Induction of the heat shock response in Arabidopsis by chlorinated 1,4-naphthoquinones

ORIGINAL PAPER

Title

Induction of the heat shock response in *Arabidopsis* by chlorinated 1,4-naphthoquinones.

Authors

Naoki Kato[1,2], Daiki Yamakawa[3], Naoya Yamauchi[3], Yoshihisa Hashimoto[3], Erina Matsuoka[3], Masakazu Hara[1, 3]*

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

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

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

Abstract

It is known that some plants produce 1,4-naphthoquinones (1,4NQs) to inhibit the growth of other plants as allelochemicals. Here, we report that a chlorinated 1,4NQ enhanced the heat tolerance of *Arabidopsis*, and this enhancement was associated with induction of the heat shock response (HSR) in the plant. Nine 1,4NQs were subjected to the HSR assay by using the promoter of a small heat shock protein (*HSP17.6C-CI*) gene of *Arabidopsis*. The results indicated that chlorinated 1,4NQs, i.e., 2,3-dichloro-1,4-naphthoquinone (DNQ) and 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (DDNQ), apparently showed HSR-inducing activities five to six times greater than a positive control, geldanamycin. However, nonchlorinated 1,4NQs such as lawsone, juglone, plumbagin, and menadione showed much lower activities than DNQ and DDNQ. The administration of DDNQ increased the accumulation of the *HSP17.6C-CI* and *HSP90.1* transcripts as well as of the corresponding proteins in the *Arabidopsis* seedlings. DDNQ significantly ameliorated the reductions of fresh weight and chlorophyll contents of the plant due to heat. These results suggest that the chlorinated 1,4NQs are potent HSR inducers that can enhance the heat tolerance of plants.

Keywords *Arabidopsis*; Heat shock protein; Heat shock response; Heat tolerance enhancers; 1,4-Naphthoquinones

Introduction

Heat is a crucial environmental cue affecting plant growth and crop productivity (Deryng et al. 2014). Extreme heat stress promotes protein denaturation, membrane fluidization, photosynthetic damage, and reactive oxygen species (ROS) generation in plants (Wahid et al. 2007; Ruelland and Zachowski 2010). Diverse responses occur to decrease physiological disorders (Kotak et al. 2007; Bita and Gerats 2013), such as the production of compatible solutes, the improvement of the ROS-scavenging system, and the expression of heat shock proteins (HSPs). HSPs, which facilitate the folding of proteins, prevent protein denaturation by heat. HSPs are supposed to be involved in the

establishment of heat tolerance, because plants highly expressing HSPs showed more heat tolerance than normal plants (Waters 2013; Masand and Yadav 2016; Wang et al. 2016).

Although heat is the principal stimulus for the induction of heat shock response (HSR), chemicals are also known to induce HSR. Plant-derived products, such as salicylic acid (Clarke et al. 2004), benzyl alcohol (Saidi et al. 2005), celastrol (Saidi et al. 2007), isothiocyanates (Hara et al. 2013), sanguinarine (Hara and Kurita 2014), monoterpenes (Hara et al. 2017), and (*E*)-2-hexenal (Yamauchi et al. 2015) have been shown to induce plant HSR. Intriguingly, some of them, e.g., isothiocyanates (Björkman et al. 2011), sanguinarine (Wink and Twardowski 1992), and monoterpenes (Macías et al. 2007), are known as allelochemicals, which inhibit plant growth. Together, these results suggest that allelochemicals may enhance the heat tolerance of plants by inducing HSR. However, it has not been investigated whether other allelochemicals can enhance the heat tolerance of plants.

A 1,4-naphthoquinone (1,4NQ) derivative juglone (5HNQ in Table 1) is a plant secondary metabolite known as a classic allelochemical released from black walnut (*Juglans nigra*) (Soderquist 1973). Other 1,4NQ derivatives, such as lawsone (2HNQ) from *Lawsonia inermis* and plumbagin (HMNQ) from *Plumbago indica*, also show allelopathic activities (Widhalm and Rhodes 2016). Juglone showed phytotoxicity by inducing apoptosis-like cell death followed by ROS generation (Babula et al. 2009; 2014). The *para*-benzoquinone moiety of 1,4NQ can be converted to the semiquinone radical by electron donation, which reduces oxygen to superoxide anion and other ROS (Bolton and Dunlap 2017). Besides the plant-derived 1,4NQs, various kinds of 1,4NQs have been chemically synthesized mainly for pharmacological purposes (Bolton and Dunlap 2017). However, it has not been documented whether 1,4NQs are involved in the enhancement of heat tolerance in plants. In this paper, we report that chlorinated 1,4NQs showed remarkably more potent HSR-inducing activities in *Arabidopsis* than the natural 1,4NQs including juglone, lawsone, and plumbagin. The pre-treatment of *Arabidopsis* with chlorinated 1,4NQ enhanced heat tolerance. These results indicate that chlorinated 1,4NQs can increase the heat tolerance of plants by promoting HSR. The underlying mechanism was also discussed.

Materials and methods

Chemicals

1,4-Naphthoquinone (1,4NQ), 2-hydroxy-1,4-naphthoquinone (2HNQ, lawsone), 2-methyl-1,4-naphthoquinone (MNQ, menadione), 2-methoxy-1,4-naphthoquinone (MONQ), 5-hydroxy-1,4-naphthoquinone (5HNQ, juglone), 5-hydroxy-2-methyl-1,4-naphthoquinone (HMNQ, plumbagin), and 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (DDNQ) were purchased from Sigma (Tokyo, Japan). 2,3-Dichloro-1,4-naphthoquinone (DNQ, Phygon) was obtained from Kanto Chemical (Tokyo, Japan). 2-Amino-3-chloro-1,4-naphthoquinone (ACNQ), 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). Geldanamycin (GDA) was from LC Laboratories (Woburn, MA, USA).

GUS assay for HSR

We used the transgenic *Arabidopsis* designated as HSP17.6C-CIProGUS in which the *GUS* gene expression was controlled by the promoter of a small *HSP* (*HSP17.6C-CI*, *At1g53540*) gene of *Arabidopsis*. The HSR was analyzed according to the whole-plant GUS reaction method (Weigel and Glazebrook 2002), with small changes (Murano et al. 2017). Briefly, surface-sterilized seeds of HSP17.6C-CIProGUS were sown on a 1/5 MS medium (1% sucrose and 0.8% agar) in 9-cm plates. The plates were kept under 22 ^{o}C with a 16-h day (60 μmol m^{-2} s^{-1})/8-h night cycle. At 7 days after germination (DAG), two seedlings were soaked in 300 μL of aqueous solutions with 1% (v/v) DMSO containing 1,4NQs at concentrations of 0 (control), 1, 10, and 100 μM in 1.5-mL microtubes. GDA was applied at 50 μM with 1% DMSO. They were incubated for 6 h under the same conditions as above. The seedlings were rinsed twice with deionized water and transferred to wells (two seedlings per well) of a 96-well microplate 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-MUG. The GUS reaction proceeded at 22 ^{o}C for 16 h in the dark and then was terminated by 100 μL of 1 M Na_2CO_3. The 4-MU production was determined by fluorescence (Ex 365 nm and Em 455 nm, Varioskan Flash, Thermo Fisher Scientific, Yokohama, Japan). The calibration curve was produced with an authentic 4-MU.

