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Title

A purine-type heat shock protein 90 inhibitor promotes the heat shock response in *Arabidopsis*

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Abstract Geldanamycin is a macrocyclic heat shock protein 90 (HSP90) inhibitor that suppresses cancer cell proliferation. Since geldanamycin also promotes the heat shock response (HSR) in cells, this compound is used as a chemical inducer of the HSR in *Arabidopsis*. Although many types of HSP90 inhibitors that are different from the macrocyclic types have been developed in pharmaceutical research, non-macrocyclic HSP90 inhibitors have not been investigated in terms of whether they can induce the HSR in plants. Here, we determined the HSR-inducing activities in *Arabidopsis* of 10 non-macrocyclic HSP90 inhibitors including 2 benzamide derivatives, 3 purine derivatives, and 5 resorcinol derivatives. Among the tested inhibitors, PU-H71, which is a purine derivative, showed the highest HSR-inducing activity. The activity of PU-H71 was significantly higher than that of geldanamycin. The application of PU-H71 induced the HSR in all *Arabidopsis* seedlings. The HSP17.6C-CI and HSP70 proteins accumulated after the treatment with PU-H71. The seedlings treated with PU-H71 maintained more chlorophyll than the control seedlings after the heat stress. These results suggest that the purine-derivative HSP90 inhibitor PU-H71 enhanced the heat tolerance of *Arabidopsis* by promoting the HSR in the plant.

Keywords: *Arabidopsis thaliana*, geldanamycin, heat shock protein 90 inhibitors, heat shock response, PU-H71.

Introduction

Heat stress promotes diverse symptoms such as protein denaturation and degradation, increased fluidity of membrane lipids, the production of reactive oxygen species, and the suppression of photosynthesis (Wahid et al. 2007; Allakhverdiev et al. 2008; Ruelland and Zachowski 2010). Physiological responses such as the production of compatible solutes, the activation of antioxidative systems, and the expression of heat shock proteins (HSPs) occur in plants that are exposed to heat stress (Kotak et al. 2007; Bita and Gerats 2013). Among these responses, the expression of HSPs is the most common response to heat in plants. HSPs are molecular chaperones which prevent protein aggregation and mediate the refolding of heat-denatured proteins. The expression levels of HSP genes in plants are positively correlated with the degree of heat tolerance (Wang et al. 2004; Wahid et al. 2007; Waters 2013), suggesting that HSPs participate in establishing the heat tolerance.

Generally, the heat shock response (HSR) is induced by diverse stresses which include not only heat but also other stresses (i.e., osmotic stress, oxidative stress, wounding, and chemicals) (Sørensen et al. 2003; Al-Whaibi 2011). Regarding the chemicals, many natural products, such

as salicylic acid (Dat et al. 1998), benzyl alcohol (Saidi et al. 2005), celastrol (Saidi et al. 2007), geldanamycin (GDA) (Yamada et al. 2007), phenethyl isothiocyanate (Hara et al. 2013), sanguinarine (Hara and Kurita 2014), and (*E*)-2-hexenal and its related compounds (Yamauchi et al. 2015), have been demonstrated to induce the HSR in plants. However, little was understood about the mechanism of the induction of the HSR by these compounds, except in the case of GDA. GDA is a macrocyclic, ansamycin-type heat shock protein 90 (HSP90) inhibitor found in *Streptomyces hygroscopicus*. Yamada et al. (2007) reported that GDA potently induced the HSR in *Arabidopsis* and proposed the following induction mechanism. In non-stressed plants, the active HSP90 may inhibit heat shock factors (HSFs) which promote the transcription of HSP genes. When GDA is added to plants, it can activate HSFs by inhibiting the HSP90 activity. Therefore GDA can induce the HSR in the absence of heat shock (HS) (Yamada and Nishimura 2008). Other macrocyclic HSP90 inhibitors, such as radicicol and monocillin I, have also been applied to induce the HSR in plants (McLellan et al. 2007; Yoshida et al. 2011).

It has been documented that HSP90 is a promising target of cancer therapy. Because HSP90 maintains many oncoproteins, the inactivation of HSP90 results in degrading the oncoproteins and suppressing cancer cell proliferation. Such roles of HSP90 in cancer cells were established by using macrocyclic HSP90 inhibitors like GDA (Jhaveri et al. 2012; Garcia-Carbonero et al. 2013). However, GDA has not been applied to cancer therapy due to its water insolubility and toxicity. Recently, many types of HSP90 inhibitors, such as benzamide derivatives, purine derivatives, and resorcinol derivatives have been developed and clinically tested (Jhaveri et al. 2012; Garcia-Carbonero et al. 2013). These non-macrocyclic HSP90 inhibitors, however, have not been applied to plant systems. Here we determined the HSR-inducing activities of 10 non-macrocyclic HSP90 inhibitors and found that PU-H71, a purine-derivative HSP90 inhibitor, showed higher HSR-inducing activity than GDA. PU-H71 may be utilized as a potent molecular probe for inducing the HSR in plants.

Materials and methods

Chemicals

GDA was purchased from LC Laboratories (Woburn, MA, USA). AT-13387, AUY922, BIIB021, NVP-BEP800, KW-2478, MPC-3100, PF-04929113, PU-H71, SNX-2112 and Ganetespib were obtained from AdooQ BioScience (Irvine, CA, 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).

