Heat shock induced resistance in tomato

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学　位　論　文　要　旨

Heat shock induced resistance in tomato - molecular mechanism and utilization in practical field -
トマトにおける熱ショック誘導抵抗性—分子機構と実用—

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Due to their sessile nature, plants are often exposed to environmental stress. The acquisition of higher levels of stress tolerance is of utmost importance to plants for survival. Unlike any other stress condition, plants under heat stress must act quickly to survive. However, this fast response to heat stress leads to the acquisition of tolerance against many other stressful conditions, such as water deficiency, high salt, chemical pollutants, oxidative stress, nematodes, herbivores, extreme temperatures, and pathogens. Known as “heat shock-induced resistance” (HSIR), short and acute exposure of plants to hot water induces plant resistance against pathogens. In the present study, I focused on the molecular mechanism of HSIR by studying the regulation of the heat stress response by heat shock transcription factors (Hsfs). In addition, I also examined the possibility for a practical application of heat shock treatment (HST) to induce pathogen resistance in tomatoes. To gain insight into the mechanism of regulation, I used tomato seedlings, for which the genome was fully sequenced and well annotated. I investigated the role of Hsfs during induction of defense response by HST.

Leaf disease symptoms were significantly reduced at 12 and 24 h after HST, consistent with the upregulation of pathogenesis-related (PR) genes *PR1a2* and *PR1b1* peaking 24 h after treatment. These genes were upregulated at the treatment application site, but not in untreated leaves. In contrast to HST, inoculation of the first leaf induced the systemic upregulation of acidic PR genes in uninoculated second leaves. Furthermore, heat shock element motifs were found in upstream regions of *PR1a2*, *PR1b1*, *Chitinase 3* (*Chi3*), *Chitinase 9* (*Chi9*), *Glucanase A* (*GluA*), and *Glucanase B* (*GluB*) genes. The relative expression of *HsfA2* and *HsfB1* peaked at 6 h after HST, which was 6 h earlier than the time when salicylic acid accumulation was observed. Foliar spray of heat shock protein 90 (Hsp90) inhibitor geldanamycin

(GDA) induced both acidic (*PR1a2*, *Chi3*, and *GluA*) and basic (*PR1b1*, *Chi9*, and *GluB*) PR gene expression, comparable to HST. PR gene expression and defense response against *Pseudomonas syringae* pv. *tomato* (*Pst*) decreased when combining HST with Hsfs inhibitor KRIBB11. The Hsfs and PR gene expression induced by heat or GDA, together with the suppression of HSIR against *Pst* by KRIBB11, suggested a direct contribution of Hsfs to HSIR regulation in tomato plants.

Practical field application of HST for inducing plant resistance against pathogens was tested using an improved hot water sprayer device against powdery mildew in a tomato nursery. In plant nurseries, reducing the frequency of chemical application is becoming a challenge owing to the appearance of hard to control pathogens, spread of diseases, and demand by farmers. This study was therefore conducted to develop a practical alternative fungal control strategy against powdery mildew by using a hot water sprayer in a tomato nursery. The expected effects of the hot water spray treatment were to induce resistance and disinfection. Gray mold was used as an experimental model to determine the conditions for a practical application of the hot water spray for inducing resistance to plant fungi by heat shock treatment. Hot water dipping of tomato seedlings at 50 °C for 20 s induced resistance against gray mold and increased the expression of some PR genes, viz., pathogenesis-related protein 1a (PR1a), GluB, and Chi9. A prototype of a towable hot water sprayer was developed, and its performance was tested in the field. This sprayer was rolled on a rail, using an electric winch installed at the end of the nursery bench. A temperature higher than 50 °C for 20 s is required to attain the optimum conditions, because of heat loss due to vaporization. Moreover, heating time must be a 20 s duration at the target leaf. In other words, at least one part of the seedling must fall under the moving spray area of hot water during hot water spray treatment (HWS) + 20 s.

The severity of powdery mildew in HWS was significantly lower than that in control seedlings. The results of tomato HWS confirmed that partial achievement of optimum conditions in the whole plant succeeded in preventing powdery mildew. The possibility that Hsfs function as triggering molecules in HSIR provides new insights into the molecular mechanisms of plant defense systems against pathogens, as well as the opportunity to develop new approaches for crop protection. Further, if Hsfs were also induced by infection, they can be proposed as a universal trigger for the activation of a defense response. Application of HSIR by hot water spraying is suggested as an effective technique for inducing resistance against powdery mildew in tomato nurseries and reducing the frequency of chemical application.

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ABBREVIATIONS

Apx : ascorbate peroxidase

AT : acquired thermotolerance

ATAF : Arabidopsis transcription activation factor

Bc : *Botrytis cinerea*

BIT : 1,2-benzisothiazol-3(2H)-one-1,1-dioxide

Chi3 : chitinase 3

Chi9 : chitinase 9

CTAD : C - terminal activation domain

CUC : cup-shaped cotyledon

DBD : DNA - binding domain

DI : disease index

DMSO : dimethyl sulfoxide

ER : endoplasmic reticulum

GAPDH : glyceraldehyde-3-phosphate dehydrogenase

GDA : geldanamycin

GluA : glucanase A

GluB : glucanase B

HS : heat shock

HSE : heat shock elements

Hsfs : heat shock transcription factors

HSIR : shock-induced resistance

Hsps : heat shock proteins

HSR : heat stress response

HST : heat shock treatment

HWD : hot water dipping

HWS : hot water spraying

JA : jasmonic acid

KB : KRIBB11 (N^2-(1H-Indazol-5-yl)-N^6-methyl-3-nitropyridine-2,6-diamine))