GUS staining

Staining was performed according to the standard method (Weigel and Glazebrook 2002). The HSP17.6C-CIProGUS seedlings at 7 DAG were treated with 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (DDNQ, 10 μM for 6 h), 2-methyl-1,4-naphthoquinone (MNQ, 10 μM for 6 h), and heat (at 37 ^{o}C for 1 h and then at 22 ^{o}C for 5 h). Seedlings with no treatment were controls.

Quantitative RT-PCR

Wild-type *Arabidopsis* Col-0 was grown under the same conditions as described in the section on the GUS assay for HSR. Aqueous solutions of DDNQ and MNQ were prepared at 10 μM with 1% (v/v) DMSO. At 7 DAG, seedlings were immersed in 4.5 mL of the test solutions in 5 mL test tubes (25 seedlings per tube). The test tubes were incubated at 22 °C for 0, 1, 6, 24, and 48 h under illumination (60 μmol m^{-2} s^{-1}). For the heat treatment, the test tubes containing 4.5 mL of water were incubated at 37 °C for 1 h and then at 22 °C for 5, 23, and 47 h under the same illumination. Total RNA was isolated from the seedlings by using TRI Reagent (Sigma, Tokyo, Japan). cDNAs were synthesized with the SuperScript VILO cDNA Synthesis kit (Thermo Fisher Scientific, Yokohama, Japan). Real-time PCR was performed with the Applied Biosystems 7300 Real Time PCR System using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). After pre-denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 15 sec and 60 °C for 1 min were performed. The experiments were repeated three times. The primers were 5’-ATGTGGCAGCGTTCACAAAC-3’ (forward) and 5’-TTGCCATCCTCAACCTCCAC-3’ (reverse) for *HSP17.6C-CI* (*At1g53540*),

5’-TGAAAGCAAAAAGGCGGTGG-3’ (forward) and 5’-TTCGTCAATCGCATCCACCA-3’ (reverse) for *HSP90.1* (*At5g52640*), and 5’-TGCTGTTGACTACGAGCAGG-3’ (forward) and 5’-CGAGGGCTGGAACAAGACTT-3’ (reverse) for a*ctin2* (*At3g18780*). The delta-delta CT method (Pfaffl 2001) was applied to evaluate the levels of gene expression. The data for the *actin2* expression were used as an internal control.

Immunoblots

The growth of *Arabidopsis*, the chemical and heat treatments, and the incubation periods were the same as described in the section on quantitative RT-PCR. The seedlings (50 mg fresh weight) were homogenized in the sample buffer (500 µl) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After separation using 12.5% (w/v) polyacrylamide gel, proteins were transferred to PVDF membrane. The primary antibodies were anti-HSP17.6C-CI (Agrisera, Vännäs, Sweden) and anti-HSP90.1 (Agrisera). The secondary antibody was horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad, Tokyo, Japan). Chemiluminescence was detected using the LAS-4000 imaging system (Fujifilm, Tokyo, Japan).

Heat tolerance

The heat tolerance of *Arabidopsis* was analyzed as described previously (Murano et al. 2017) with slight modifications. Wild-type seeds were sown on filter paper disks (12 seeds per disk) which were sterilely placed on 1/5 MS medium (Murashige and Skoog 1962) containing 1% sucrose solidified by 0.8% agar in 6-well plates (#92406; TPP, Trasadingen, Switzerland). At 7 DAG, the seedlings on the filter paper were transferred to the test medium prepared in the new plates consisting of the 1/5 MS medium with 1% sucrose, 0.8% agar, 1% DMSO, and DDNQ (0.5, 5, 50, and 500 µM). The medium without DDNQ was the control. The plates were incubated at 22 ^{o}C with a 16-h day (60 µmol m^{-2} s^{-1})/8-h night cycle. The plates were sealed in plastic bags and then heated at 44°C for 1 h in a water bath. The control was no heat treatment. Fresh weights and

chlorophyll contents were measured at 3 days after the heat treatment. Chlorophyll was quantified according to a previous report (Lichtenthaler and Buschmann 2001).

Statistical analysis

Significant differences were determined by Dunnett's test and Tukey-Kramer's test.

Results

Induction of HSR by 1,4NQs

We investigated whether 1,4NQs induced HSR in *Arabidopsis*. The nine 1,4NQs used in this study are listed in Table 1 and the corresponding structures are presented in Fig. 1. 1,4NQ has a basic structure. 2-Hydroxylated 1,4NQ (2HNQ) is called lawsone. 2-Methylated 1,4NQ (MNQ) is menadione, which is also designated as vitamin K3. MONQ is an *O*-methylated lawsone. ACNQ is 2-amino-3-chloro-1,4NQ. 5-Hydroxylated 1,4NQ (5HNQ) and 5-hydroxyl-2-methyl-1,4NQ (HMNQ) are juglone and plumbagin, respectively. 2,3-Dichloro-1,4NQ (DNQ) has the trade name of Phygon. DDNQ is 5,8-dihydroxylated DNQ.

To examine the HSR-inducing activities of the 1,4NQs, we applied the HSR assay system using transgenic *Arabidopsis* possessing the *β-glucuronidase* (*GUS*) gene controlled by the promoter of a small *HSP* gene of *Arabidopsis* (*At1g53540*, *HSP17.6C-CI*) (Matsuoka et al. 2016). We measured the production of fluorescent 4-methylumbelliferone (4-MU) by GUS, which linearly increased during the incubation period. Previously, this *HSP* gene responded remarkably to plant-derived HSR inducers, such as sanguinarine (Matsuoka et al. 2016) and monoterpenes (Hara et al. 2017). In this assay system, we used geldanamycin (GDA), a chaperone inhibitor isolated from *Streptomyces hygroscopicus*, as a positive control for HSR-inducing activity, because GDA has been used to induce the *HSP* genes in *Arabidopsis* (Yamada et al. 2007; Matsuoka et al. 2016). The concentration of GDA was 50 μM because it was the most effective concentration in this experimental system (Matsuoka et al. 2016).

