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Note

The isoquinoline alkaloid sanguinarine which inhibits chaperone activity enhances the production of heat shock proteins in *Arabidopsis*

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Abstract Sanguinarine is an isoquinoline alkaloid produced by Papaveraceae plants. Because sanguinarine has antimicrobial activity, it is believed to be related to the plants' chemical defense systems. However, its action against plants has not been well understood. A previous study reported that among 12 alkaloids, sanguinarine was the only compound which enhanced heat tolerance in *Arabidopsis*. Here we performed a promoter assay using a heat shock protein gene (*HSP17.6C-CI*) of *Arabidopsis* to assess the induction of heat shock responses by alkaloids. Although sanguinarine induced the heat shock response, the other 11 alkaloids did not. Sanguinarine promoted the production of HSP17.6C-CI protein, but berberine and papaverine, which are isoquinoline alkaloids as well as sanguinarine, did not promote it. It is known that geldanamycin, a small molecule chaperone inhibitor, activates the heat shock response in *Arabidopsis*. Although sanguinarine inhibited the chaperone activities of wheat germ extract much like geldanamycin, berberine and papaverine influenced the activities very little. These results suggest that sanguinarine may promote the heat shock response by regulating the chaperone activities in the way that geldanamycin does in plants.

Key words: *Arabidopsis thaliana*, geldanamycin, heat shock protein, heat shock response, sanguinarine.

Sanguinarine is an isoquinoline alkaloid which is produced by Papaveraceae plants such as *Sanguinaria canadensis*, *Papaver somniferum*, *Eschscholzia californica*, *Chelidonium majus*, and *Macleaya cordata*. In these plants, sanguinarine is considered to be related to the defense system in plant-microbe interaction, because this alkaloid has potent antimicrobial activity (Facchini and St-Pierre 2005). In addition, sanguinarine has been attracting attention as an active compound due to its diverse medicinal activities (antihypertensive, antiplatelet, and anticarcinogenic activities) in animals (Mackraj et al. 2008). On the other hand, the role of sanguinarine in plant-plant interaction has not been well understood, except that sanguinarine showed an allelopathic effect against *Lepidium sativum* (Wink and Twardowsky 1992). Recently, we found that sanguinarine enhanced heat tolerance in *Arabidopsis thaliana* (Hara and Kurita 2014). This effect was likely specific because only sanguinarine enhanced the heat tolerance among the 12 alkaloids we could test, including isoquinoline alkaloids (berberine, emetine, noscapine, papaverine, and sanguinarine), indole alkaloids (reserpine and yohimbine), a purine alkaloid (caffeine), a quinoline alkaloid (quinine), a pyridine alkaloid (trigonelline), and others (capsaicin and colchicine). Moreover, the heat shock response (HSR), i.e. the activation of heat shock protein (HSP) genes, occurred in *Arabidopsis* as a result of the addition of sanguinarine, but not of berberine nor papaverine. However, it was not shown whether the other 9 alkaloids promoted the HSR in *Arabidopsis*.

Generally, HSR is induced not only by heat but also by various stresses (e.g., osmotic stress, oxidative stress, irradiation, wounding, and chemicals) (Al-Whaibi 2011; Sørensen et al. 2003). Naturally occurring compounds such as salicylic acid (Dat et al. 1998), benzyl alcohol (Saidi et al. 2005), celastrol (Saidi et al. 2007), geldanamycin (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 shown to induce the HSR in plants. Thanks to previous studies, the mechanism of HSR induction by geldanamycin is better understood relative to other compounds. Geldanamycin, which was first isolated from *Streptomyces hygroscopicus*,