Transformation of *Arabidopsis*

A plasmid vector was constructed by exchanging the cauliflower mosaic virus (CaMV) 35S promoter region of pRI201-AN-GUS (Takara, Shiga, Japan) with the region 1-kb upstream from the translational start site of the *HSP17.6C-CI* (At1g53540) gene. The CaMV 35S promoter and AtADH 5'-UTR regions were removed from the pRI201-AN-GUS plasmid by double digestion with *Hin*dIII and *Nde*I. The upstream region of the *HSP17.6C-CI* gene was amplified by polymerase chain reaction and then inserted via the sticky ends of *Hin*dIII and *Nde*I to the plasmid using the In-Fusion kit (Takara). The plasmid was named HSP17.6C-CIProGUS. The *Agrobacterium*-mediated floral dip method was applied to transform *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) with the HSP17.6C-CIProGUS plasmid. The homozygotes were designated the HSP17.6C-CIProGUS *Arabidopsis* plant.

Reporter gene assay

The β-glucuronidase (GUS) activity in *Arabidopsis* seedlings was determined according to the established method of whole-plant GUS reaction (Weigel and Glazebrook 2002), with slight modifications. Seeds of the HSP17.6C-CIProGUS *Arabidopsis* were sown on a 1/5 MS medium containing 1% sucrose solidified by 0.8% agar in 6-well plates (TPP, Trasadingen, Switzerland, #92406) under sterile conditions. After vernalization (at 6 ^{o}C for 2 days), the seeds were transferred to the growth chamber (NK System, Tokyo, Japan), conditioned at 22 ^{o}C with a 16-h day (60 μmol m^{-2} s^{-1})/8-h night cycle. At 6 days after germination (DAG), whole seedlings were soaked in 250 μL of the test solutions [1% dimethyl sulfoxide (DMSO) in water (v/v) containing HSP90 inhibitors (0.005, 0.05, 0.5, 5, 50, and 500 μM)] in 1.5-mL micro test tubes (2 seedlings per tube). The test tubes were incubated at 22 ^{o}C for 6 h under illumination (60 μmol m^{-2} s^{-1}). The control solution was 1% DMSO. For HS, the 6 DAG seedlings soaked in the 1% DMSO solution of the test tubes were incubated at 22 ^{o}C for 5 h, and then the test tubes were immersed in a water bath at 37 ^{o}C for 1 h. The seedlings were rinsed with water and transferred to the wells (2 seedlings per well) in a 96-well microplate (IWAKI, Funabasi, Japan) containing 200 μL of the GUS assay solution [50-mM sodium phosphate buffer pH 7.0, 10-mM EDTA, 0.1% (w/v) Triton X-100, 0.1% (w/v) SDS, and 1-mM 4-MUG]. Seedlings were incubated at 22 ^{o}C for 0, 1, 2, 4, 16, 24, and 48 h in the dark. To stop the GUS reaction and enhance the fluorescence of the product (4-MU), 100 μL of 1 M Na_2CO_3 was added and incubated for 10 min. The fluorescence was determined at excitation and emission wavelengths of 365 and 455 nm, respectively, with Varioskan Flash (Thermo Fisher Scientific, Yokohama, Japan). The 4-MU

amounts were calculated from the calibration curve made with authentic 4-MU. For non-macrocyclic HSP90 inhibitors, the incubation period in the GUS assay solution was 16 h.

GUS tissue staining of *Arabidopsis* seedlings was performed (Weigel and Glazebrook, 2002). The 7 DAG seedlings were treated with GDA (50 μM for 6 h), PU-H71 (50 μM for 6 h), and HS (at 37 ^{o}C for 1 h). The control was no treatment. After destaining with acetone, the seedlings were stained with X-Gluc and then observed with light microscopy.

Immunoblot analysis

For immunoblot, *Arabidopsis* seedlings were cultivated as described in the reporter gene assay section. The seedlings (6 DAG) were immersed in 2 ml of test solution [0.2% DMSO in water (v/v) containing 50 μM GDA and PU-H71] in 5 ml test tubes (10 seedlings per tube). Test tubes were incubated at 22^{o}C for 0, 1, 6, 24, and 48 h under illumination (60 μmol m^{-2} s^{-1}). The control solution was no treatment (0.2% DMSO). For the HS, the seedlings at 6 DAG were immersed in 2 ml of water for 5 h in the test tubes, and then the tubes were transferred to a water bath at 37 ^{o}C for 1 h. After that the tubes were kept at 22 ^{o}C under illumination. Seedlings (25 mg fresh weight) were homogenized in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (250 μL) using a microdrill on ice. After centrifugation at 10,000 *g* for 10 min at 4^{o}C, the supernatant was heated at 95^{o}C for 5 min. The samples (10 μg protein each) were resolved by SDS-PAGE. Proteins were blotted onto a polyvinylidene difluoride membrane filter (Immun-Blot; Bio-Rad, Tokyo, Japan) by a Mini Trans-Blot Cell (Bio-Rad). The primary antibodies were anti-GUS (Sigma), anti-HSP17.6C-CI (Agrisera, Vännäs, Sweden), and anti-HSP70 (Agrisera) antibodies. The secondary antibody was horseradish peroxidase conjugated anti-rabbit IgG (Bio-Rad). The antibodies were used at the dilution of 1:5000. Positive signals were detected using the chemiluminescent Clarity Western ECL (Bio-Rad). The chemiluminescence was detected using the LAS-4000 imaging system (Fujifilm, Tokyo, Japan).