We measured the HSR-inducing activities of 1,4NQs at concentrations of 1, 10, and 100 μM (Fig. 2). Among the nine tested 1,4NQs, DNQ (10 and 100 μM) and DDNQ (10 and 100 μM) showed higher activity levels than the GDA treatment. Especially, the activity of DDNQ at 10 μM was approximately six times higher than that of GDA. 1,4NQ, 2HNQ, MONQ, ACNQ, 5HNQ, and HMNQ tended to show higher activity levels than the controls, whereas their activities did not exceed the GDA activity. MNQ had no activity.

Expression of *HSP* genes promoted by DDNQ

Since DDNQ showed the highest HSR-inducing activity, we studied the mode by which it induced HSR in *Arabidopsis*. The positive and negative controls were heat and MNQ (menadione), respectively. GUS tissue staining assays using *Arabidopsis* seedlings indicated that the administration of DDNQ at 10 μM apparently induced HSR in all parts of the plant, especially in young true leaves and radicle tips (Fig. 3). Little staining appeared in the control and MNQ-treated seedlings. Heat induced apparent *GUS* expression in all parts of the seedlings.

The endogenous *HSP17.6C-CI* gene of wild-type *Arabidopsis* was highly expressed by the addition of DDNQ (Fig. 4A). After this addition, expression occurred within 1 h and peaked at 6 h. On the other hand, MNQ induced *HSP17.6C-CI* gene expression only slightly. Heat induced potent and transient gene expression at 1 h after treatment. We also tested the *HSP90.1* gene, whose expression was enhanced by the HSR-inducing alkaloid, sanguinarine (Hara and Kurita 2014). Consequently, the induction pattern of the *HSP90.1* gene was very similar to that of the *HSP17.6C-CI* gene (Fig. 4B).

The accumulation of the HSP17.6C-CI protein in *Arabidopsis* was promoted by DDNQ administration (Fig. 5A). The protein was detected at 6 h, and more was produced at 24 h after DDNQ was added. Heat also promoted HSP17.6C-CI production. The response to heat was faster than that to DDNQ, with accumulation peaking at 6 h after the heat treatment. The HSP17.6C-CI protein was not detected in the control group or the MNQ administration group. DDNQ and heat increased the accumulation of the HSP90.1 protein, whereas control and MNQ did not affect the accumulation (Fig. 5B).

Effect of DDNQ on the heat tolerance of *Arabidopsis*

Since DDNQ promoted the production of HSPs in *Arabidopsis*, we assumed that DDNQ might affect the plant's heat tolerance. Thus we conducted a heat-tolerance assay (Fig. 6). DDNQ was applied to plants at 7 days after germination (DAG). After incubation for 24 h, the plants were heated at 44 ^{o}C for 1 h, and then fresh weights and chlorophyll contents were determined at 11 DAG. When DDNQ was not supplied (0 μM), the fresh weight and chlorophyll contents were remarkably reduced by heat. However, the addition of DDNQ suppressed heat's ability to decrease fresh weight and chlorophyll contents. This indicates that the administration of DDNQ could enhance the heat tolerance of *Arabidopsis*. The GDA application slightly promoted the plant's heat tolerance, and the moderate heat treatment also enhanced heat tolerance (Supplemental Fig. 1).

Here we used a series of DDNQ concentrations at 0.5, 5, 50, and 500 μM, because in the preliminary test 50 and 500 μM of DDNQ clearly ameliorated the fresh weight loss of the seedlings. Although DDNQ inhibited the loss of fresh weight by heat at 50 μM or more (Fig. 6B), the most effective concentration in the GUS assay was 10 μM (Fig. 2). This inconsistency may be due to differences in the methods of DDNQ treatment applied to the plant. *Arabidopsis* seedlings were totally immersed in the DDNQ solution in the GUS assay, whereas DDNQ was administered to roots on a solid medium in the heat-tolerance assay. On the other hand, 0.5 μM of DDNQ inhibited the decrease in Chl contents by heat stress, suggesting that, under heat stress, the mechanism underlying Chl degradation differed from that underlying fresh weight loss.

Discussion

1,4NQs are distributed in all kingdoms of organisms. Especially, plants produce large numbers of secondary metabolites related to 1,4NQ. It has been documented that plant-derived 1,4NQs, such as juglone (5HNQ) and plumbagin (HMNQ), have allelopathic activities (Widhalm and Rhodes 2016). The mechanisms underlying their

allelopathic activities have been investigated. Juglone and plumbagin induced apoptosis-like cell death in tobacco accompanied by ROS generation (Babula et al. 2009). Programmed cell death in lettuce seedlings was induced by juglone due to ROS generation and DNA fragmentation (Babula et al. 2014). These results suggest that the severe physiological damages caused by herbicidal 1,4NQs like juglone may be associated with ROS generation.

A milder effect of juglone than the herbicidal activity was also studied. A microarray analysis indicated that the administration of juglone upregulated the diverse HSPs of rice seedlings (Chi et al. 2011). This indicates that juglone induced not only apoptosis but also HSR in plants. However, it has not been demonstrated whether HSR-inducing activities are common characteristics of 1,4NQs.

In the present study, most of the tested 1,4NQs exhibited HSR-inducing activities in *Arabidopsis,* although the activity levels varied highly (Fig. 2). The 2,3-dichlorinated 1,4NQs (DNQ and DDNQ) showed remarkable activity levels, but the monochlorinated and nonchlorinated 1,4NQs had low levels. This difference can be explained by the redox potential of 1,4NQs (Widhalm and Rhodes 2016; Bolton and Dunlap 2017). The aromatic B-ring of 1,4NQ can be converted to the corresponding semiquinone radical by accepting an electron from an electron donor (Supplemental Fig. 2). The semiquinone radical transfers an electron to oxygen, and then superoxide anion and other ROS generate. The B-ring of DNQ and DDNQ possesses two chlorines, both of which are electron-accepting species, suggesting that the B-ring facilitates the formation of semiquinone radicals because the dichlorinated B-ring has the potential to show a strong electron-accepting power. If this suggestion is correct, the high levels of HSR-inducing activity by DNQ and DDNQ may be due to efficient ROS production by these compounds via the semiquinone radicals. Indeed, the generation of hydrogen peroxide was apparently detected in the *Arabidopsis* seedlings treated with DNQ and DDNQ (Supplemental Fig. 3). However, hydrogen peroxide was generated only slightly in the MNQ-treated seedlings. In the case of ACNQ, the B-ring was chlorinated but possessed the electron-donating amino group as well. For this reason, it is likely that ACNQ showed low levels of HSR-inducing activity.