Abbreviations: DAG, days after germination; GUS, β-glucuronidase; HSP, heat shock protein; HSR, heat shock response.
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is a chaperone inhibitor, especially showing a specific inhibition of HSP90. Under normal conditions, HSP90 binds to heat shock factors (HSFs). This binding reduces the transcription-regulating activities of the HSFs. If HSP90 is inactivated by geldanamycin, the HSFs are activated via their release from the complex with HSP90, resulting in the induction of a strong HSR in plants (Yamada et al. 2007). The addition of geldanamycin to *Arabidopsis* induces the expression of many HSP genes, including small HSP genes such as the *HSP17.6C-CI* (*At1g53540*) gene (Yoshida et al. 2011). The *HSP17.6C-CI* gene was also activated by the addition of sanguinarine (Hara and Kurita 2014) and (*E*)-2-hexenal (Yamauchi et al. 2015), suggesting that this gene is a common regulon controlled by small molecule HSR inducers in *Arabidopsis*. Accordingly, we first performed the promoter assay by using a heat shock protein gene (*HSP17.6C-CI*) of *Arabidopsis* to assess induction of the HSRs by alkaloids.

A plasmid vector for the transformation of *A. thaliana* was constructed by exchanging the cauliflower mosaic virus (CaMV) 35S promoter region, including AtADH 5′-UTR of pRI201-AN-*β*-glucuronidase (GUS) (Takara, Shiga, Japan), to the 1-kb upstream region from the translational start site of the *HSP17.6C-CI* (*At1g53540*) gene. The pRI201-AN-GUS plasmid was digested by *Hin*dIII and *Nde*I to remove the CaMV 35S promoter and AtADH 5′-UTR regions. The upstream region of the *HSP17.6C-CI* gene, which was amplified by polymerase chain reaction, was inserted via the sticky ends of *Hin*dIII and *Nde*I into the plasmid using the In-Fusion kit (Takara) according to the manufacturer's instructions. Finally, the *HSP17.6C-CI* gene promoter was located adjacent to the 5′-terminus of the GUS gene of the pRI201-AN-GUS plasmid (Figure 1A). We designated the plasmid HSP17.6C-CIProGUS. *A. thaliana* (L.) Heynh. ecotype Columbia (Col-0) was transformed with the HSP17.6C-CIProGUS plasmid using the *Agrobacterium*-mediated floral dip method. The 7 homozygous lines were obtained and named the HSP17.6C-CIProGUS *Arabidopsis* plants. We used one of them whose GUS expression strongly responded to geldanamycin.

The GUS activity assay was performed according to the established method of whole-plant GUS reaction (Weigel and Glazebrook 2002), with slight modifications. Seeds of the HSP17.6C-CIProGUS *Arabidopsis* were sown on a 1/5 MS medium containing 1% sucrose solidified by 0.8% agar in 9-cm plates under sterile conditions. The plates were kept at 6°C for 2 days (vernalization), and then transferred to the growth chamber (NK System, Tokyo, Japan), conditioned at 22°C with a 16-h day (60 μmol $m^{-2} s^{-1}$)/8-h night cycle. At 7 days after germination (DAG), whole seedlings were soaked in 500 μl of test solution in 1.5-ml micro

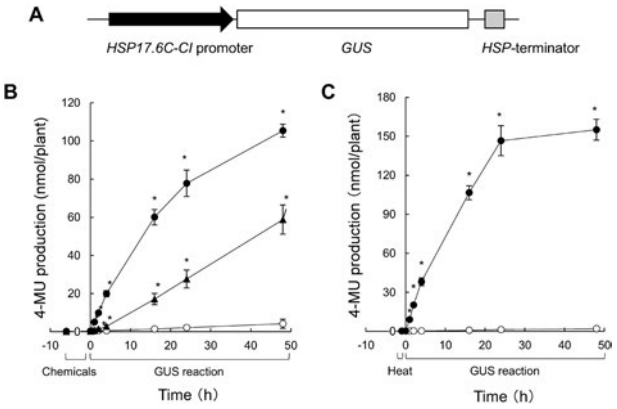

Figure 1. GUS reporter assay. (A) Construct of the *HSP17.6C-CIProGUS* gene. (B) Time-course of 4-MU production by the HSP17.6C-CIProGUS *Arabidopsis* plants. Geldanamycin (50 μM, closed circles) and sanguinarine (50 μM, closed triangles) were administered. Controls (no treatment, open circles) are also shown. (C) Heat shock (37°C for 1 h) was applied to the HSP17.6C-CIProGUS plants. Closed and open circles represent heat shock and controls, respectively. The values and bars are means and SD (four individual experiments), respectively. Asterisks show significant differences ($p<0.05$) as determined by Student's *t*-test in a comparison between the controls (open circles) and treatments.