NAM : non-apical meristem

NLS : nuclear localization signal

NT : non-treated

OD : oligomerization domain

PE1b1 : pathogenesis related protein protein 1b1

PR : pathogenesis-related

PR1a2 : pathogenesis related protein 1a2

Pst : *Pseudomonas syringae* pv. *tomato*

qPCR : quantitative real-time polymerase chain reaction

SA : salicylic acid

SAG : salicylic acid β-D-glucoside

SAR : systemic acquired resistance

sHsps : small Hsps

WIR : wound-induced resistance

CHAPTER ONE

General Introduction

1.1. Stress response in plants

Plants are constantly faced with a variety of environmental stresses. Because of their sessile nature, they have evolved to respond rapidly and efficiently to adverse conditions in order to survive and reproduce. These stresses often coincide, leading to compounded effects of low and unreliable yields. Abiotic stress factors, such as high temperature, low temperature, drought, and salinity have a considerable bearing on world agriculture and are thought to reduce average yields by over 50% for most major crop plants (Wang et al., 2003). Additionally, plants must defend themselves from attack by a vast range of pests and pathogens, including fungi, bacteria, viruses, nematodes, and herbivorous insects

(Hammond-Kosack and Jones, 2000). Each stress elicits a complex cellular and molecular stress response system, activated within plants to prevent damage.

1.2. Abiotic stress

Abiotic stress is caused by physical or chemical components of the environment (Bray et al., 2000). Abiotic stress elicits various responses in plants that encompass a sequence of physicochemical and molecular events. Multiple stress response mechanisms often function coordinately or synergistically to avoid cellular damage (Ahuja et al., 2010). Many abiotic stresses, such as drought, salinity, oxidative stress, and heat stress, have a similar effect on plants, thus eliciting a similar molecular stress response. For example, drought and salt stress both exert oxidative stress on plant cells, leading to the buildup of reactive oxygen species (ROS) (Smirnoff, 1993), which are important as signaling molecules that regulate and maintain normal physiological and metabolic functions (Mittler, 2017). Also, drought, salinity, and flooding all result in cellular osmotic stress (Wang et al., 2003). Plants have developed several resistance mechanisms for minimizing the effects of abiotic stress and preventing damage. These can be categorized into avoidance or tolerance mechanisms.

Avoidance depends on strategic adaptations that prevent exposure to stress. In the case of dehydration avoidance, these may include longer roots, a waxy cuticle, sunken stomata, or early flowering (Taiz and Zeiger, 1991; Bray et al., 2000). In arid environments, plants are adapted to complete their life cycle in a short time when water is available (Chaves et al., 2003). Stress tolerance mechanisms allow plants to withstand stress, which involves processes such as stress perception, signaling, and cellular osmotic adjustment (Bartels and Sunkar, 2005). The heat stress response (HSR) is characterized by inhibition of normal transcription and translation, higher expression of heat shock proteins (Hsps), and induction of thermotolerance. If stress is too severe, signaling pathways leading to apoptotic cell death are also activated. Furthermore, the buildup of ROS, which induces programed cell death in plants in response to abiotic stress (Petrov et al., 2015), could lead to the

activation of Hsfs and downstream gene expression (Driedonks et al., 2015). These reports suggest that Hsfs activation by heat shock could produce similar responses as other abiotic stresses that trigger ROS accumulation.

1.3. Plant response to heat stress

A plant undergoes heat stress when the temperature rises beyond an optimal threshold level for a period of time, and this could lead to irreversible damage to growth and development. A transient increase in temperature of approximately 10–15 °C above ambient is considered a heat shock (HS). It is a complex interaction of heat intensity, duration, rapidity, and stage of growth. Conversely, heat tolerance is the ability of the plant to overcome this adverse effect and produce economic yields. Although some researchers have reported that the night temperature is more critical (Willits and Peet, 1998), it is generally accepted that daily mean temperatures are better indicators of plant HSR. The ability of an organism to cope with high temperatures has two components: inherent and acquired thermotolerance (AT). Inherent or basal thermotolerance is a constitutive component resulting from the evolutionary thermal adaptation of a species.

AT is the ability of a plant to survive normally lethal temperatures after exposure to mild stress. AT relies on the induction of a specific pathway during the acclimation period and subsequent acquisition of thermotolerance. The intraspecies difference is significant in AT, but not significant in inherent thermotolerance (Klueva et al., 2001). Thus, the measurement of AT is a more useful tool in crop breeding and selection (Klueva et al., 2001). The extent of damage caused by any stress depends on the crop species, the stage of growth, and the adapted geographical zone. Seedling emergence, flowering, and seed filling are the most critical stages for all crop species (Wahid et al., 2007). However, as plant development is dependent on several environmental conditions, it is difficult to determine a consistent upper threshold temperature (Miller et al., 2001). Several internal factors dictate a plant's ability for thermotolerance, such as antioxidant capacity, accumulation and stability of

proteins and enzymes, signaling cascade, membrane composition, and maintenance of transcript functions (Klueva et al., 2001). Heat stress can cause damage at the whole plant and cellular level. It can manifest as morphological, physiological, and molecular responses.