The hypothetical mode of action of 1,4NQs in plants is summarized in Fig. 7. The

311 allelopathic 1,4NQs induced apoptosis in plant cells (Babula et al. 2009; Babula et al.
312 2014). Besides that, some 1,4NQs can enhance the heat tolerance of plants via HSR
313 induction (this study). This indicates that 1,4NQs have double-edged functions in plants.
314 Since it has been reported that ROS induced not only apoptosis but also HSR in
315 *Arabidopsis* (Tiwari et al. 2002, Volkov et al. 2006), 1,4NQs may induce different
316 responses by promoting ROS generation. Interestingly, DDNQ, which generated ROS
317 efficiently in *Arabidopsis* seedlings, could induce a strong HSR without herbicidal
318 damage (Fig. 6B and C, white bars). This suggests that there may be a controller that
319 determines the priority between apoptosis and HSR during the response to 1,4NQs in
320 plants.
321 Chlorinated 1,4NQs have been studied for agricultural and medicinal purposes. DNQ
322 (trade name, Phygon) is a fungicide that has been widely used in the storage and
323 germination of crop seeds. The practical advantage is that DNQ shows fungicidal
324 activity without inhibiting the growth of crops (Khan et al. 2001). DDNQ induced
325 apoptosis in cancer cells (Kang et al. 2001; Copeland et al. 2007) and inhibited Fanconi
326 anemia (Landais et al. 2009) and *Leishmania* growth (Lezama-Dávila et al. 2012).
327 Recently, acaricidal and insecticidal activities of DDNQ have been reported (Shang et al.
328 2018; Kim and Lee 2016). However, physiological responses of plants to the
329 chlorinated 1,4NQs have been little studied. From the results of this study, we propose
330 that the chlorinated 1,4NQs may be useful for protecting agricultural crops from
331 extreme high temperatures.
332
333 **References**
334
335 Babula P, Adam V, Kizek R, Sladký Z, Havel L (2009) Naphthoquinones as
336 allelochemical triggers of programmed cell death. Environ Exp Bot 65:330-337
337
338 Babula P, Vaverkova V, Poborilova Z, Ballova L, Masarik M, Provaznik I (2014)
339 Phytotoxic action of naphthoquinone juglone demonstrated on lettuce seedling roots.
340 Plant Physiol Biochem 84:78-86
341

342 Bita CE, Gerats T (2013) Plant tolerance to high temperature in a changing
343 environment: scientific fundamentals and production of heat stress-tolerant crops. Front
344 Plant Sci 4:273 (1-18)
345
346 Björkman M, Klingen I, Birch AN, Bones AM, Bruce TJ, Johansen TJ, Meadow R,
347 Mølmann J, Seljåsen R, Smart LE, Stewart D (2011) Phytochemicals of Brassicaceae in
348 plant protection and human health - influences of climate, environment and agronomic
349 practice. Phytochemistry 72:538-556
350
351 Bolton JL, Dunlap T (2017) Formation and biological targets of quinones: cytotoxic
352 versus cytoprotective effects. Chem Res Toxicol 30:13-37
353
354 Chi WC, Fu SF, Huang TL, Chen YA, Chen CC, Huang HJ (2011) Identification of
355 transcriptome profiles and signaling pathways for the allelochemical juglone in rice
356 roots. Plant Mol Biol 77:591-607
357
358 Copeland RL Jr, Das JR, Bakare O, Enwerem NM, Berhe S, Hillaire K, White D,
359 Beyene D, Kassim OO, Kanaan YM (2007) Cytotoxicity of
360 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone in androgen-dependent and
361 -independent prostate cancer cell lines. Anticancer Res 27:1537-1546
362
363 Clarke SM, Mur LA, Wood JE, Scott IM (2004) Salicylic acid dependent signaling
364 promotes basal thermotolerance but is not essential for acquired thermotolerance in
365 *Arabidopsis thaliana*. Plant J 38:432-447
366
367 Deryng D, Conway D, Ramankutty N, Price J, Warren R (2014) Global crop yield
368 response to extreme heat stress under multiple climate change futures. Environ Res Lett
369 9:034011 (1-13)
370
371 Hara M, Harazaki A, Tabata K (2013) Administration of isothiocyanates enhances heat
372 tolerance in *Arabidopsis thaliana*. Plant Growth Regul 69:71-77

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

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

Kang KH, Lee KH, Kim MY, Choi KH (2001) Caspase-3-mediated cleavage of the NF-κB subunit p65 at the NH_2 terminus potentiates naphthoquinone analog-induced apoptosis. J Biol Chem 276:24638-24644

Khan MA, Gul B, Weber DJ (2001) Seed germination characteristics of *Halogeton glomeratus*. Can J Bot 79:1189-1194

Kim MG, Lee HS (2016) Insecticidal toxicities of naphthoquinone and its structural derivatives. Appl Biol Chem 59:3-8

Kotak S, Larkindale J, Lee U, von Koskull-Döring P, Vierling E, Scharf KD (2007) Complexity of the heat stress response in plants. Curr Opin Plant Biol 10:310-316

Landais I, Sobeck A, Stone S, LaChapelle A, Hoatlin ME (2009) A novel cell-free screen identifies a potent inhibitor of the Fanconi anemia pathway. Int J Cancer 124:783-792

Lezama-Dávila CM, Isaac-Márquez AP, Kapadia G, Owens K, Oghumu S, Beverley S, Satoskar AR (2012) Leishmanicidal activity of two naphthoquinones against *Leishmania donovani*. Biol Pharm Bull 35:1761-1764

Lichtenthaler HK, Buschmann C (2001) Chlorophylls and carotenoids - measurement and characterization by UV-VIS. In: Current protocols in food analytical chemistry (CPFA), (Supplement 1). John Wiley, New York, F4.3.1-F4.3.8

Macías FA, Molinillo JM, Varela RM, Galindo JC (2007) Allelopathy - a natural alternative for weed control. Pest Manag Sci 63:327-348

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

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 Biotechnol 33:409-413

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 Biotechnol Rep 11:107-113

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473-497

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res 29:e45-e45

Ruelland E, Zachowski A (2010) How plants sense temperature. Environ Exp Bot 69:225-232

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 Environ 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 Mol Biol 59:697-711

Shang XF, Liu YQ, Guo X, Miao XL, Chen C, Zhang JX, Xu XS, Yang GZ, Yang CJ, Li JC, Zhang XS (2018) Application of sustainable natural resources in agriculture: acaricidal and enzyme inhibitory activities of naphthoquinones and their analogs against *Psoroptes cuniculi*. Sci Rep 8:1609 (1-9)

Soderquist CJ (1973) Juglone and allelopathy. J Chem Educ 50:782-783

Tiwari BS, Belenghi B, Levine A (2002) Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death. Plant Physiol 128:1271-1281

Volkov RA, Panchuk II, Mullineaux PM, Schöffl F (2006) Heat stress-induced H_2O_2 is required for effective expression of heat shock genes in *Arabidopsis*. Plant Mol Biol 61:733-746

Wahid A, Gelani S, Ashraf M, Foolad MR (2007) Heat tolerance in plants: An overview. Environ Exp Bot 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. J Exp Bot 64:391-403

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

Widhalm JR, Rhodes D (2016) Biosynthesis and molecular actions of specialized 1,4-naphthoquinone natural products produced by horticultural plants. Hortic Res 3:16046 (1-17)

Wink M, Twardowski T (1992) Allelochemical properties of alkaloids. Effects on plants, bacteria and protein biosynthesis. In: Rizvi SJH and Rizvi V (eds) Allelopathy. Basic and Applied Aspects. Chapman & Hall, London, pp 129-150

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*. J Biol Chem 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. Sci Rep 26:8030 (1-8)

Figure legends

Fig. 1 Chemical structures of 1,4NQs used in this study. Abbreviations and common names are shown.

