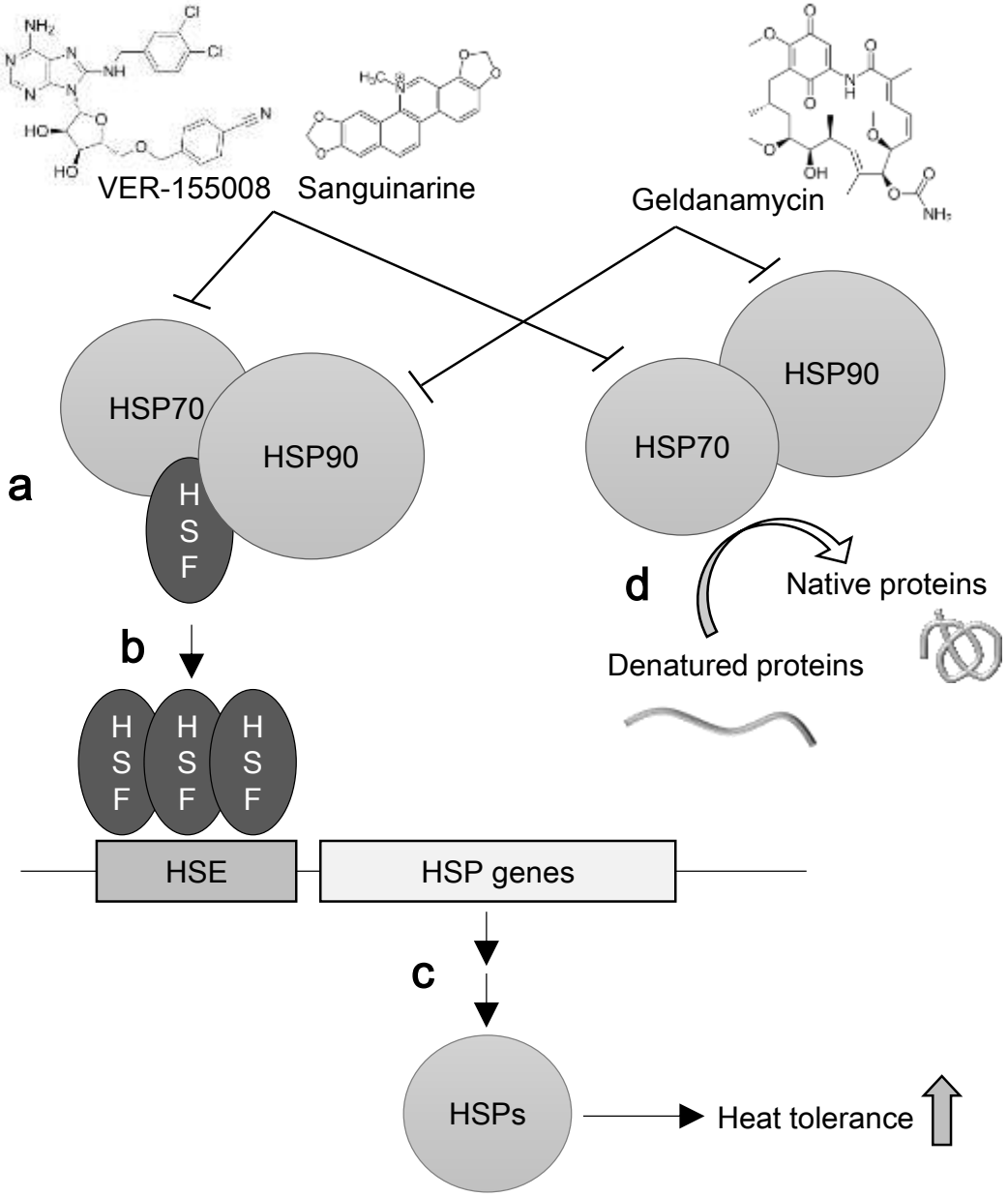


Fig. 6 Matsuoka et al.

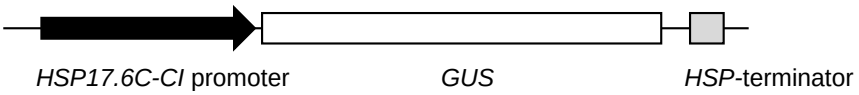


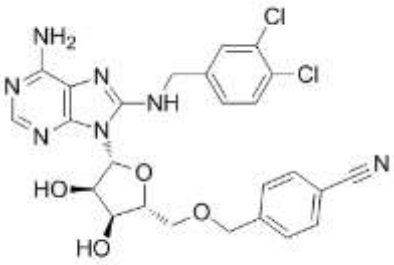
Figure S1. A scheme of the HSP17.6C-CIProGUS construct. The *β-glucuronidase* (*GUS*) gene was controlled by the *HSP17.6C-CI* promoter.

