Induction of Systemic Acquired Resistance by Heat Shock Treatment in Arabidopsis

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Systemic acquired resistance (SAR) is a potent innate immunity system in plants and has been used in rice fields. Development of SAR, involving priming, is achieved by activation of salicylic acid (SA)-mediated pathway. To determine whether heat shock (HS) treatment can induce SAR, we analyzed the effects of HS on Arabidopsis. HS treatment induced disease resistance, expression of SAR marker genes, and SA accumulation in wild-type but not in SA-deficient sid2 and NahG plants, indicating induction of SAR. Time course analysis of the effects of HS indicated that SAR was activated transiently, differently from biological induction, with a peak at 2–3 d after HS, and that it ceased in several days. Production of reactive oxygen species was observed before SA biosynthesis, which might be a trigger for SAR activation. The data presented here suggest that HS can induce SAR, but there exist unknown regulation mechanisms for the maintenance of SAR.

Key words: systemic acquired resistance; heat shock; salicylic acid; disease resistance; Arabidopsis

Induced resistance is an inducible defense mechanism of plants that is effective against various pathogens. It is activated by stimuli such as pathogen infection, microbial symbiosis, and wounding. Systemic acquired resistance (SAR) is a form of induced resistance, that is induced after a hypersensitive response (HR) caused by pathogens. It plays an important role in defending plants from further attack by pathogens.1,2 Several synthetic chemicals capable of inducing SAR, plant activators, have been developed and are used in rice fields in Japan.2-3 The modes of action of some plant activators have been characterized in Arabidopsis and tobacco, and have been used as chemical probes to clarify the priming mechanisms of SAR.2-9

The mechanisms of SAR in Arabidopsis, tobacco, and rice have been well-characterized. A set of pathogenesis-related (PR) genes have been identified as SAR marker genes, and the involvement of salicylic acid (SA) in SAR development was confirmed by analysis using mutants and transgenic plants defective in SAR-related signaling pathways.10,11

In the practical cultivation of vegetables in greenhouses, high temperature treatment, at 45 °C, for 1–2 h has been developed to protect plants from insects and diseases.12,13 The protective effects are due not only to the direct action of high temperature on insects and microbes, but also to the activation of defense mechanisms of the plants. Elevated levels of SA and the expression of defense-related genes after heat shock (HS) treatment have been observed in cucumber and tomato, however, the involvement of SA in the HS-induced defense mechanism remains to be determined.14 In melon a short-duration HS, dipping in hot water at 50 °C for 20 s, induced the resistance to Botrytis cinerea only when inoculation was performed within 1 d of HS treatment.15 Under this experimental condition, induction of defense related genes was detected 12 h after HS treatment, whereas no information was obtained on the hormonal signaling pathway that figured in this resistance. Because control of diseases is economically important, molecular evidence of the induction of disease resistance by HS treatment ought to be determined.

Plant heat stress responses have been extensively studied to clarify the mechanism of thermotolerance.16 Many important molecules functioning in physiological events activated by heat have been identified and characterized, however, the relationship between thermotolerance and disease resistance is poorly understood. To determine the molecular mechanism of resistance induced by HS treatment, we characterized the induction and maintenance of induced resistance in Arabidopsis. We found that HS treatment induced SAR through activation of SA biosynthesis.

Materials and Methods

Plant materials. Arabidopsis thaliana was grown in sterilized potting soil (Kureha, Tokyo, Japan) in pots (5 × 5 × 5 cm³) in a growth

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Abbreviations: dpt, days post treatment; HS, heat shock; PR, pathogenesis relate; Pst, Pseudomonas syringae pv. tomato DC3000; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance
HS treatment. Aerial HS treatment was performed by placing 4-week-old plants for 1, 2, or 3 h at 45 °C in a growth chamber preheated to 45 °C. Hot water dipping method was applied by immersing 4-week-old plants in hot water preheated to various temperatures. After HS treatment, the plants were cultured in a growth chamber under normal conditions.

Challenge inoculation. For the Pseudomonas syringae pv. tomato DC3000 (Pst) infection assay, plants were subjected to HS treatment. Pst was inoculated by dipping the plants in the bacterial solution (2 × 10^5 colony-forming units per mL). After incubation for 3 d at 22 °C, leaves were harvested from the plants. Three leaves were homogenized together in 10 mM MgCl2. Homogenate was then plated on nutrient broth agar containing rifampicin (50 μg/mL) at appropriate dilutions. After incubation at 28 °C, the number of rifampicin-resistant bacterial colonies was counted. The experiment was repeated three times.