Fig. 2 Effects of 1,4NQs on HSR induction in *Arabidopsis*. The HSR-inducing activities of nine 1,4NQs were determined by using HSP17.6C-CIProGUS plants. The concentrations of 1,4NQs were 1 μM (light gray bars), 10 μM (dark gray bars), and 100 μM (black bars). The results of control (0 μM) and geldanamycin (GDA, 50 μM) are shown by white bars. The GUS activities are expressed as 4-MU production during incubation for 16 h. Means and SD are denoted (three individual experiments). Asterisks indicate significant differences (*$p < 0.05$ and **$p < 0.01$) as determined by Dunnett's test in comparisons of control and other treatments.

Fig. 3 GUS staining of the HSP17.6C-CIProGUS seedlings. Plants were treated with

DDNQ (10 μM for 6 h), MNQ (10 μM for 6 h), or heat (HS, 37 ºC for 1 h followed by 22 ºC for 5 h). The scale bars are 1 mm.

Fig. 4 Expression levels of *HSP17.6C-CI* and *HSP90.1* genes in wild-type *Arabidopsis*. Seedlings were treated with DDNQ (10 μM), MNQ (10 μM), or HS (37 °C for 1 h). A real-time PCR system was applied to determine the relative mRNA amounts for *HSP17.6C-CI* (*At1g53540*) and *HSP90.1* (*At5g52640*) by using *actin2* (*At3g18780*) as an internal standard. Means and SD are shown (three individual experiments). Asterisks indicate significant differences ($p < 0.01$) as determined by Dunnett's test in comparisons of zero-time control and treatments.

Fig. 5 Immunoblots for HSP17.6C-CI and HSP90.1 proteins. HSP17.6C-CI (A) and HSP90.1 (B) were detected after seedlings were treated with DDNQ (10 μM), MNQ (10 μM), or heat (HS, 37 ºC for 1 h). Control was 1% (v/v) DMSO. A Rubisco large subunit stained with Coomassie Brilliant Blue is shown in C as a protein loading control.

Fig. 6 Fresh weight and chlorophyll contents of heat-stressed *Arabidopsis* seedlings pre-treated with DDNQ. (A) The experimental schedule. Treatment of DDNQ was administered at 7 days after germination (DAG). Heat (44 ºC for 1 h) was applied at 8 DAG. This heat treatment caused severe damage to control seedlings (0 μM DDNQ). Measurements were taken at 11 DAG. Fresh weight (B) and chlorophyll (Chl) contents (C) are shown. White and gray bars represent plants not exposed to heat and exposed to heat, respectively. Means and SE are denoted (six individual experiments). Tukey-Kramer's test was applied to judge significant differences ($p < 0.01$).

Fig. 7 A hypothetical scheme showing action modes of 1,4NQs in plants. Reactive oxygen species (ROS) generated from 1,4NQs can induce apoptosis and heat shock response (HSR). Apoptosis and HSR may promote herbicidal damage and heat tolerance, respectively.

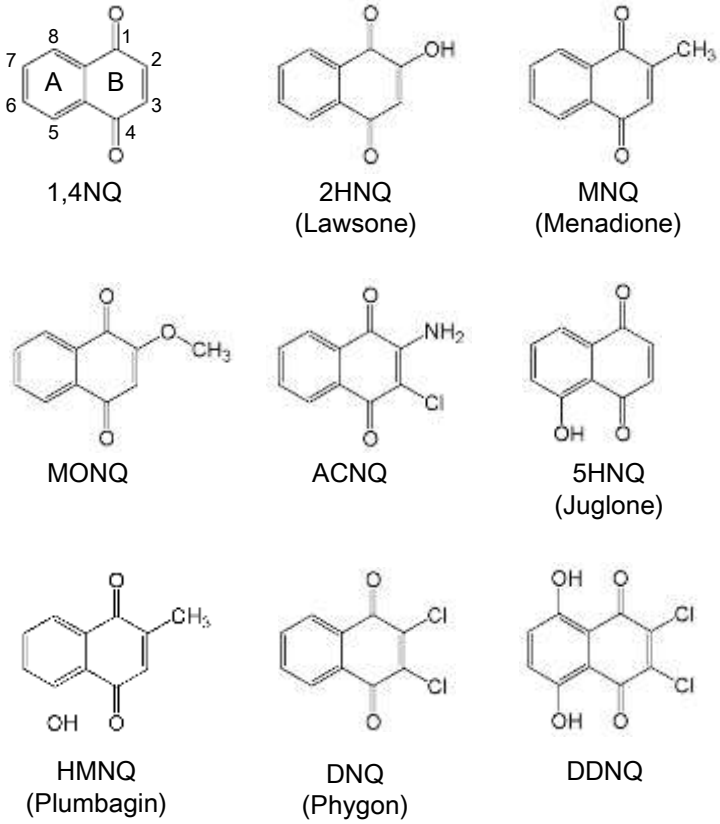


Figure 1 Kato et al.