Heat-tolerance assay

Seeds of the wild-type *Arabidopsis* were sown on filter paper disk (No. 1, Advantec, Tokyo, Japan), which was then plated on the 1/5 MS medium with 0.8% agar and 1% sucrose in 6-well plates (TPP, #92406) under sterile conditions. After vernalization, the plates were placed in the growth chamber (NK System) conditioned at 22 ^{o}C with a 16-h day (60 μmol m^{-2} s^{-1})/8-h night cycle. The seedlings, which were thinned out to 6 per well, were inoculated onto the test medium in the 6-well plates with the filter paper. GDA and PU-H71 were prepared as DMSO

solutions at concentrations of 0.25, 2.5, 25, and 250 mM. The test medium (4 ml each) was prepared by combining the HSP90 inhibitor solutions (8 µl) and the 1/5 MS medium with 0.8% agar and 1% sucrose (3992 µl). At this step, the final concentrations of the test compounds were 0.5, 5, 50, and 500 µM, respectively. After incubation for 24 h under the same condition as above, the plates were sealed with a polyethylene zipper bag (4-F, 120 nm x 170 mm, System Polymer, Tokyo, Japan) and soaked in water at 46 ^{o}C for 1 h. Control plates were not heat-treated. The plates were cultivated for 3 days under the described conditions. At 10 DAG, the seedlings were harvested, and the fresh weights were measured. The contents of photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, and carotenoids) were determined spectrophotometrically (Lichtenthaler and Buschmann 2001).

Statistical analysis

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

Results

An HSP17.6C-CIProGUS *Arabidopsis* plant was produced to perform the *GUS* reporter assay for measuring the level of the HSR. We applied the whole-plant GUS assay system, which was established previously (Weigel and Glazebrook 2002). The transgenic *Arabidopsis* possessed the *HSP17.6C-CIProGUS* construct (Supplemental Fig. 1), which contained the promoter region (1 kb) of a small HSP gene, i.e., the *HSP17.6C-CI* (*At1g53540*) gene of *Arabidopsis.* We chose the HSP gene because it was reported that the gene was upregulated by the addition of GDA (Yoshida et al. 2011). Figure 1a indicates that the apparent GUS activities were detected in the HSP17.6C-CIProGUS *Arabidopsis* plants by the addition of GDA at the concentration of 50 µM. Heat at 37^{o}C also strongly induced the HSR in the plants. Although a dose response was observed when GDA was applied at concentrations up to 50 µM, the highest concentration (500 µM) showed weaker activity than the 50 µM concentration (Fig. 1b).

By using the reporter assay system, we tested 10 non-macrocyclic HSP90 inhibitors including 2 benzamide derivatives (PF-04929113 and SNX-2112), 3 purine derivatives (MPC-3100, BIIB021 and PU-H71), and 5 resorcinol derivatives (AT13387, AUY922, NVP-BEP800, KW-2478, and Ganetespib) (Fig. 2a). The chemical structures of the HSP90 inhibitors are shown (Supplemental Fig. 2). The result was that PU-H71 showed the highest HSR-inducing activity, which was approximately three times higher than the activity of GDA. The PU-H71 concentration showing the maximum activity was 50 µM (Fig. 2b). The dose-response curve of

PU-H71 was similar to that of GDA, suggesting that PU-H71 and GDA were similar in their function in the HSR induction.

The GUS staining was seen in the cotyledons, first true leaves, bases of the true leaves, and roots of the *Arabidopsis* seedlings after the administration of PU-H71 and GDA (Fig. 3). Heat also induced a similar GUS expression pattern, whereas the expression induced by heat was more intensive than those induced by PU-H71 and GDA. Control plants showed little expression. Immunoblot analysis indicated that the GUS protein was produced by the addition of PU-H71 and GDA to the HSP17.6C-CIProGUS plants (Fig. 4a). The production of HSP17.6C-CI and HSP70 in wild-type plants was also enhanced by PU-H71 and GDA, indicating that the accumulation of endogenous HSPs was promoted during the HSR induction by these HSP90 inhibitors in *Arabidopsis* (Fig. 4b, c). The expression of the HSP17.6C-CI and HSP70 proteins tended to be maintained for 48 h, because the seedlings were incubated in the PU-H71 and GDA solutions during the incubation periods. It is likely that the promotion of HSP17.6C-CI production was concomitant with the up-regulated expression of the corresponding gene when PU-H71 and GDA were added to the plant (Supplemental Fig. 3). Heat (37^{o}C) potently promoted the production of GUS, HSP17.6C-CI, and HSP70 in the plants. In this experimental condition the production of the proteins by heat was transient, because the duration of the heat treatment (37^{o}C) was 1 h. The continuous treatment with heat caused damage to the seedlings.