1.4. The molecular response to heat stress

Exposure of plants to elevated temperatures for a short time results in the expression of a complex set of genes and selective translation of messenger RNA encoding Hsps, a molecular chaperone that plays an important role in enhancing thermotolerance and improving cellular survival to subsequent heat stress (Nover et al., 1989; Waters et al., 1996; Gong et al., 1997, 1998). Molecular chaperones are defined as a family of unrelated classes of proteins that mediate the correct assembly of other polypeptides but that are not components of the functional assembled structures (Ellis and Hemmingsen, 1989). In addition, chaperones play an essential role in re-solubilization and degradation of proteins partially denatured and/or aggregated by mutation or environmental stresses, such as high temperature and oxidative conditions. Moreover, protein denaturation is a direct or indirect effect of any stress. Therefore, any biotic or abiotic stress that induces protein misfolding requires chaperones to maintain those proteins (Jacob et al., 2017). Many molecular chaperones are stress proteins that are abundant even in the absence of stress. Thus, the stress response can be viewed as an amplification of the primary chaperone function. It is possible that all stress-related proteins act as molecular chaperones, and many chaperones were originally identified as Hsps. Hsps are the major class involved in acquired thermotolerance (Vierling, 1991), though other proteins, such as late embryogenesis abundant, dehydrins, and ubiquitins, also play a role. Furthermore, it was found that the role of Hsps is not limited to the heat stress response. Hsps also play an important role in response to other stresses, such as cold, osmotic, drought, salt, UV, high light, and oxidative stress and pathogen infection (Swindell et al., 2007; Park et al., 2015).

Five major families of chaperones are conservatively recognized: chaperonins/Hsp60, Hsp70, Hsp90, Hsp100, and the small Hsps (sHsps) family (Wang et al., 2004). sHsps are low molecular weight proteins of 15–30 kiloDalton that can increase as much as 200-fold under stress. All sHsps in plants are encoded by six nuclear gene families, with each gene family corresponding to proteins found in distinct cellular compartments, such as the cytosol, chloroplast, endoplasmic reticulum (ER), mitochondria, and membranes. The expression of Hsps in response to the heat stress condition is controlled by heat shock transcription factors (Hsfs).

Hsfs are regulatory proteins that control the transcription of Hsps encoding genes (Baniwal et al., 2004), serving as terminal components of signal transduction (Kotak et al., 2007). Detailed analysis of Hsfs, including the terminal components of the signal transduction pathway for gene activation in response to heat stress, and a large number of chemical stressors have been reported (Zhu et al., 2006; Nover et al., 2001). The basic modular structure of Hsfs includes a highly conserved DNA-binding domain (DBD), oligomerization domain (OD), nuclear localization signal (NLS), and the least conserved C-terminal activation domain (CTAD). The transcription-activating function of Hsfs is related to the short peptide motifs (AHA motifs) within the CTADs. The palindromic and heat-responsive heat shock elements (HSE) are always present in the promoter regions of heat shock genes. The HSE (5-AGAAnnTTCT-3) is the binding target and recognition site for the trans-active Hsfs (Nover and Scharf, 1997; Schöffl et al., 1998).

Plant Hsfs, consisting of 21 members in Arabidopsis, more than 18 and approximately 23 in tomato and rice, respectively, comprise three conserved evolutionary classes: A, B, and C (Nover et al., 2001). The essential role of HSE for heat-dependent transcriptional activation in plants via Hsfs has been established by promoter deletion analysis in tomatoes. Hsps activation mechanism by Hsfs is highly conserved and includes the dissociation of a negative regulatory molecule R (repressor) from the Hsfs in monomer form followed by the oligomerization of Hsfs molecules. Subsequently, monomeric Hsfs with low affinity for

DNA binding are converted into trimers with high affinity for DNA binding. The trimeric Hsfs then bind to the HSE of Hsps and activate their transcription. Biosynthesis of Hsps feeds back to the regulation of Hsfs expression negatively.

HsfA1a, HsfA2, and HsfB1 form a regulatory network (Baniwal et al., 2004), in which HsfA1a is constitutively expressed and regulates the expression of the other two. Therefore, it is defined as the master regulator of heat stress response in plants (Mishra et al., 2002; Hahn et al., 2011; Ohama et al., 2017). The knockout mutant analysis shows that HsfA1a and HsfA1b are essential for the initial phase of gene expression and that HsfA2 controls expression under prolonged heat shock and recovery (Lohmann et al., 2004; Schramm et al., 2006; Nishizawa et al., 2007). In Arabidopsis, HS-induced expression of HsfA2 is not regulated by HsfA1a or HsfA1b, unlike in that in tomatoes (Busch et al., 2005). Neither single nor double mutants of *AtHsfA1a* and *AtHsfA1b* (Lohmann et al., 2004) affected the response to HS and long-term thermotolerance of Arabidopsis. The role of HsfB1 underlines a pre-programmed recovery period for rapid resumption of housekeeping and developmental gene expression (Bharti et al., 2004; Baniwal et al., 2004).