530
531

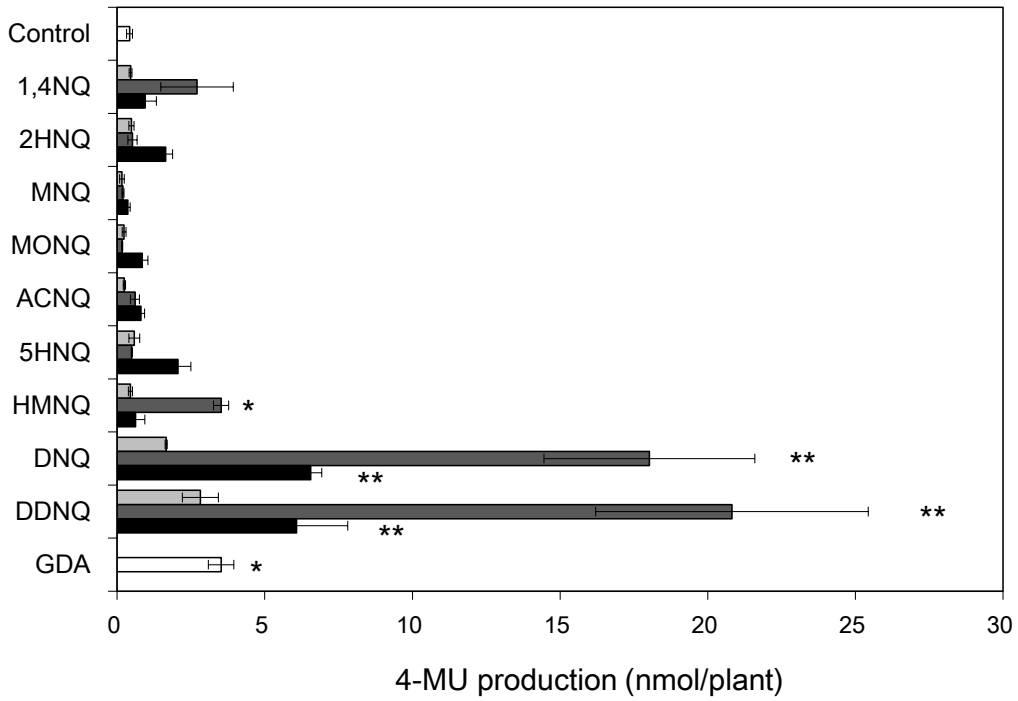


Figure 2 Kato et al.

532

533

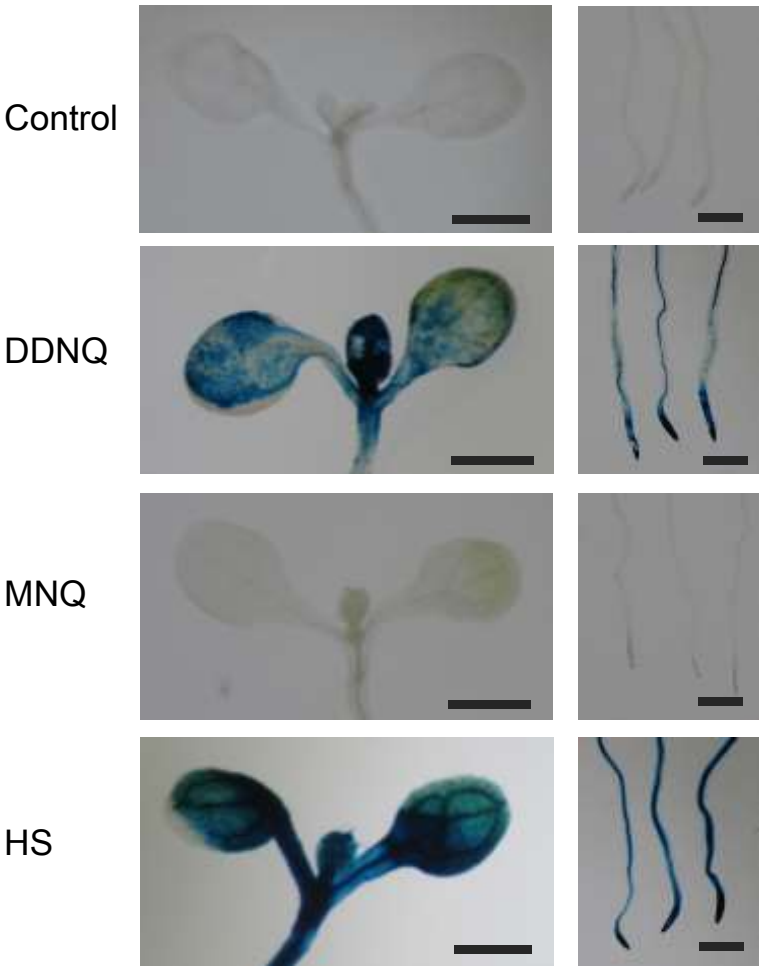


Figure 3 Kato et al.

534
535

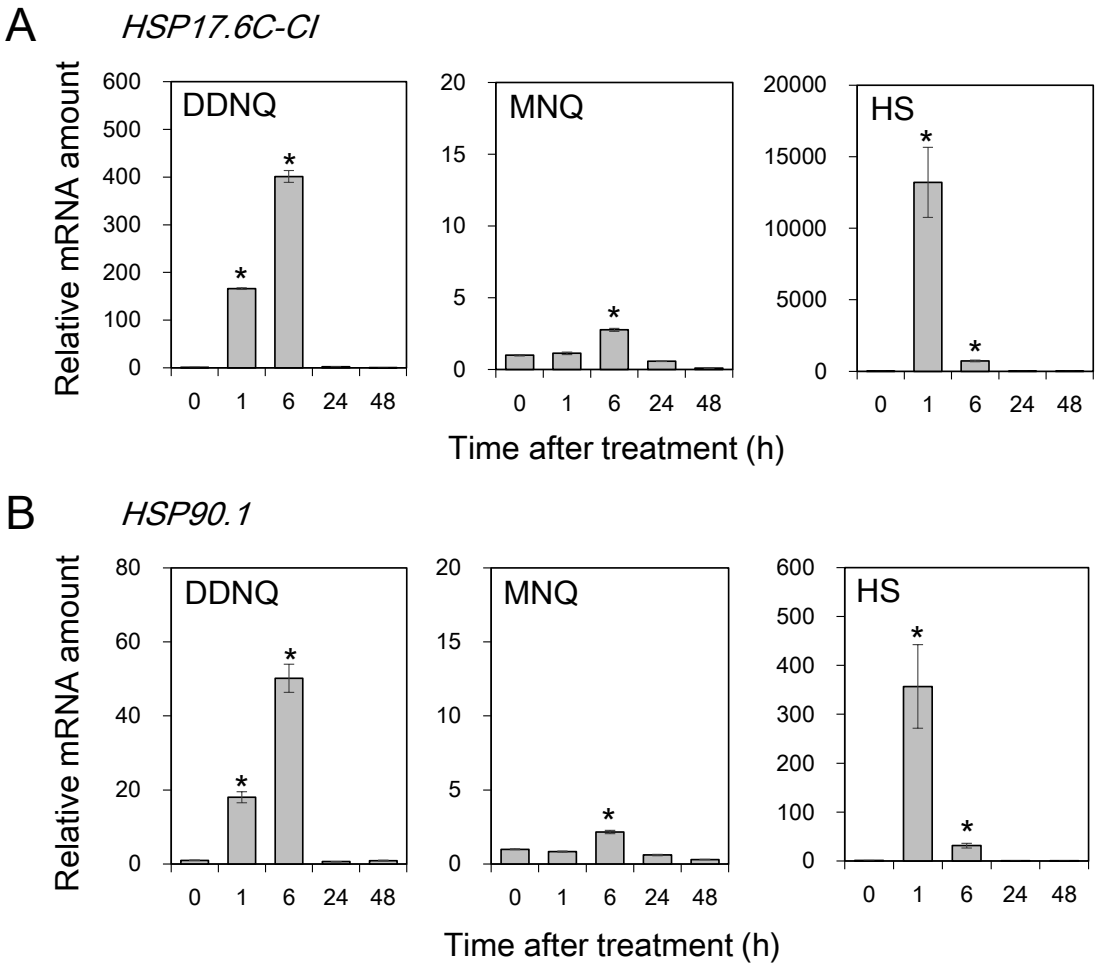


Figure 4 Kato et al.