Real-time PCR analysis. Total RNA was extracted using Sepasol-RNA I super reagent (Nacalai Tesque, Kyoto, Japan), followed by phenol-chloroform mixture purification, according to the manufacturer’s instructions. Total RNA (0.5 μg) was converted into cDNA with PrimeScript RT Reagent Kit (Takara Bio) according to the manufacturer’s instructions, yielding 10 μL cDNA solution. Real-time PCR was performed using the GeneAmp SDS 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) with SYBR Premix Ex Taq (Takara Bio). The PCR reaction contained 1 μL SYBR Premix Ex Taq, 0.2 mM of each primer, and the appropriate dilution of cDNA in a final volume of 24 μL. The following PCR program was used: initial denaturation at 94 °C for 10 s, then 40 cycles of 94 °C for 5 s and 60 °C for 34 s. The expression level of each sample was normalized to ubiquitin (UBQ). Gene-specific primer pairs for PR-1, PR-2, PR-5 and ICSl, previously described and 5'-GGTGTTCTAAACGATCTCCCG-3' and 5'-TGGCAGAAGAAGGCCTGGAAT-3' for BSMT1 were used in real-time PCR.17,18

Measurement of salicylic acid (SA) level. Leaf tissues (approximately 0.3 g fresh weight) frozen in liquid nitrogen were ground with a mortar and pestle, homogenized, and extracted with 5 mL of 90% methanol and then with 5 mL of 100% methanol. These two extracts were combined, and 2 mL of the mixture was dried at 40 °C. The SA and SAG (free SA and SA-glucoside) contents were measured as previously described.5-7

Histochemical detection of ROS production. Leaves were harvested at 1, 3, 6, 12, 24, and 48 h after treatment and placed immediately in 1 mg/mL of Diaminobenzidine (DAB)-HCl pH 3.8, and this was incubated for 6 h in the dark at room temperature. The stained leaves were cleared in boiling ethanol.

Results

Induction of disease resistance in Arabidopsis by aerial heating

HS treatment at 45 °C for 1–2 h in a greenhouse induced disease resistance in cucumber and tomato. The expression of defense related genes and the accumulation of SA in these plants suggest that the SA-mediated signaling pathway functions in resistance induction, but the details of the mechanisms remained to be clarified. Hence, we examined the effect of HS treatment on Arabidopsis, a model plant for the analysis of plant defense mechanisms. First, we analyzed the effect of HS treatment at 45 °C for 1 h on Arabidopsis wild-type plants (Col-0) in a growth chamber. Disease resistance was examined by challenge inoculation with a virulent bacterial pathogen, Pseudomonas syringae pv. tomato DC3000, 3 d post treatment (dpt). By 3 d post challenge inoculation, bacterial growth in the plant tissues of the HS-treated plants was about 3 times lower than that of the control plants, indicating that HS treatment induced disease resistance (Fig. 1A). Real-time PCR analysis indicated that HS treatment for 1 h induced accumulation of the transcripts for PR-1, a maker gene of systemic acquired resistance (Fig. 1B). These results suggest that HS treatment can induce SAR-like disease resistance also in Arabidopsis. However, analysis of PR-1 gene expression over a 3-d time course indicated that the efficacy of HS treatment is not stable enough for quantitative investigation, suggesting that a more effective, reproducible experimental system is needed to investigate the molecular mechanism of HS-induced resistance (Fig. 1B). Hence, for further investigation, we utilized the hot water dipping method, capable of transferring heat rapidly to plant tissues, which has been used for post-harvest treatment of fruits for enhanced storability.9,20

Induction of systemic acquired resistance in Arabidopsis by HS treatment

First, we optimized HS treatment conditions by hot water dipping for Arabidopsis. The expression of PR-1 was analyzed after HS treatment at various temperatures for 2.5 min. Treatment at 45 °C induced a high level of accumulation of PR-1 transcripts, whereas very slight induction was observed in the plants treated at 35 to 42.5 °C (Fig. 2A). High-level expression of PR-1 was also detected in plant samples treated at 47.5 °C, but plant growth was heavily affected, with wilting (data not shown). Next, various treatment periods were assessed
by dipping in 45°C water, followed by analysis of PR-1 expression. Accumulation of the PR-1 transcripts increased gradually depending on the treating period, from 1 to 3.5 min, indicating that treatment for 2, 2.5, or 3 min induced maximum levels of PR-1 expression without wilting (Fig. 2B). Based on these results, we used dipping at 45°C for 2.5 min as the optimal experimental condition for further analysis.