HsfA2 is important for abiotic stress tolerance as is evident from its involvement in the regulation of *APX2*, which is key to oxidative stress regulation (Nishizawa et al., 2007). *DREB2A*, a transcription factor (TF) that regulates dehydration-responsive genes, has been shown to regulate the Arabidopsis *HsfA3* (Sakuma et al., 2006). Although HsfA4a and HsfA8 may act as ROS sensors (Davletova et al., 2005), in sunflowers, HsfA9 appears to be unique to seed development and not required for stress tolerance (Kotak et al., 2007; Prieto-Dapena et al., 2006). A point mutation in *HsfA4a* induces spontaneous necrotic lesions in rice leaves suggesting a role as an antiapoptotic factor (Yamanouchi et al., 2002). Overexpression of *GmHSFA1* in soybeans – enhanced thermotolerance, activated *GmHSP70* under normal temperature and enhanced its expression under high temperature (Zhu et al., 2006). Non-apical meristem (NAM), Arabidopsis transcription activation factor (ATAF), and the cup-shaped cotyledon (CUC) gene, which are known as NAC (from first letters of each gene), is

a plant-specific TF family with transactivation activity and has diverse roles in development and stress regulation. Transgenic rice plants overexpressing a stress-responsive NAC gene (*SNAC2*) displayed various stress tolerances (Hu et al., 2008). Microarray analysis revealed upregulation of stress response and adaptation genes, such as peroxidase and Hsps, unique to SNAC2 among SNAC genes. A putative membrane tethered transcription factor called basic leucine zipper (bZIP)28 was shown to be upregulated in response to heat (Gao et al., 2008). The heat-sensitive null mutant shows attenuation of heat-inducible expression of the ER chaperone BiP2 and HSP26.5-P. HS releases the TF from the ER membrane and redistributes it to the nucleus. TFs are attractive candidates for genetic engineering because a single TF can result in multiple stress tolerance (Yamaguchi-Shinozaki and Shinozaki, 2005). Furthermore, by studying the TF binding site in a stress-related gene, the mode of action of a certain stress response might be elucidated. In the case of plant response to HS and the following downstream event, Hsfs is most likely involved in regulation.

1.5. Heat shock-induced plant resistance

The acquisition of higher levels of stress tolerance is of utmost importance to plants for survival. Plants adapt to heat stress through the long-term evolutionary manifestation of developmental and morphological changes and short-term acclimation mechanisms, such as leaf orientation and transpirational cooling (Hanumappa and Nguyen., 2012). Similar mechanisms may be used to overcome other stresses, overlapping the response ranges and placing emphasis on cellular and physiological strategies that have a broad and overreaching adaptation mechanism. Plant HSR leads to the acquisition of tolerance against many other stress conditions. Cross-protection has been reported between heat stress, dehydration/drought (Sato and Yokoya, 2008), cold/chilling/freezing (Sabehat et al., 1998), salt stress (Fu et al., 2016), and disease (Widiastuti et al., 2013a). The enhanced disease resistance in plants following the heat stress condition has been known as heat shock-induced resistance (HSIR) (Widiastuti et al., 2011). HS treatment is reported to accumulate salicylic acid (SA), an important signal molecule in systemic acquired resistance (SAR), as

well as to induce resistance against crown rot fungus *Colletotrichum gleosporioides* in strawberries (Widiastuti et al., 2013b), and *Botrytis cinerea* in melons (Widiastuti et al., 2011) and cucumbers (Yoshino et al., 2012).

Defense responses are largely mediated through the accumulation of the phytohormones, SA, jasmonic acid (JA), and ethylene. The precise interplay of these compounds can dictate the nature of the defense response induced, allowing specificity against different types of pathogens (De Vos et al., 2005; Pieterse et al., 2009). The dissection of hormone-mediated defense pathways has been enabled through analysis of hormone signaling mutants (Jalali et al., 2006). SA accumulates locally during pathogen attack, as well as systemically. Immediately following pathogen recognition by R-gene products, the expression of signal molecules EDS5, SID1, and PAD4 are induced. This leads to SA production, which acts as a signaling molecule for the activation of downstream resistance genes, such as pathogenesis-related (PR) genes, through the expression of transcriptional activator NPR1. Therefore, PR genes can be used as markers of SA signaling (Cao et al., 1997; Kaloshian, 2004; Jalali et al., 2006; van Loon et al., 2006). Mutants deficient in SA signaling show susceptibility to pathogens, such as *Pseudomonas syringae*. In addition to the local pathogen-induced defense response, a signal travels to distal parts of the plant where SA again accumulates, establishing a distal defense response to protect remote parts of the plant from secondary infection (Bostock, 2005). This protection system is known as SAR. The identity of the SAR signal has long been in question (Heil and Ton, 2008), but now, a diverse set of SAR inducers has already been identified, including hormones (salicylic acid, methyl salicylate), primary/secondary metabolites (nitric oxide, reactive oxygen species, glycerol-3-phosphate, azelaic acid, pipecolic acid, dihyroabetinal), fatty acid/lipid derivatives (18 carbon unsaturated fatty acids, galactolipids), and proteins (DIR1-Defective in Induced Resistance 1, AZI1-Azelaic acid Induced 1) (Shine et al., 2018). Among them, Azelaic acid has been identified as a mobile metabolite that primes tissues to accumulate SA (Jung et al., 2009; Parker, 2009). Recent findings showed that azelaic acid induced 1 (AZI1) mediated the

signal mobilization for priming the systemic defense response in Arabidopsis (Cecchini et al., 2015).