test tubes (2 seedlings per tube). The test solutions were 5% dimethyl sulfoxide (DMSO) in water (v/v) containing geldanamycin (Tokyo Kasei) and alkaloids (Sigma or Wako) (0.005, 0.05, 0.5, 5, 50, and 500 μM). The test tubes were incubated at 22°C for 6 h under illumination (60 μmol $m^{-2} s^{-1}$). The control solution was 5% DMSO. For heat shock (HS), the 6 DAG seedlings soaked in water in the test tubes (2 seedlings per tube) were incubated at 22°C for 5 h, and then the test tubes were immersed in a water bath at 37°C for 1 h. The seedlings that were rinsed with water were transferred to wells (2 seedlings per well) in a 96-well microplate (IWAKI, Funabasi, Japan) containing 200 μl of the GUS assay solution composed of 50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA, 0.1% (w/v) Triton X-100, 0.1% (w/v) SDS, and 1 mM 4-methylumbelliferyl-*β*-D-glucuronide (4-MUG, Wako). The seedlings were incubated at 22°C for 0, 1, 2, 4, 16, 24, and 48 h in the dark. At the end of the incubations, 100 μl of 1 M Na_2CO_3 was added and incubated for 10 min to stop the GUS reaction and enhance the fluorescence of the reaction product (4-methylumbelliferone, 4-MU). It has been established that GUS activity can be detected without extracting the GUS protein from the seedlings, because both 4-MUG and 4-MU can pass through the plant cell membranes under the reported conditions (Weigel and Glazebrook 2002). The solutions were appropriately diluted with 0.33 M Na_2CO_3. 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 (Wako). When the activities between the geldanamycin and isoquinoline alkaloids were compared, the incubation period in the GUS assay solution was 16 h.

For immunoblot analysis, cultivation of the *Arabidopsis* seedlings was basically the same as described in the GUS reporter assay section. The seedlings at 7 DAG were immersed in 3 ml of test solution in 5 ml test tubes (10 seedlings per tube). The test solutions were 5% DMSO in water (v/v) containing 50 μM geldanamycin and 5 μM isoquinoline alkaloids. Test tubes were incubated at 22°C for 0, 1, 6, 24, and 48 h under illumination (60 μmol $m^{-2} s^{-1}$). The control solution was 5% DMSO. 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°C, the supernatant was heated at 95°C for 5 min. The protein amounts were determined by SDS-PAGE stained with Coomassie Brilliant Blue by using Image-J software. Bovine serum albumin was a standard protein. The samples (10 μg protein each) were resolved by SDS-PAGE. Proteins in the gel 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), anti-HSP70 (Agrisera), and anti-HSP90.1 (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).

Chaperone inhibition assay was performed according to the method used in a previous report (McLellan et al. 2007) with modifications. Recombinant luciferase (QuantiLum; Promega, Tokyo, Japan), which was diluted with the supplied buffer (CCLR; Promega) to the concentration of 0.3 μg ml^{-1}, was denatured by incubation at 40°C for 5 min. Wheat germ extract (Promega), which was double-diluted with distilled water (45 μl), was combined with the test DMSO solution (0.5 μl). After being incubated for 30 min at 25°C, the denatured luciferase (5 μl) was added to start the renaturation of luciferase. The luciferase activities were determined using the LUC assay system (Promega) with the chemiluminescence detection apparatus (LAS-4000) after incubations for 0 (0 time control), 20, 40, and 60 min at 25°C. The renaturation activities were calculated from the initial velocities. The values with no treatment (0 mM) were standardized to 100%.