By hot water dipping treatment at 45°C for 2.5 min, significant induction of PR-2 and PR-5, in addition to PR-1, was detected 2 dpt (Fig. 2C, D). Next, to confirm induction of disease resistance by the hot water dipping method, challenge inoculation with Pst was done 3 dpt. By 3 d post challenge inoculation, the bacterial growth in the tissues of the HS-treated plants was about 10-fold lower than in the control plants (Fig. 2E). A yellowish disease symptom appeared in the non-treated control plants by 5 d after the inoculation, but this was suppressed in the HS-treated plants (Fig. 2F). The induction of disease resistance and the expression of PR-1, PR-2, and PR-5 indicated that HS treatment induced SAR in Arabidopsis.

To clarify whether SA was involved in the induction of disease resistance by HS treatment, we examined the effects of HS treatment on the sid2 mutant, defective in SA biosynthesis, and a NahG transgenic plant that is unable to accumulate SA due to expression of SA hydroxylase. In the HS-treated sid2 and NahG plants, PR-1 expression and enhanced resistance to Pst were not detected (Fig. 3A, B), indicating that SA biosynthesis is required for the induction of disease resistance by HS treatment. These results indicate that HS treatment can induce SAR through activation of SA biosynthesis. In general, SAR is activated by hypersensitive responses against pathogen infection and lasts for a long period to protect the plant from further attack by pathogens. However, in contrast with the challenge inoculation of 3 dpt, no induction of disease resistance was detected when the inoculation was performed 1 or 5 dpt, indicating an unknown regulation mechanism for the maintenance of HS-induced SAR.

To achieve a better understanding of SAR activated by HS treatment, the levels of free SA and total SA (free SA and SA glucoside) and the expression of SA-related

Fig. 2. Induction of SAR in Arabidopsis by Hot Water Dipping.

Four-week-old wild-type plants (Col-0) were immersed into hot water preheated to various temperatures for optimization and 45°C for other experiments. Real-time PCR was performed as described in “Materials and Methods.” Transcript levels were normalized to the expression of UBQ measured in the same samples. Relative mRNA levels between the control and HS-treated plants of the genes tested are presented. Values are shown as averages with standard deviation of three independent experiments. The experiments were performed with five or more plants, and were repeated 3 times with similar results. A, PR-1 expression by HS treatment at various temperatures. HS treatment was performed at 35, 37.5, 40, 42.5, 45, and 47.5°C for 2.5 min 2 d prior to analysis of PR-1 expression. B, PR-1 expression by HS treatment at 45°C for various times. HS treatment was performed at 45°C for 1, 1.5, 2, 2.5, 3, 3.5 min 2 d prior to analysis of PR-1 expression. C, PR-2 expression by HS treatment at 45°C for 2.5 min. D, PR-3 expression by HS treatment at 45°C for 2.5 min. E, Induction of disease resistance against Pst. Challenge inoculation was performed 3 d after HS treatment at 45°C for 2.5 min. Evaluation of the induction of disease resistance was done as described in “Materials and Methods” and Fig. 1. F, Disease symptoms by inoculation of Pst. Photograph was taken 5 d after inoculation.
genes were analyzed in HS-treated and non-treated Arabidopsis over a 9-d and a 7-d time course, respectively. Induction of SA biosynthesis gene ICS1, which encodes isochorismate synthase 1, was transiently observed only at 2 dpt (Fig. 4A). The accumulation of both free SA and total SA started from 2 dpt (Fig. 4C, D). The levels of free SA reached a peak at 3 dpt and then rapidly decreased in the next day, and to the normal level by 7 dpt. The levels of total SA also reached a peak at 3 dpt and then gradually decreased. Induction of PR-1 was observed also from 2 dpt, and gradually decreased from the next day to the normal level by 7 dpt (Fig. 4B). Thus, HS treatment triggers the transient induction of ICS1, initiating an accumulation of free SA, but the responses in terms of total SA levels and PR-1 expression, downstream of free SA, were also transient and relatively slower. This indicates that physiological changes relating SAR were transient and effective only on 2 dpt and probably also on 3 dpt, which might be the reason for the failure to detect disease resistance on 1 and 5 dpt.