536

537

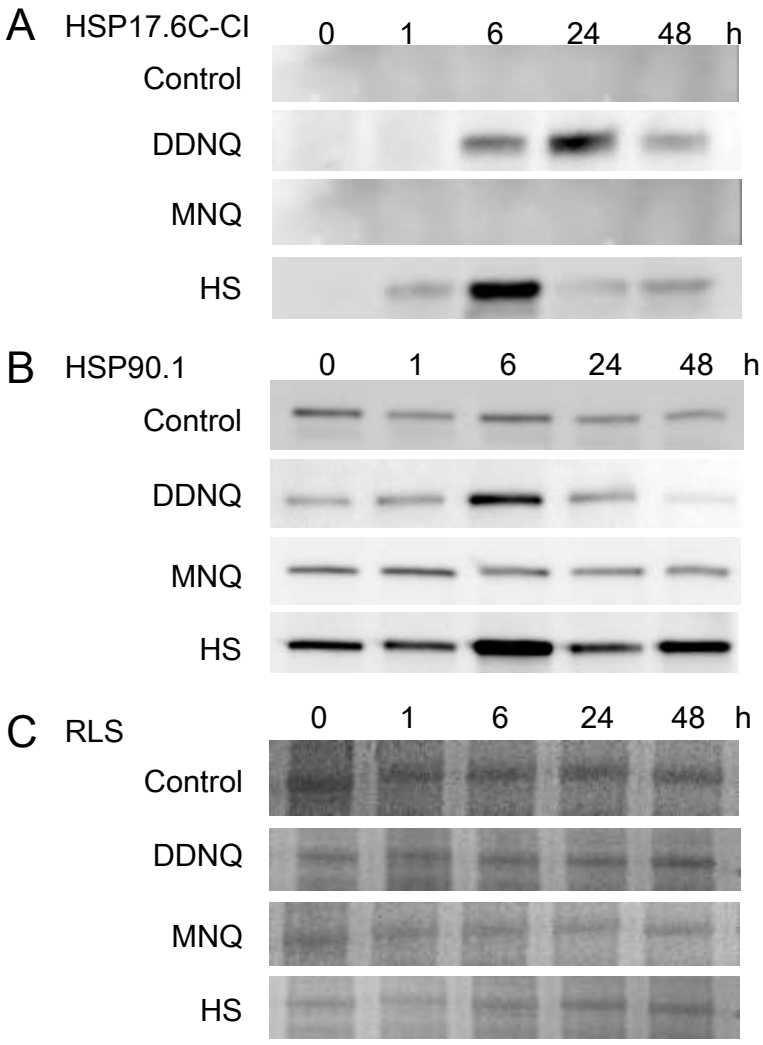


Figure 5 Kato et al.

538

539

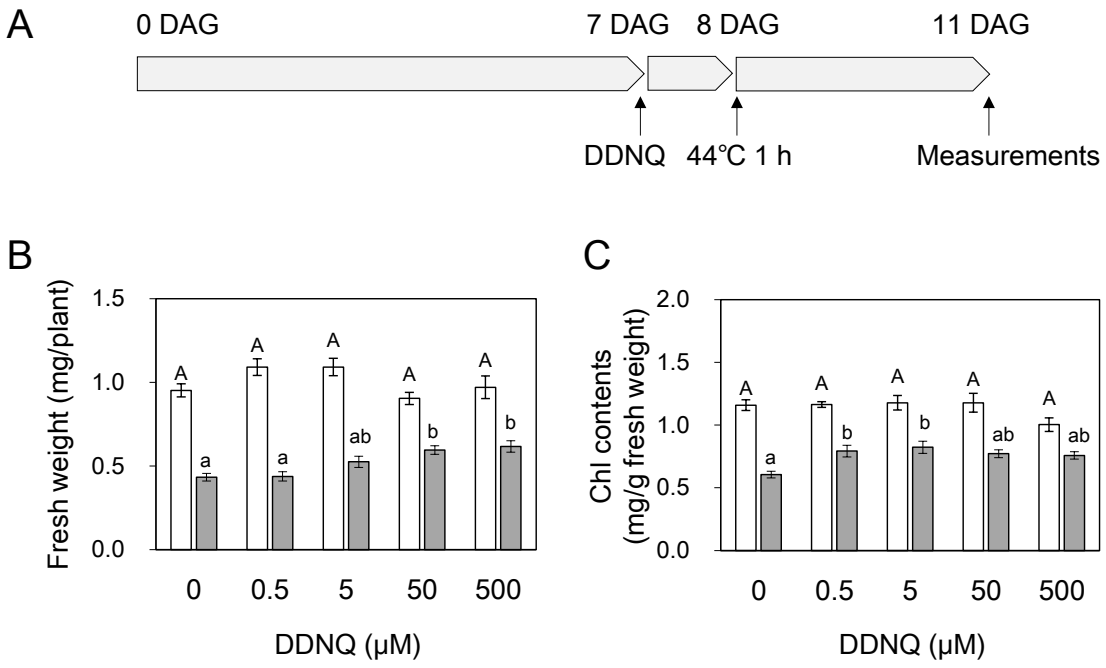


Figure 6 Kato et al.

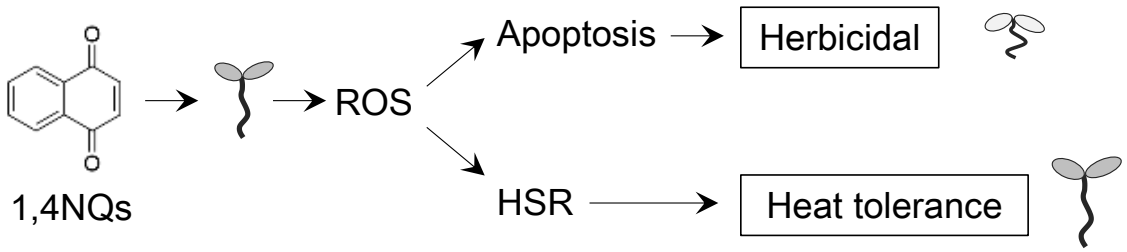
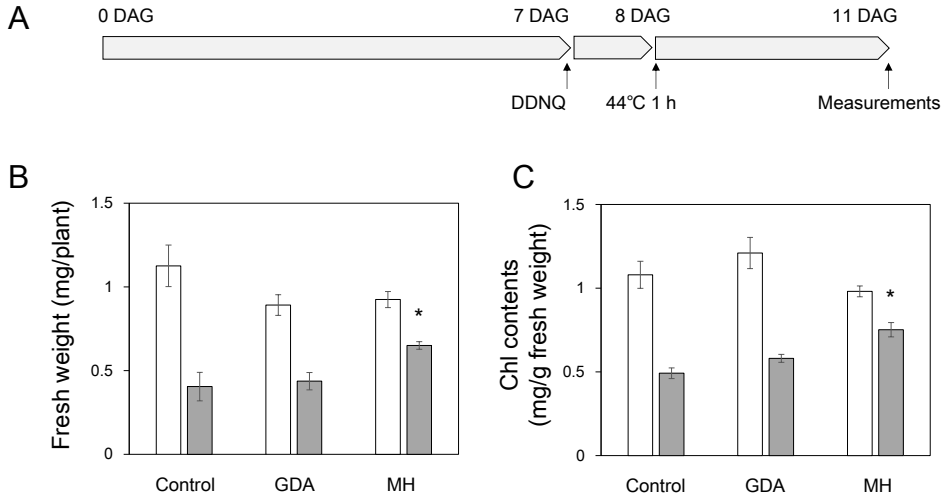