On the other hand, JA is an oxylipin that is rapidly produced by plants in response to mechanical wounding or insect herbivory (Koo and Howe, 2009). It has a crucial role in defense, and when applied exogenously it can protect plants from herbivore attack (Baldwin, 1998). JA acts through activation of the transcription factors MYC2 and ERF1 to induce the transcription of downstream defense genes, such as *PDF1.2* and *VSP2* (Koo and Howe, 2009; Pieterse et al., 2009). There is evidence that JA is also essential for and in fact mediates the long-distance SAR signal (Truman et al., 2007). JA and ethylene function synergistically in defense signaling, activating the same downstream defense genes and providing resistance to necrotrophic pathogens, such as *Botrytis cinerea* and *Erwinia carotovora*. In contrast, SA mediates the response to biotrophic pathogens, such as *Pseudomonas syringae* (Anderson, 2004; Pieterse et al., 2009).

The interaction between the SA defense pathway and the JA-ethylene pathway is mainly antagonistic, as SA-induced transcription factors can suppress JA-dependent gene expression, whilst JA-induced MYC2 is involved in the suppression of the SA response (Pieterse et al., 2009). The complex crosstalk between defense-induced hormone signaling pathways becomes increasingly more apparent as further studies are conducted.

Until now, SA has been considered a critical signal molecule in SAR, which correlates with the activation of local and/or systemic defense responses (Derksen et al., 2013; Kumar, 2014). Park et al. (2007) reported the methylated derivative of SA, methyl salicylate (MeSA) is required for signal induction in systemic tissue, which is not the site of the primary infection. In tomatoes, SA has been reported to induce the expression of pathogenesis-related (PR) protein: PR1b, PR1b1, PR2a, PR2b, PR3, and PR7 (Jordá et al., 1999; Meichtry et al., 1999; van Kan et al., 1995). Tomato PR1b1 gene was transcriptionally up-regulated after tomato mosaic virus infection, and the promoter activity was enhanced by SA and ethylene (Tornero et al., 1997). However, SA was reported to accumulate after the expression of PR

genes in HS treated melons (Widiastuti et al., 2013a). These results suggest that in the case of HSIR, PR genes could be regulated without the direct involvement of SA. Therefore, an alternate triggering mechanism to regulate PR genes expression that leads to induced resistance might exist in HSIR. Additionally, because HSIR is closely related to HSR, the mechanism might be elucidated by referring to the well-studied mechanism of HSR in tomatoes.

1.6. Practical application of HS treatment

High-temperature treatment has been suggested as an alternative technique for inducing plant resistance. Hot water spraying (HWS) against powdery mildew (Sato et al., 2017; Ogawara et al., 2012; Yamagishi et al., 2009) and gray mold (Yoshino et al., 2012); hot water dipping (HWD) against powdery mildew (Schweizer et al., 1995), and heat shock treatment by greenhouse closure against cucumber downy mildew (Ding et al., 2016) have been reported. Although the mechanism is not clearly understood, high-temperature treatments have been shown as prospective methods to protect plants against diseases as an alternative to chemicals or fungicides.

Effective control of disease has generally been achieved by hot water treatment, which consists of the exposure of plant material to water at a predetermined temperature for a predetermined time. Widiastuti et al. (2011) demonstrated that melon leaves subjected to high temperatures (50 °C) for short periods (20 s) exhibited maximum *Botrytis cinerea* (*Bc*) symptom reduction and peroxidase gene expression. Also, Widiastuti et al. (2013b) reported that HS treatment at 50 °C for 20 s reduced the disease index of strawberry crown rot fungus and increased chitinase gene expression, as well as free SA accumulation. Yoshino et al. (2011) mentioned that HWD at 40 °C for 2 minutes enhanced cucumber resistance against *Bc*, as well as peroxidase gene expression.

HWS has been suggested as a practical means of activating HSIR and directly inhibiting pathogen growth (Yamagishi et al., 2009; Ogawara et al., 2012; Yoshino et al., 2011; Sato et

al., 2017). The prototype of a hot water sprayer device has been developed in a previous study (Yoshino et al., 2012), and it was proven to effectively protect cucumbers against gray mold, as well as induce the accumulation of SA and the expression of the peroxidase gene in leaves. However, the implementation of a sprayer prototype under field conditions was laborious because the sprayer had to be manually operated and was not designed to spray multiple seedlings simultaneously. In this study, an improved design of hot water sprayer device was evaluated.

1.7. Research objectives

The objective of this study was to (i) determine the effect of HS treatment against *Pst* in tomatoes, (ii) assess the role of Hsfs in the regulation of HSIR, and (iii) develop a practical method for the application of HSIR in a tomato nursery.

1.8. Research outline

First, we confirmed whether HS treatment could induce a tomato defense response against the *Pst* model pathogen. After HS treatment, temporal development of resistance against *Pst*, gene expression profiles of tomato PR protein and Hsfs, as well as SA accumulation were evaluated. In addition, we studied the existence of HSEs in the upstream area of PR genes. Hsp90 inhibitor geldanamycin and Hsfs inhibitor KRIBB11 were applied to provide evidence for the regulation of HSIR via Hsfs. The application of HS treatment in the practical field was examined using an improved hot water spraying device against powdery mildew in a tomato nursery. Gray mold was used as an experimental model to confirm the efficacy of HWD for inducing resistance to plant fungi in different tomato cultivars. Thus, a prototype of a towable hot water sprayer device was developed, and its performance was tested in the field against powdery mildew.