Since PU-H71 induced the HSR in *Arabidopsis*, we assessed whether PU-H71 enhanced the heat tolerance of the plant. PU-H71 and GDA were supplied to the seedlings at 6 DAG, and the heat treatment (46^{o}C for 1 h) was administered at 7 DAG. This heat treatment was the lowest lethal condition for the control seedlings. After incubation for 3 days, the fresh weights and photosynthetic pigment contents were measured (Fig. 5a). The additions of PU-H71 and GDA at the concentrations of 50 and 500 μM tended to reduce the growth of the seedlings without HS (Fig. 5b, white columns). However, PU-H71 and GDA did not affect the fresh weights of the seedlings that were subjected to HS (Fig. 5b, gray columns). The chlorophyll *a* and *b* contents of the PU-H71-treated seedlings were higher than those of the non-treated seedlings after the HS (Fig. 5c, gray columns). A similar tendency was observed in the case of GDA, whereas PU-H71 maintained slightly more chlorophyll *a* and *b* contents than GDA. The carotenoid contents remarkably decreased due to the heat treatment (Fig. 5d). However, neither PU-H71 nor GDA maintained the carotenoid contents after the heat treatment (Fig. 5d, gray columns). Maintenance of the chlorophyll contents was also shown in the photographs of the seedlings at 10 DAG (Supplemental Fig. 4).

Discussion

GDA was the first compound found to inhibit cancer cell proliferation by reducing the chaperone activity of HSP90 (Blagg and Kerr 2006; Jhaveri et al. 2012). GDA binds to the N-terminal ATP binding pocket of HSP90 and inhibits the maturation of client proteins including many oncoproteins. Thus, cancer cells are suppressed by the administration of GDA. However, GDA has not been used for cancer therapy because this molecule is poorly water soluble and toxic to the liver. Given this background, many kinds of non-macrocyclic HSP90 inhibitors have been developed as anti-cancer drugs (Jhaveri et al. 2012).

GDA induces the HSR in cancer cells, because HSF1, which is suppressed by HSP90 under normal conditions, is released by GDA and promotes the expression of HSP genes without heat stress (Blagg and Kerr 2006). A similar mechanism is assumed to function in *Arabidopsis* (Yamada and Nishimura 2008). Our results indicate that the non-macrocyclic HSP90 inhibitors showed the HSR-inducing activities in *Arabidopsis*, whereas their activities were very different (Fig. 2a). Generally, the synthesized HSP90 inhibitors showed more potent inhibiting activities than GDA. The IC_{50}s of the inhibitors of human HSP90s were 70 nM (GDA), 60 nM (MPC-3100), 58 nM (NVP-BEP800), 51 nM (PU-H71), 3.8 nM (KW-2478), and 1.7 nM (BIIB021) (database web sites on HSP90 inhibitors, e.g. http://www.selleckchem.com/HSP-90.html). This means that in *Arabidopsis* the HSR-inducing activities of the HSP90 inhibitors were little correlated with their HSP90 inhibition activities. It is supposed that this discrepancy is due to the differences in the efficiency of absorption, excretion, and degradation of the inhibitors by *Arabidopsis* cells. Moreover plant HSP90s may show different levels of susceptibility to these inhibitors from animal HSP90s. Further studies are needed to elucidate the reason for the different HSR-inducing activities of the HSP90 inhibitors in the plant system.

PU-H71 showed the most potent HSR-inducing activity among the HSP90 inhibitors tested in this study. However, other purine derivatives (MPC-3100 and BIIB021) did not show such strong activities. Moreover, cytokinins, which are purine-type plant hormones, showed little activity (data not shown). This indicates that the presence of the purine moiety is not an essential condition for the HSR-inducing activity.

Although PU-H71 and GDA induced HSR in the whole seedlings of *Arabidopsis*, the strong expression of the GUS gene was observed in the base of the first true leaves and the root tips, suggesting that HSP90 and its client HSFs accumulate in meristems. This may facilitate the immediate expression of the HSP genes in the meristems when plants are exposed to stresses. We are investigating the effect of HSP90 inhibitors on the HSP90 gene expression including post-transcriptional regulation.

In this study we found that some non-macrocyclic HSP90 inhibitors can induce the HSR in *Arabidopsis* as well as traditional macrocyclic inhibitors like GDA. Recent studies have indicated that HSP90 was not only related to plant immunity (Shirasu 2009) but also to the signal transduction of plant hormones such as auxin (Wang et al. 2016), abscisic acid (Clément et al. 2011), brassinosteroid (Samakovli et al. 2014), and jasmonate (Zhang et al. 2015). These physiological responses were modulated by the addition of macrocyclic HSP90 inhibitors. Purine-derivative HSP90 inhibitors were the first class of fully synthetic inhibitors for HSP90. PU-H71 is now under clinical examination due to its specific inhibition of human HSP90 and its remarkable preclinical activities in a variety of cancer types (Trendowski 2015). PU-H71 may be utilized as a potent molecular probe for studying physiological mechanisms such as HSR, immunity, and hormonal actions in plants.

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Figure legends

Fig. 1 GUS reporter assay. **a** Time-course of 4-MU production by the HSP17.6C-CIProGUS *Arabidopsis* plants. GDA and HS represent geldanamycin (50 μM) and heat shock (37 ^{o}C for 1 h), respectively. Closed and open circles represent treatment and no treatment, respectively. **b** GUS activities in the HSP17.6C-CIProGUS plants treated with GDA at different concentrations. The incubation period was 16h. The activities are represented in terms of 4-MU production. A picture showing the fluorescence of 4-MU produced by the plants is shown above the graph. The values and bars are means and SD (three individual experiments), respectively. Asterisks show significant differences ($p < 0.05$) as determined by Student's t-test in a comparison between no treatment and treatments.