Figure 7 Kato et al.

Table 1 1,4-Naphthoquinones used in this study

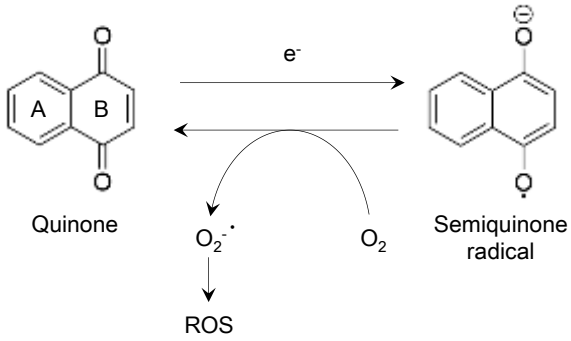
Chemical name	Abbreviation	Common name
1,4-Naphthoquinone	1,4NQ	
2-Hydroxy-1,4-naphthoquinone	2HNQ	Lawsone
2-Methyl-1,4-naphthoquinone	MNQ	Menadione
2-Methoxy-1,4-naphthoquinone	MONQ	
2-Amino-3-chloro-1,4-naphthoquinone	ACNQ	
5-Hydroxy-1,4-naphthoquinone	5HNQ	Juglone
5-Hydroxy-2-methyl-1,4-naphthoquinone	HMNQ	Plumbagin
2,3-Dichloro-1,4-naphthoquinone	DNQ	Phygon
2,3-Dichloro-5,8-dihydroxy-1,4-naphthoquinone	DDNQ	

Table 1 Kato et al.



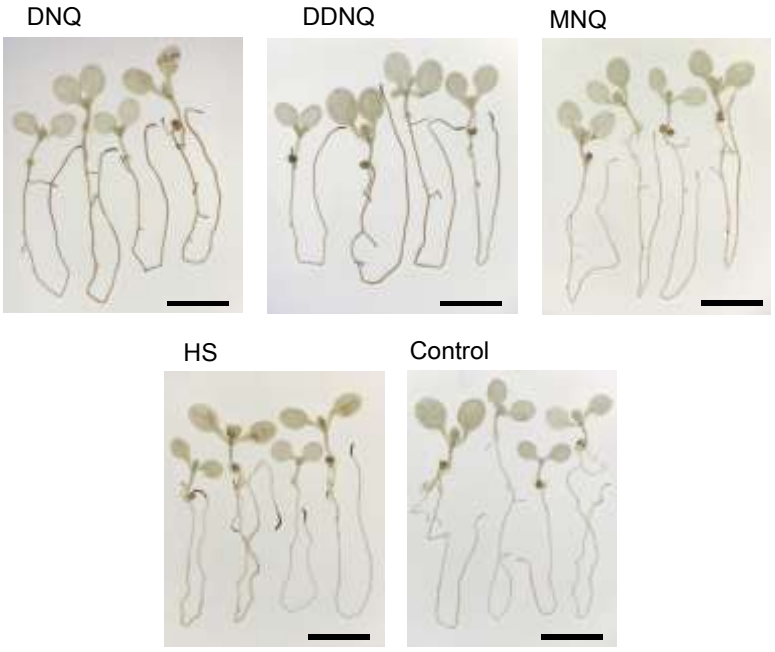
Supplemental Fig. 1 Fresh weight and chlorophyll contents of heat-stressed *Arabidopsis* seedlings which were pre-treated with geldanamycin (GDA, 50 μM) and moderate heat (MH, 37 ºC for 1 h). The experiments were conducted under the same condition as in Fig. 6 in the text (A). Treatments of GDA and MH were done at 7 days after germination (DAG). Heat stress (44 ºC for 1 h) was applied at 8 DAG. Fresh weight (B) and chlorophyll (Chl) contents (C) were measured at 11 DAG. White and gray bars represent plants not exposed to heat and exposed to heat, respectively. Means and SE are denoted (four individual experiments). *Student's t-test was employed to judge significant differences ($p < 0.01$) between control and treatments.

Authors: Naoki Kato, Daiki Yamakawa, Naoya Yamauchi, Yoshihisa Hashimoto, Erina Matsuoka, Masakazu Hara*
*E-mail: hara.masakazu@shizuoka.ac.jp



Supplemental Fig. 2 A putative action of 1,4NQs. A semiquinone radical is formed from a quinone by electron acceptance. The semiquinone radical converts oxygen to superoxide anion by electron addition. Other reactive oxygen species (ROS) are produced from the superoxide anion.

Authors: Naoki Kato, Daiki Yamakawa, Naoya Yamauchi, Yoshihisa Hashimoto, Erina Matsuoka, Masakazu Hara*
*E-mail: hara.masakazu@shizuoka.ac.jp



Supplemental Fig. 3 Detection of hydrogen peroxide by using 3,3′-diaminobenzidine (DAB). *Arabidopsis* seedlings at 7 days after germination were subjected to various treatments for 60 min. DNQ (2,3-dichloro-1,4-naphthoquinone, 10 μM), DDNQ (2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone, 10 μM), MNQ (2-methyl-1,4-naphthoquinone, 10 μM), HS (37 ºC), and control (no treatment). After each treatment, the seedlings were incubated in 4.7 mM DAB solution for 4 h at 22 ºC in the dark, and then photographs were taken. Bars represent 5 mm. The DNQ-, DDNQ-, and HS-treated root tips were densely stained with DAB (dark brown). However, the control root tips and the MNQ-treated root tips were little stained.

Authors: Naoki Kato, Daiki Yamakawa, Naoya Yamauchi, Yoshihisa Hashimoto, Erina Matsuoka, Masakazu Hara*
*E-mail: hara.masakazu@shizuoka.ac.jp