CHAPTER TWO

Heat shock-induced resistance against *Pseudomonas syringae* via heat shock transcription factors in tomatoes

2.1. Introduction

Plant disease resistance acquired under heat stress is known as heat shock-induced resistance (HSIR) (Widiastuti et al., 2011). Heat shock (HS) reportedly triggers defensive responses against *Colletotrichum gloeosporioides* in strawberry (Widiastuti et al., 2013b), as well as against *Botrytis cinerea* (*Bc*) in melon (Widiastuti et al., 2011) and cucumber (Yoshino et al., 2011). Furthermore, HS-treated plants accumulated salicylic acid (SA) (Widiastuti et al., 2011), the primary signaling molecule in systemic acquired resistance (SAR), which plays a role in the initial activation of the heat stress response (HSR) by inducing higher levels of heat shock protein 70 (Hsp70) (Cronje´ and Bornman, 1999; Cronje´ et al., 2004). In the

present study, we focused on HSIR by studying the regulation of HSR by heat shock transcription factors (Hsfs).

Upon exposure of an organism to heat stress, Hsfs activate gene expression of heat shock proteins (Hsps) by binding the heat shock element (HSE) in the promoter DNA sequence of the corresponding gene (Liu et al., 2011; Liu and Charng 2012; Xue et al., 2014). HSEs are trinucleotide core sequences, 5′-nGAAn-3′ or 5′-nTTCn-3′, in alternating orientation, separated by two nucleotides (Basra, 2000). Plants have several homologous Hsfs classified as A, B, and C subfamilies (von Koskull-Döring et al., 2007). Tomato has eleven A-, three B-, and one C-class Hsfs (von Koskull-Döring et al., 2007; Nover et al., 2001) among which HsfA2 and HsfB1 are heat-stress-inducible proteins themselves (Treuter et al., 1993). HsfA1 is a master regulator that interacts with HsfA2 and HsfB1 to regulate gene expression in response to heat stress (Chan-Schaminet et al., 2009). According to Hahn et al. (2011), the formation of the HsfA1/HsfA2 super activator complex is responsible for enhancing the expression of Hsps in tomato cell suspension cultures. Under normal conditions, Hsp90 and Hsp70 bind HsfA1, thereby inactivating HsfA1. In contrast, under HS, the complex dissociates, and free HsfA1 enters the nucleus, where it binds HsfA2 to form a super-activator complex that regulates gene expression by binding HSEs located in the upstream regions of genes that are essential for survival under heat stress (Hahn et al., 2011).

Certain stress-related genes with HSEs regulated by Hsfs have been reported. For instance, Storozhenko et al. (1998) reported that cloned tomato HsfB1 bound the HSE of ascorbate peroxidase (apx) 1 gene and activated its transcription under HS in Arabidopsis. Similarly, an *apx* bearing a similar HSE motif was also upregulated after exposure of rice seedlings to 42 °C (Sato et al., 2001). Moreover, HsfB1 regulated the expression of defensin-like protein 16 (*Pdf1.2a)* and pathogen resistance in Arabidopsis (Kumar et al., 2009). These results suggest that the transcription of pathogenesis-related (PR) genes is regulated by Hsfs and that Hsfs are involved in defense responses.

Inhibitors of Hsps and Hsfs have been used to investigate the role of Hsfs in the activation of gene expression after HS. For example, geldanamycin (GDA; NSC 122750) was applied for suppressing the Hsp90 chaperone ATPase cycle in eukaryotic cells by binding the ATP-binding site of the N-terminal domain of Hsp90 (Prodromou et al., 1997). Similarly, the application of GDA on leaves of heat-treated tomato induced accumulation of HsfA2 (Moshe et al., 2016). Further, pretreatment of tomato cell cultures with GDA before HS enhanced the endogenous levels of HsfA2 and HsfB1, relative to the control treatment (Hahn et al., 2011).

In another case, Hsfs-HSE binding was hindered by KRIBB11 (N^2-(1H-Indazol-5-yl)-N^6-methyl-3-nitropyridine-2,6-diamine ; KB), the only known transcription factor inhibitor that binds directly to HSF1, a transcription factor that regulates Hsps in human cancer cells (McConnell et al., 2015). KB showed a maximal inhibitory concentration (IC_{50}) of 1.2 μM in Hsps transcription inhibition assay (Yoon et al., 2011). Induction of Hsp70 was blocked in human colon cancer 116 cells through inhibition of HSF1, thereby completely suppressing the expression of Hsp70 when cells were exposed to HS in the presence of KB. The ability to suppress Hsp70 upon HS exposure suggested that KB might be successfully applied to suppress HSIR.

Based on the foregoing discussion, we hypothesized that if Hsfs are involved in the activation of HSIR, the inhibition of Hsp90 will release HsfA1 and induction of *HsfA2* and *HsfB1*, as well as induction of PR genes, will follow. On the other hand, inhibition of Hsfs will suppress the enhanced expression of PR genes as well as disease resistance after HS. Moreover, Hsfs gene expression should be upregulated earlier than that of PR gene and the induction of disease resistance.