Fig. 2 Induction of HSRs by HSP90 inhibitors in *Arabidopsis*. **a** GUS activities in the HSP17.6C-CIProGUS plants treated with HSP90 inhibitors (50 μM). **b** Effect of different concentrations of PU-H71 on the GUS expression in the plants. The incubation period was 16 h. The activities are presented in terms of 4-MU production. The values and bars are means and SD (three individual experiments), respectively. Asterisks show significant differences ($p < 0.05$) as determined by Student's t-test in comparisons between the GDA treatment and other treatments (**a**), and between no treatment (0 μM) and treatments (**b**).

Fig. 3 GUS staining of *Arabidopsis* seedlings. Seedlings were treated with PU-H71 (50 μM for 6 h), GDA (50 μM for 6 h), and heat (HS, 37 ^{o}C for 1 h), respectively. No treatment was shown (C). Shoots and roots are exhibited. The scale bars are 1 mm.

Fig. 4 Accumulation of HSPs in *Arabidopsis*. GUS (**a**), HSP17.6C-CI (**b**), and HSP70 (**c**) were detected by immunoblot. HSP17.6C-CIProGUS plants (for GUS) and wild-type plants (for HSP17.6C-CI and HSP70) were used. Seedlings were treated with PU-H71 (50 μM), GDA (50 μM), and heat (HS, 37 ^{o}C for 1 h), respectively. No treatment was shown (C).

Fig. 5 Effects of pre-treatments with PU-H71 and GDA on fresh weight and photosynthetic pigment contents of *Arabidopsis* seedlings after heat treatment (46 ^{o}C for 1 h). This heat treatment was the lowest lethal condition for the control seedlings (0 μM). **a** The scheme of the experimental schedule. DAG means days after germination. The fresh weight (**b**), chlorophyll *a* and *b* contents (**c**), and carotenoid content (**d**) of the seedlings. White and gray bars represent plants not exposed to heat and plants exposed to heat, respectively. The values and bars are means and SD (n=12 in fresh weight and n=4 in photosynthetic pigment contents), respectively. Asterisks show significant differences ($p < 0.05$) as determined by Student's t-test in a comparison between no treatment (0 μM) and treatments.

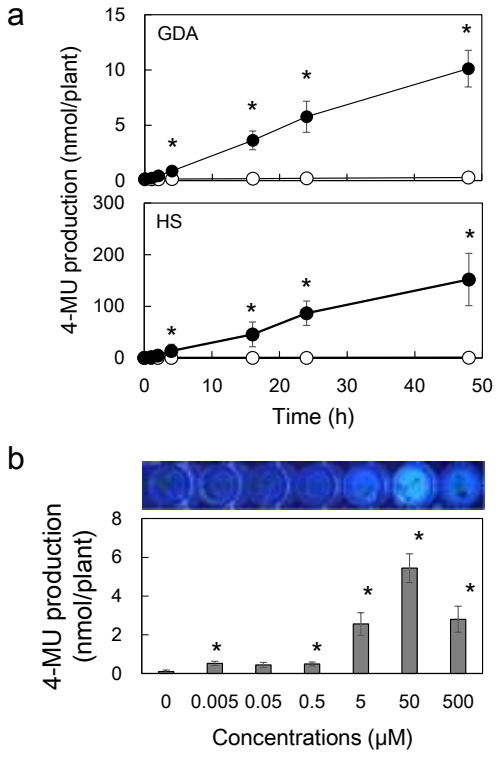


Fig. 1 Murano et al.

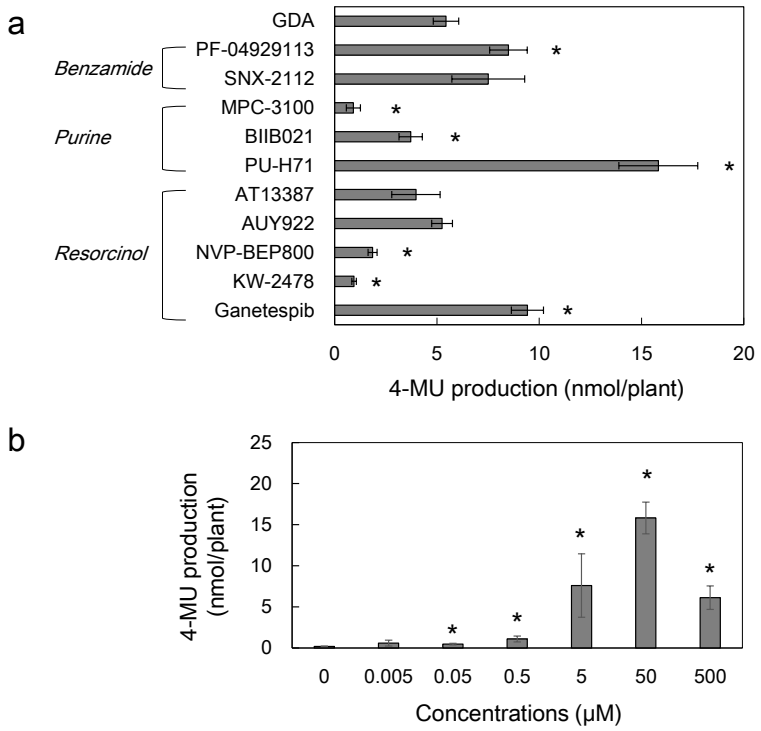


Fig. 2 Murano et al.