The HSRs were induced by treatments with geldanamycin and sanguinarine (50 μM each) in the HSP17.6C-CIProGUS plants (Figure 1B). The blank control (0 μM) enhanced 4-MU production very little. Heat (37°C for 1 h) also induced the 4-MU production in the HSP17.6C-CIProGUS plant (Figure 1C). These data indicate that the reporter assay system is useful to determine the dose responses for the activation of the *HSP17.6C-CI* promoter by means of alkaloid treatments. We tested the HSR-inducing activities of the 13 compounds, i.e., geldanamycin, berberine, emetine, noscapine, papaverine, sanguinarine, reserpine, yohimbine, caffeine, quinine, trigonelline, capsaicin, and colchicine, at the concentrations of 5, 50, and 500 μM by using the reporter assay system, and the results indicated that geldanamycin and sanguinarine showed the HSR-inducing activity. Dose dependencies of the HSRs by the alkaloids are shown in Figure 2. From here, we added chelerythrine to the alkaloids list to obtain information on structure–activity relationship. The order of the HSR-inducing activity was geldanamycin> sanguinarine>chelerythrine, whereas other 11 alkaloids (berberine shown as example in Figure 2) had little activity. The concentrations of the maximum activities

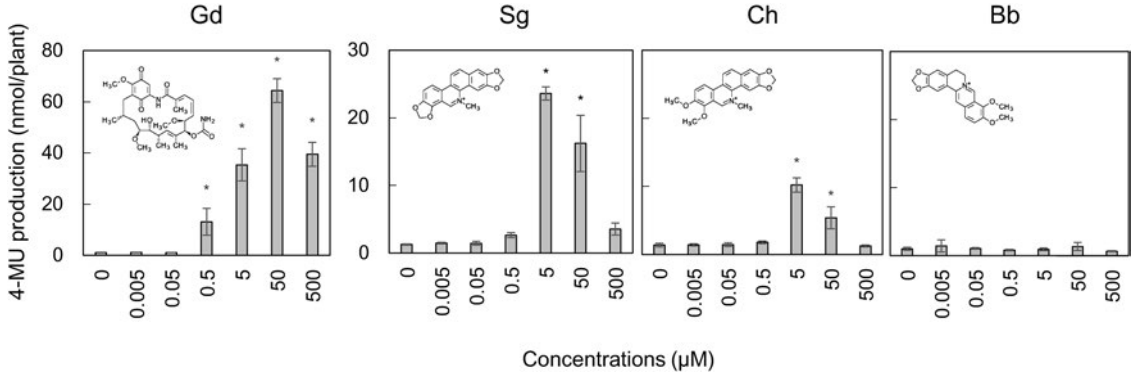

Figure 2. Induction of HSRs by chemicals in *Arabidopsis*. GUS activities in the HSP17.6C-CIProGUS plants treated with geldanamycin (Gd), sanguinarine (Sg), chelerythrine (Ch), and berberine (Bb). Incubation period was 16 h. The activities are represented in terms of 4-MU production. The values and bars represent means ± SD (four individual experiments), respectively.

were 50 μM for geldanamycin and 5 μM for sanguinarine and chelerythrine. It was likely that the concentrations of these compounds higher than those provided the maximum activities were toxic to the cells.

The GUS protein synthesis was apparently induced by the administration of geldanamycin and sanguinarine to the HSP17.6C-CIProGUS plant (Figure 3A). However, control plants produced little GUS protein. When geldanamycin and sanguinarine were administered to the wild-type plants, HSP17.6C-CI, HSP70, and HSP90.1 proteins were produced. The HSP17.6C-CI production in the wild-type plants was slightly enhanced by chelerythrine, but little promoted by berberine and papaverine (Figure 3B). Heat efficiently induced the production of the GUS protein in the HSP17.6C-CIProGUS plant and the HSPs (HSP17.6C-CI, HSP70, and HSP90.1) in the wild-type plants (Supplemental Figure 1).