The objective of this study was to assess the role of Hsfs in the regulation of HSIR. Tomato PR protein 1a2 (PR1a2) and PR protein 1b1 (PR1b1) were chosen as resistance marker genes because of their distinctive characteristics. An earlier study showed that tomato PR1a2 and PR1b1 genes were upregulated by different modes of action: Upon

infection by tobacco mosaic virus, the PR1b1 gene is strongly activated locally in tissues undergoing the hypersensitive response but not systemically in uninoculated tissues (Tornero et al., 1997). In addition, wounding was shown to induce PR1b1 expression and wound-induced resistance (WIR) against *Pseudomonas syringae* pv. *tomato* (*Pst*) (Francia et al., 2007), suggesting that PR1b1 tended to be expressed after physical damage via jasmonic acid and ethylene pathway, whereas PR1a2 is positively activated by reactive oxygen species (Di Baccio et al., 2012), independently from ethylene (Tornero et al., 1997). Gene expression profile of PR1a2 and PR1b1, SA accumulation, as well as the appearance of resistance against *Pst* after HS treatment were evaluated. In addition, we studied the time-course of the expression of Hsf genes and the existence of HSEs in the upstream area of PR genes. GDA and KB were applied to provide evidence for the regulation of HSIR via Hsfs.

2.2. Materials and Methods

2.2.1. Growth and maintenance of pathogens and plant materials

Cultures of *Pst* strain MAFF302666 with pathogenicity of bacterial speck leading to straight-edged brown spots or circular spots with yellow haloes were obtained from the culture collection of the Genetic Resources Center, National Agriculture and Food Research Organization, Tsukuba, Japan. The stock *Pst* culture was stored in glycerol solution at -80 °C. For the experiments designed, the culture was maintained on King's B agar slant at 4 °C and subcultured every four weeks. Pathogenicity of *Pst* was confirmed by challenge inoculation prior to the experiment. Plants of the tomato cultivar Natsunokoma were grown in soil (Super Mix A, Sakata Co., Ltd., Yokohama, Japan) at room temperature under a 16:8 h (light:dark) regime, until the 2nd true leaf was fully expanded. Square plastic propagation liner tray arranged in a 2 × 2 cell configuration (4 plant/replication) or in a 3 × 2 cell configuration (6 plants/replication) was used. For partial treatment, plants were grown in individual pots. Plants with abnormal leaves were excluded.

2.2.2. Pathogen inoculation

Pst cultures were grown overnight at 28 °C in King's B broth in a shaker. Next, 10 mL of each culture was pelleted by centrifuging at 3200 × *g* for 20 min and resuspended in 500 μL of 10 mM $MgCl_2$. Cell concentration was measured by determining optical density at 600 nm using the GeneQuant Pro DNA/RNA Calculator (Amersham Biosciences, Little Chalfont, UK). These *Pst* cultures were diluted to 2 × 10^7 colony-forming units/mL in inoculation buffer containing 10 mM $MgCl_2$ plus 200 μL/L Silwett-L77 (Momentive Performance Materials Inc., New York, USA). For whole plant treatment, seedlings were inoculated by dipping upside down into the bacterial suspension at different times after HST. For partial treatment, only the 1st leaf was inoculated in the same manner. Plants dipped into inoculation buffer were used as mock. Then, mock and inoculated plants were separated for incubation in different growth chambers and maintained under the same

conditions described above. Disease severity was estimated by the extent of leaf area showing a lesion at 3 days after inoculation according to the following scale: rank 0, without injuries; rank 1, less than 10%; rank 2, from 10% to 20%; rank 3, from 20% to 40%; and rank 4, above 40% of leaf tissue affected. Disease index (DI) was calculated as follows: $DI = [\Sigma(n \times v)/N \times Z]$, where n is the lesion score as ranked, v is the number of samples in the score category, N is the highest score value, and Z is the total number of samples. The experiment was repeated thrice; DI data shown are means of both 1st and 2nd leaves from three plants per replicate.

2.2.3. Heat shock, GDA, and KB treatments

Whole seedlings or only the 1st leaf of tomato seedlings at the second-leaf stage, were dipped upside down into water at 45 °C for 2 min as described by Sato et al. (2005) (Heat shock treatment, HST). Non-treated (NT) plants were used as negative controls. GDA (Tokyo Chemical Industry co., ltd., Tokyo, Japan) and KB (Tocris Bioscience, Bristol, UK) were dissolved into dimethyl sulfoxide (DMSO) at 10 and 20 mM, respectively and diluted by ion exchange water up to the desired concentration. GDA and KB were applied manually by foliar spray of 2.5 ml solution each plant using a spray bottle. KB was applied three times on the leaves at 6, 12, and 18 h after HST. Mock plants treated with 0.1% (v/v) aqueous DMSO were used as negative controls.