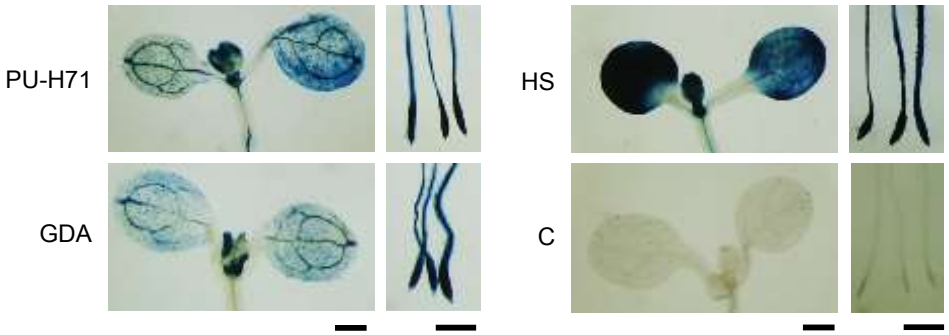


Fig. 3 Murano et al.

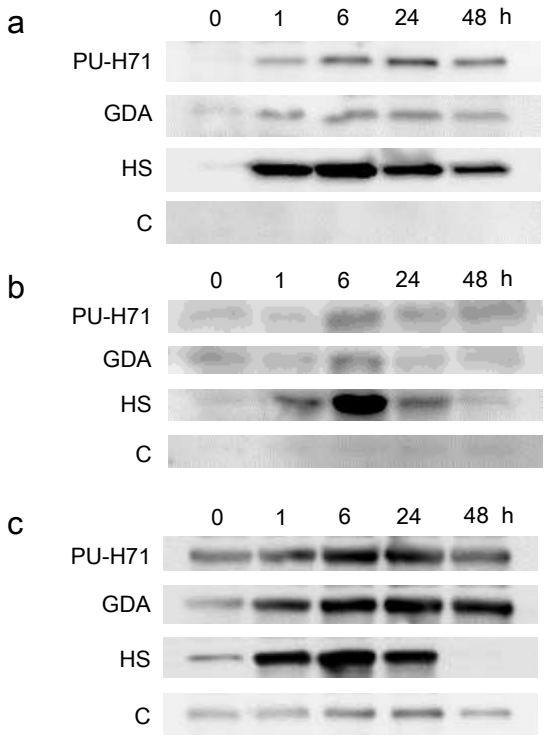


Fig. 4 Murano et al.