Production of reactive oxygen species by HS treatment

It has been reported that the production of reactive oxygen species (ROS) was involved in SAR induction stimulated by pathogenic infection. On the other hand, ROS production was also activated by HS treatment. We examined to determine whether ROS was produced before SA biosynthesis in the leaves of HS-treated wild-type plants. DAB staining of leaf tissues indicated that ROS production started 1 h after treatment, reached a maximum at 6 h (Fig. 5), and gradually decreased to a very low level by 48 h after treatment (data not shown). A similar level of ROS production was observed 6 h after treatment in the sid2 mutant (Fig. 5). These data confirm that ROS production before SA biosynthesis might be a trigger for SAR induction by HS treatment.

Induction of benzoic acid/SA carboxyl methyltransferase by HS treatment

SA is metabolized by benzoic acid/SA carboxyl methyltransferase (BSMT1) to methyl salicylate (MeSA), a volatile ester, which can be an airborne
Heat shock stress has been reported to induce multiple signaling pathways containing SA-, abscisic acid (ABA)-, JA-, and ethylene-mediated signals. Endogenous SA was reported to promote basal thermotolerance by inhibiting oxidative stress by the same signaling pathway as for SAR induction. The cessation of ROS production before SA accumulation in our study can be explained by the thermotolerance mechanism by SA, whereas ROS can be a trigger for activation of the SAR signaling pathway, as reported previously. Further investigation is needed to clarify the relationship between ROS production and SA biosynthesis in HS-induced SAR.

The signaling pathway for SAR including SA biosynthesis started at least 24 h after HS and was detected from 2 d after HS, following ROS production with a peak at 6 h after HS. Considering the activation patterns of ROS production and SA biosynthesis, it is plausible that ROS is an intermediate signal for SAR induction. ROS production was quickly activated by HS treatment, similarly to that by the pathogenic infection, however, PR-1 expression was detected later, at least 24 h after treatment, whereas induction by pathogenic infection is detected at 6 h. Multiple responsive events activated by HS must have influenced on the initiation of the SAR signaling pathway, however, further study is need to explain the delayed initiation. In addition to hormonal signaling pathways, heat shock transcription factors (HSFs), DNA binding transcriptional regulators, and multiprotein bridging factor 1c (MBF1c), non-DNA-binding transcriptional co-activator, have been identified as key components coordinating the multiple response to heat stress. Thus HSFs and MBF1c might play important roles also in the induction or regulation of disease resistance by HS treatment. This is currently under investigation.

It has also been reported that SA is not essential for acquired thermotolerance or heat acclimation, by investigation using several SA-related mutants. Induction of PR-1 was detected after 5 h of incubation at 38 °C, but no induction of SA accumulation by heat was observed in Arabidopsis. We detected SA accumulation, however, which was transient and clearly detected only for a limited duration. The elevated SA levels after HS were observed in some plant species, but their contribution to thermotolerance remains to be determined. On the other hand, at the practical research level, SA accumulation and disease resistance were observed after heat treatment, but the detailed mechanism of the resistance induction is unknown due to lack of genetic information. Using Arabidopsis, a model plant with much genetic information, by an optimized hotwater dipping method, we found that HS treatment activated the SAR signaling pathway through SA biosynthesis.

During the transient activation of HS-induced SAR, even upregulated free SA levels, in addition to ICS1 levels, decreased to normal levels in a short time, suggesting that not only inactivation of SA biosynthesis but also activation of SA metabolism is involved in the regulation of HS-induced SAR. It has been reported that the ABA-mediated signaling pathway was also activated by HS treatment. We confirmed that under our experimental conditions, pretreatment with ABA (0.1 mg/pot) reduced HS-induced PR-1 expression by 85% as compared to HS treatment without ABA. Expression of the ABA responsive RAB18 gene 2 dpt by the hot water dipping method was 7 times greater than in the control plants, which might contribute to the
inactivation of SA biosynthesis by the SA-ABA cross-talk mechanism.\textsuperscript{17,22} On the other hand, we detected induction of benzoic acid/SA carboxyl methyltransferase (BSMT1) under HS treatment, which started at 1 d after HS, earlier than the activation of SA biosynthesis (Fig. 6). Recent studies have indicated that overexpression of OsBSMT1 in Arabidopsis reduced SAR induction by inhibiting the accumulation of SA.\textsuperscript{33} Thus it is possible that conversion of SA to methyl-SA contributes to decrease in SA levels.

HS, then, can activate SAR, however, there exist complicated physiological mechanisms for the up- and downregulation of SAR. Clarification of the regulation mechanisms of HS-induced SAR at the molecular level is expected to yield useful information in the effort to exploit this resistance for various crops.

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**References**