Title: Induction of the heat shock response in *Arabidopsis* by heat shock protein 70 inhibitor VER-155008

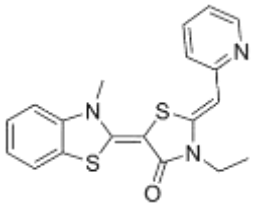
Authors: Matsuoka E, Kato N, Hara M*

*hara.masakazu@shizuoka.ac.jp

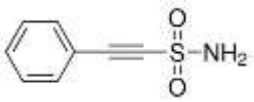
HSP70 inhibitors



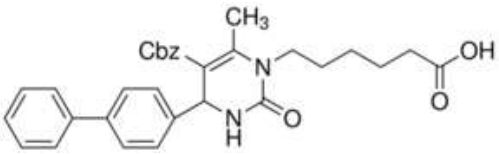
VER-155008



YM-08

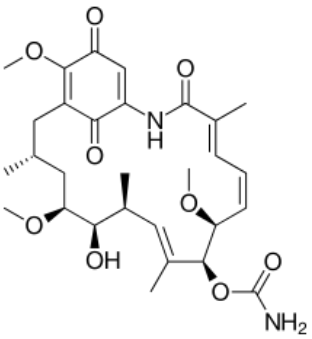


Pifithrin-μ

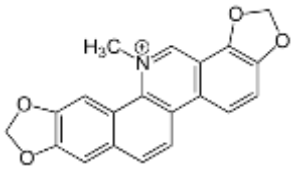


116-9e

Plant chaperone inhibitors



Geldanamycin



Sanguinarine

Figure S2. HSP70 inhibitors and plant chaperone inhibitors used in this study.
Title: Induction of the heat shock response in *Arabidopsis* by heat shock protein 70 inhibitor VER-155008
Authors: Matsuoka E, Kato N, Hara M*
*hara.masakazu@shizuoka.ac.jp

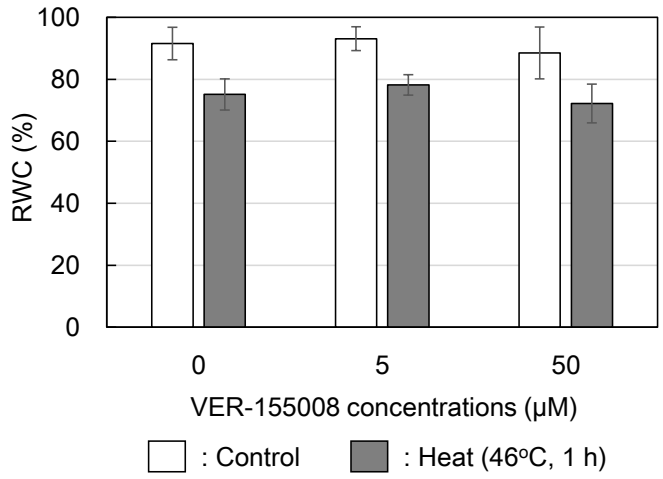


Figure S3. Relative water contents (RWCs) of *Arabidopsis* seedlings at 10 DAG in the same experimental conditions as in Fig. 4. The fresh, turgid, and dry weights of the seedlings were measured. RWCs were determined as the following equation. RWC = (fresh weight - dry weight) / (turgid weight - dry weight). Values and bars are means and SD (n=4).

Title: Induction of the heat shock response in *Arabidopsis* by heat shock protein 70 inhibitor VER-155008

Authors: Matsuoka E, Kato N, Hara M*

*hara.masakazu@shizuoka.ac.jp

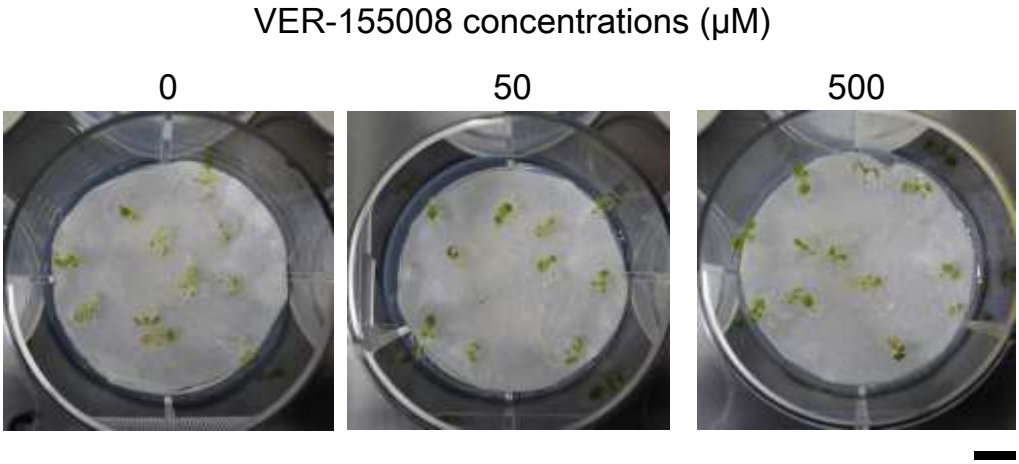


Figure S4. Photographs of *Arabidopsis* seedlings at 10 DAG in the same experimental conditions as in Fig. 4. Bar represents 1 cm.
Title: Induction of the heat shock response in *Arabidopsis* by heat shock protein 70 inhibitor VER-155008
Authors: Matsuoka E, Kato N, Hara M*
*hara.masakazu@shizuoka.ac.jp