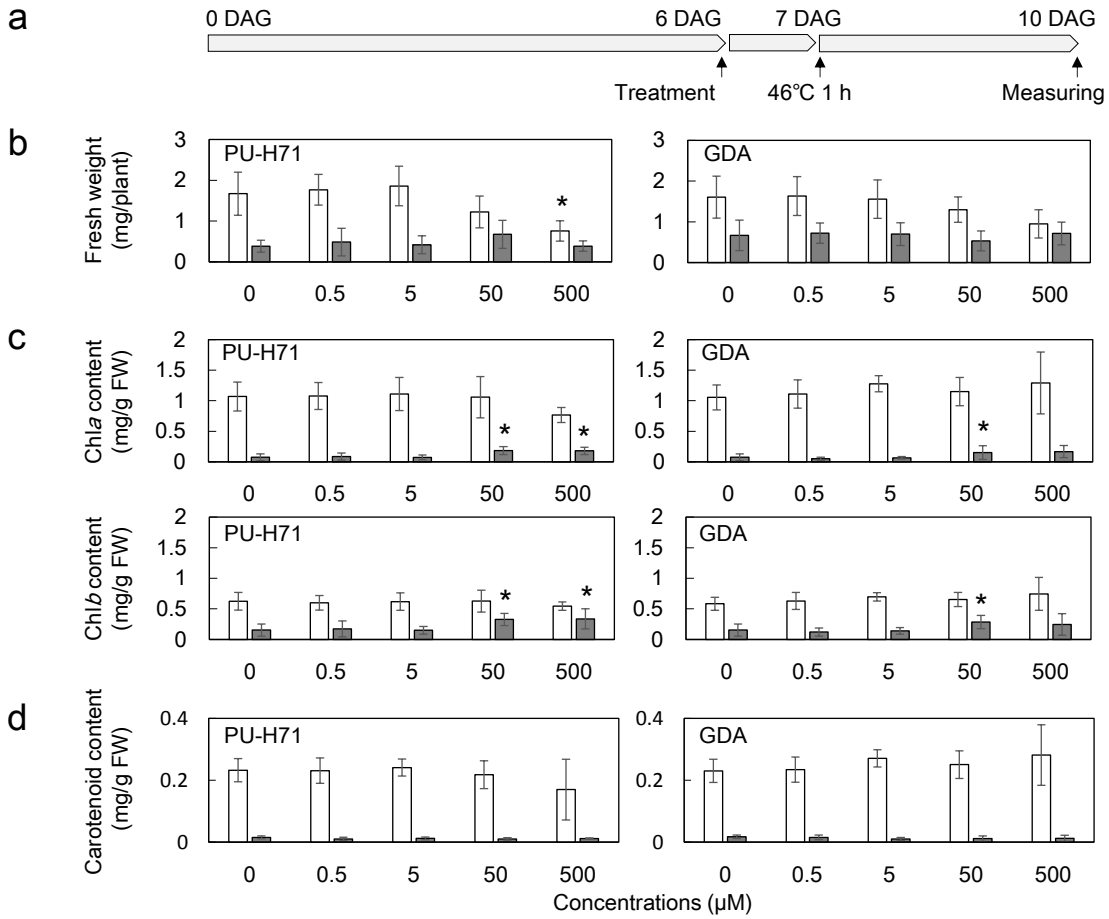
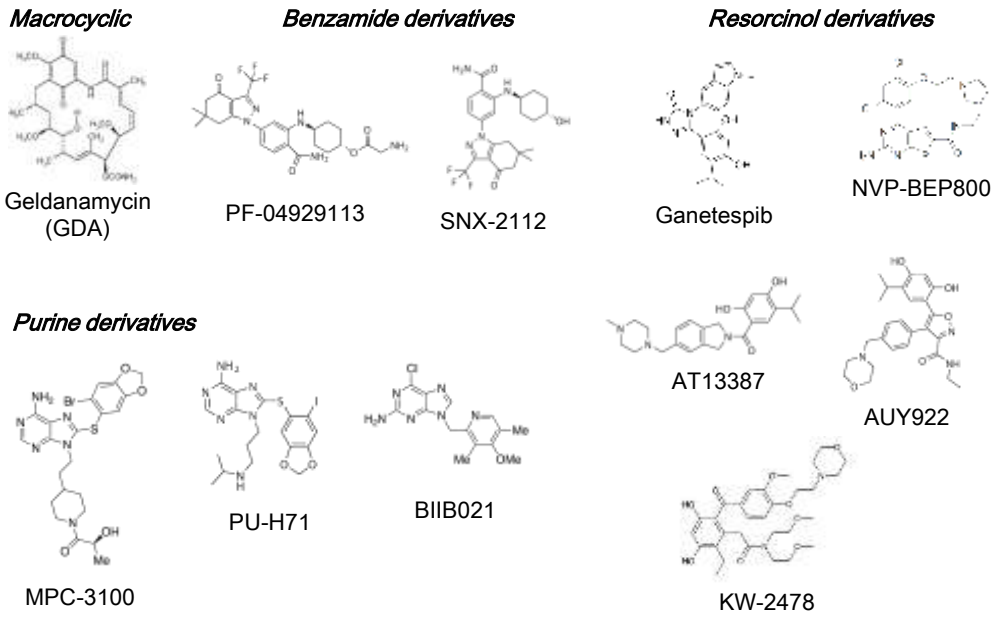


Fig. 5 Murano et al.

Supplemental Fig. 1 Construct of the transgene in the HSP17.6C-CIProGUS plasmid for the *β-glucuronidase* (*GUS*) reporter assay.
Title: A purine-type heat shock protein 90 inhibitor promotes the heat shock response in *Arabidopsis*
Authors: Murano H, Matsubara T, Takahashi I, Hara M*
*hara.masakazu@shizuoka.ac.jp