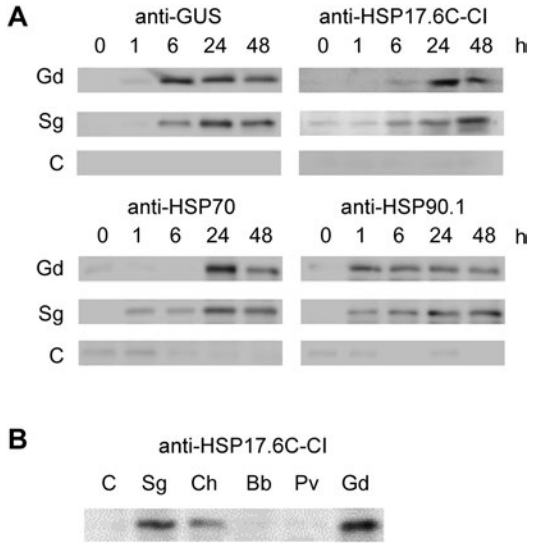

Figure 3. Accumulation of HSPs in *Arabidopsis*. (A) GUS, HSP17.6C-CI, HSP70, and HSP90.1 were detected by immunoblot. Geldanamycin (Gd, 50 μM) and sanguinarine (Sg, 5 μM) were treated. HSP17.6C-CIProGUS plants (for GUS) and wild-type plants (for HSP17.6C-CI, HSP70, and HSP90.1) were used. The results of no treatment (C) are shown. (B) Detection of HSP17.6C-CI in wild-type *Arabidopsis* to which 50 μM geldanamycin (Gd) and 5 μM isoquinoline alkaloids (Sg, Ch, Bb, and Pv) were treated for 24 h. The control received no treatment (C).

It is postulated that geldanamycin induces the HSR by affecting the HSP90-HsfA1 system in *Arabidopsis* (Yamada et al. 2007). Geldanamycin, which inhibits the activity of chaperone HSP90, is thought to release the active HsfA1 to induce the HSR. Since it was shown that the expression of the *HSP17.6C-CI* gene was under the control of the HSP90-HsfA1 system (Yoshida et al. 2011), we hypothesized that sanguinarine may show chaperone-inhibiting activity like geldanamycin does. A previous study indicated that HSP90 inhibitors attenuated the chaperone activity of wheat germ extract, and this inhibition could be detected by refolding heat-denatured luciferase (McLellan et al. 2007). Thus, we subjected geldanamycin, sanguinarine, chelerythrine, berberine, and papaverine to a refolding assay (Figure 4). Sanguinarine remarkably inhibited the chaperone activity of wheat germ extract in a dose-dependent manner as did geldanamycin, whereas sanguinarine showed more potent inhibition than geldanamycin. On the other hand, chelerythrine, berberine and papaverine showed slight or no inhibition of the chaperone activity. This suggests