Supplemental Fig. 2 HSP90 inhibitors tested in this study.
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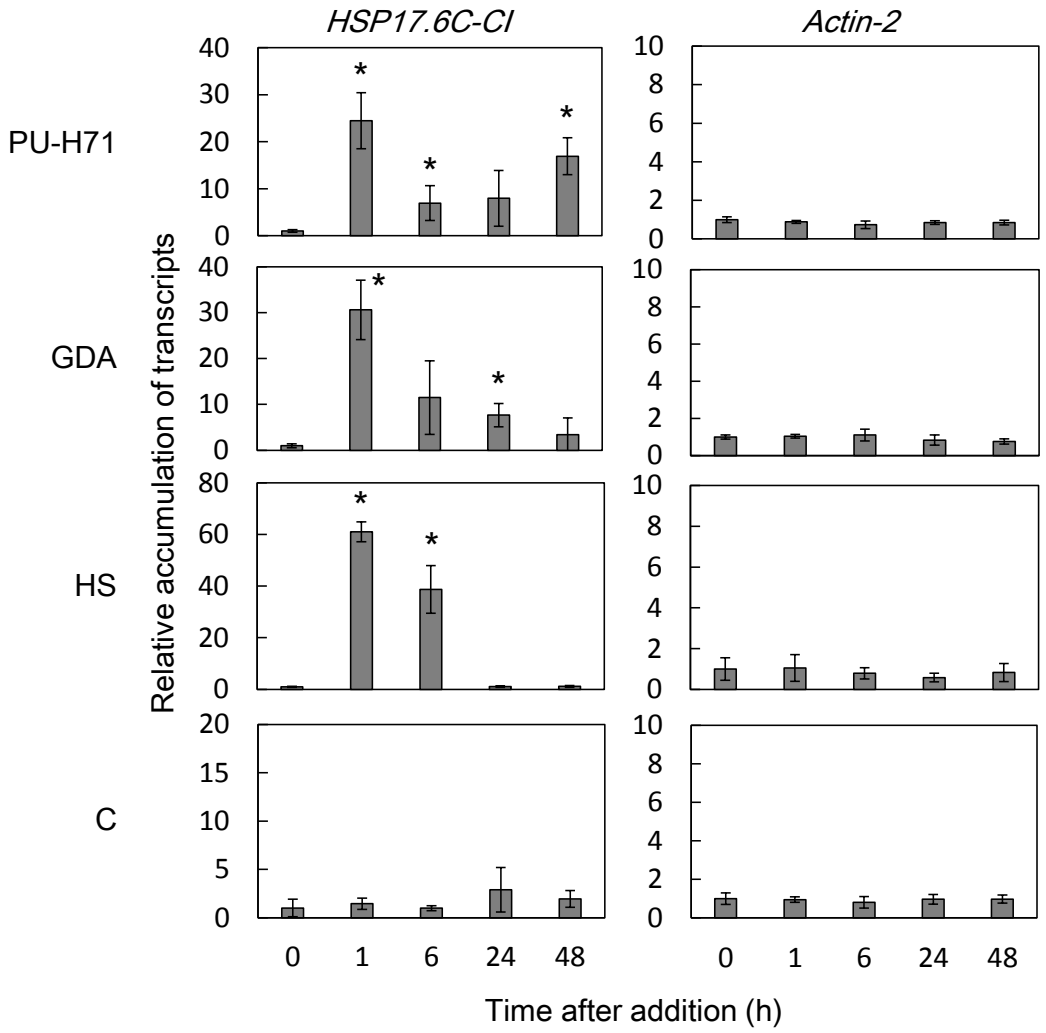

Supplemental Fig. 3 Effects of test compounds and heat on the expression of *HSP17.6C-CI* and *actin-2* genes in *Arabidopsis* seedlings. The seedlings (6 DAG) were treated with PU-H71 (50 μM for 48 h), GDA (50 μM for 48 h), and heat (HS, 37°C for 1 h), respectively. Treatments were initiated at 0 time. No-treatment (C) was a negative control. The relative accumulation of transcripts was determined by RT-PCR. Total RNA was extracted from the whole seedlings with the RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). Total RNA (390 ng/μl) was subjected to reverse transcription (45°C for 30 min), and then underwent PCR with cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s (18-22 cycles for *HSP17.6C-CI* and 30 cycles for *actin-2*). The primers were 5'-CTCTAATTCCAAGCATC-3' (sense) and 5'-CCAGAGATATCAATGGAC-3' (antisense) for *HSP17.6C-CI*, and 5'-ACCTTGCTGGACGTGACCTTACTGAT-3' (sense) and 5'-GTTGTCTCGTGGATTCCAGCAGCTT-3' (antisense) for *actin-2*. Values at 0 h were standardized. Values and bars represent mean ± SD (n = 3). Asterisks show significant differences ($P < 0.05$) as determined by Student's t test in a comparison between no incubation (0 h) and incubation.

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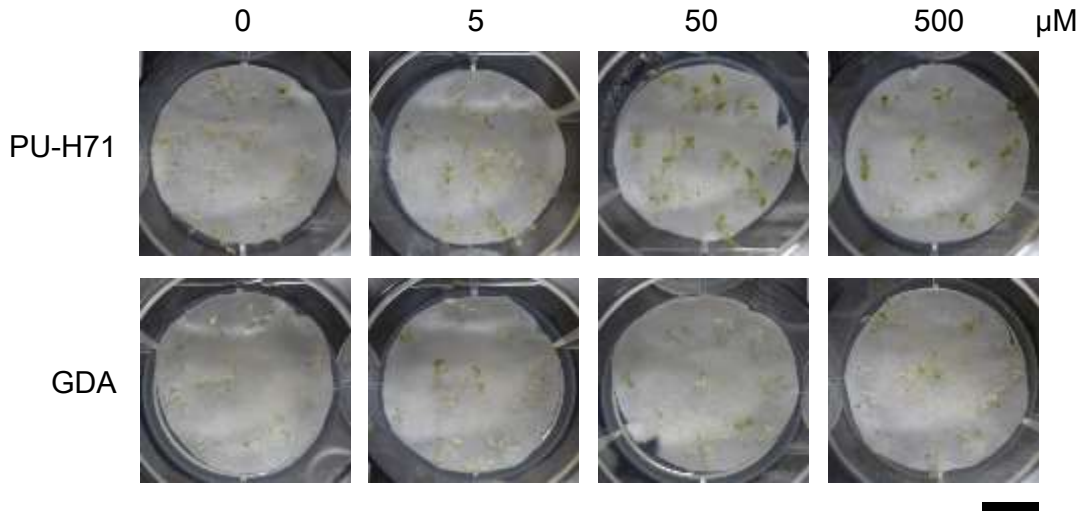

Supplemental Fig. 4 Photographs of *Arabidopsis* seedlings at 10 DAG in the same experimental condition as Fig. 5. A bar represents 1 cm.

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