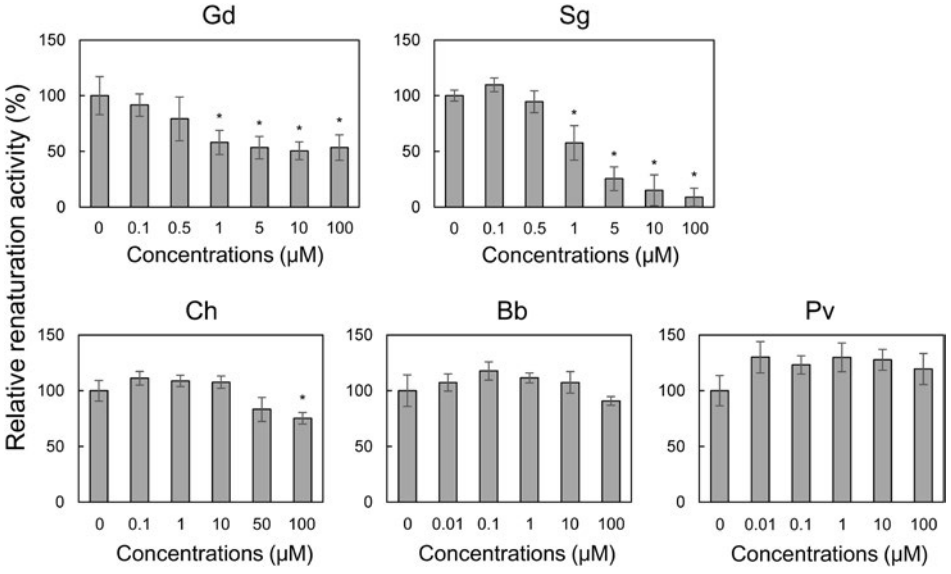

Figure 4. Chaperone inhibition by compounds. The renaturation of luciferase by wheat germ extract was determined. Geldanamycin (Gd), sanguinarine (Sg), chelerythrine (Ch), berberine (Bb), and papaverine (Pv) were used. The concentrations of the chemicals in the chaperone inhibition assay are shown. The mean values of no treatment (0 μM) were standardized to 100% in each compound. The values and bars represent means±SD (4 individual experiments), respectively. Asterisks show significant differences ($p<0.05$) as determined by Student's *t*-test in a comparison between no treatment (0 μM) and the treatments.

that sanguinarine may act as a chaperone inhibitor in plants. Moreover, if sanguinarine inhibited HSP90, sanguinarine might activate the *HSP17.6C-CI* gene by regulating the HSP90-HsfA1 system. It is intriguing that chelerythrine slightly inhibited chaperone, but berberine and papaverine did not. The structural difference between sanguinarine and chelerythrine is that the former has two methylenedioxy bridges and the latter has one methylenedioxy bridge and one dimethoxy group (Matsushima et al. 2012). This indicates that the small structural difference between the two alkaloids apparently affected their chaperone inhibition activities. Berberine and papaverine did not show chaperone inhibition because they have basic structures that are distinct from that of sanguinarine.

A recent paper demonstrated that expression of the gene for HSP70 chaperone was up-regulated concomitant with the gene expression of norcoclaurine:SAM 4′ *O*-methyltransferase, which is a rate-limiting biosynthetic enzyme of sanguinarine in *E. californica* (Angelova et al. 2010). This result suggested that sanguinarine biosynthesis may be related to the HSR in Papaveraceae plants. This may be due to a self-defense reaction in Papaveraceae plants against the chaperone inhibition induced by sanguinarine. Further studies of the molecular targets of sanguinarine in the process of chaperone inhibition in plants will provide better insight into the physiological and ecological functions of this alkaloid.

Acknowledgements

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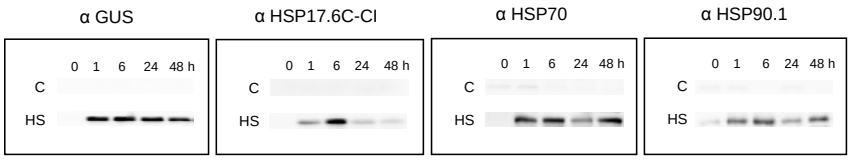

Supplemental Fig.1 Accumulation of HSPs in *Arabidopsis* treated with heat. GUS, HSP17.6C-CI, HSP70, and HSP90.1 were detected by immunoblot. Heat shock (HS) and control (C, no heat shock) were shown. HSP17.6C-CIProGUS plants (for GUS) and wild-type plants (for HSP17.6C-CI, HSP70, and HSP90.1) were used. Heat treatment (at 37ºC for 1 h) was initiated at 0 time.

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