2.2.4. Gene expression analysis

For time course sampling, total RNA was isolated from the 1st leaf at different time intervals (3, 6, 12, 24, 48, or 72 h after HST) using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan), following manufacturer instructions. Thereafter, mRNA was reverse transcribed using the Prime Script RT Reagent Kit (Takara Bio Inc., Shiga, Japan). The synthesized first strand cDNA was used as a template for quantitative real-time polymerase chain reaction (qPCR) analysis. The reaction was performed using the KOD SYBR qPCR Mix (TOYOBO, Osaka, Japan) on a CFX Connect Real-Time PCR system (Bio-Rad, California, USA)

according to the user´s manual. The gene-expression levels in each sample were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Gene-specific primers used for qPCR were designed using the National Center for Biotechnology Information (NCBI; Maryland, USA) program Primer Blast, with the parameters set to create a product of 200–500 base pairs (bp) within the region of the target mRNA (Table 2.1). In a preliminary experiment, no significant changes were observed in the expression levels of *PR1b1* and *HsfA2* from 0 to 3 h after HST. In partial-treatment tests, only the 1st leaf of tomato was treated by HS or inoculation, and gene-expression levels in treated (1st leaf) and untreated (2nd leaf) plants were independently analyzed using the protocol described above. The 2nd leaves of untreated plants were also analyzed as references. Three technical replicates of qPCR were performed for each biological replicate.

Table 2.1. Primers used to amplify Hsfs, PR, and GAPDH genes from tomato leaves.

Target gene	Location	Accession Number	Properties	Primer Name	Sequence (5′ to 3′)
PR1a2	chr 9	NM_001321040.1	Pathogenesis-related leaf protein 4 Acidic	SLPR1a2 F	TGTTACTTATGACTTGTCTCATGGT
				SLPR1a2 R	CGACCCAATTGCCTACAGGA
PR1b1	unplaced scaffold	NM_001247385.2	Pathogenesis-related leaf protein 6 Basic	SLPR1b1 F	ACATCTCATTGTTACTCACTTGTCT
				SLPR1b1 R	GACGTTGTCCGATCCAGTT
GluA	chr 1	NM_001247869.2	Glucan endo-1,3-beta-D-glucosidase Acidic	SLGluA F	GGTCTCAACCGCGACATATT
				SLGluA R	CACAAGGGCATCGAAAAGAT
GluB	chr 1	NM_001247876.2	β-1,3-glucanases Basic	SLGluB F	TCTTGCCCCATTTCAAGTTC
				SLGluB R	TGCACGTGTATCCCTCAAAA
Chi3	chr 2	NM_001247475.2	Class III endochitinase Acidic	SLChi3 F	TGCAGGAACATTCACTGGAG
				SLChi3 R	TAACGTTGTGGCATGATGGT
Chi9	chr 10	NM_001247474.2	Class I chitinase Basic	SLChi9 F	CTCCAATGGCTCTTCCACAT
				SLChi9 R	GAAATTGCTGCTTTCCTTGC
HsfA2	chr 8	XM_010326728.2	Heat shock transcription factor A2	SLHsfA2 F	GCGAATGGAGGTTTTCTGGG
				SLHsfA2 R	GTCACAACAGAATCCGGCCT
HsfB1	chr 2	NM_001321450.1	Heat shock transcription factor B1	SLHsfB1 F	CTATACGCCGTCGGAAGACC
				SLHsfB1 R	TACCACGTCCACCACAAGTC
GAPDH	chr 5	NM_001279325.2	Glyceraldehyde-3-phosphate dehydrogenase	SLGAPDH F	GGTTTGGCATTGTTGAGGGTC
				SLGAPDH R	TCGACAACGGAGACATCAGC

2.2.5. Measurement of SA and SAG levels

Samples were collected from the 1st leaves at 6, 12, 24, and 48 h after HST. NT were used as a control. We used 3 independent biological replicates for each time point of the experiment. Each biological replicate is a pool of 3 leaves from independent plants, one leaf from each plant was cut, combined and weighed immediately. SA was extracted as described by Widiastuti et al. (2011).

SA and salicylic acid β-D-glucoside (SAG) analyses were performed on Shimadzu UFLC system (Shimadzu Corp., Kyoto, Japan) equipped with a SIL-20AC autosampler and a Shim-pack XR-ODS (2.0 mm id x 30 mm) column (Shimadzu Corp., Kyoto, Japan). A standard methanolic solution was prepared by diluting SA (Kanto Chemical Inc., Tokyo, Japan) and SAG (Santa Cruz Biotechnology Inc., Texas, USA). The injection volume was 5 µl and the temperatures of the analytical column and autosampler were set at 40 °C. The elution gradient was obtained with a binary solvent system consisting of 0.1% CH_3COOH in H2O (solvent A) and MeOH (solvent B) at a total flow rate of 0.2 ml/min. The gradient program was as follows: 0-5 min, linear gradient of 5-100% solvent B; 5-8 min, isocratic elution of 100% solvent B.

The mass spectrometer was operated using an AB Sciex 3200 QTRAP LC-MS/MS System (AB Sciex LLC, Massachusetts, USA) equipped with a Turbo V electrospray ionization (ESI) source in negative mode. For best selectivity and sensitivity multiple reaction monitoring (MRM) mode was used for detection. The source parameters were set as follows: curtain gas, 15 psi; temperature, 600 °C; spray gas, 70 psi; dry gas, 80 psi; ion spray voltage, -4500 V; and declustering potential, -25 V. SA and SAG were measured by monitoring the following transitions (in parentheses, entrance potential, EP; collision cell exit potential, CEP; collision energy, CE): SA: 136.9-93.0 (EP -2.5 V,-16 V, -24 V); SAG: 299.0-136.9 (EP -2 V, CEP -20 V, CE -22 V). MRM data was acquired and the chromatograms were integrated using the Analyst 1.5.1 software.

2.2.6. *Identification of HSEs in the upstream regions of PR genes*

Sequences of PR1a2, PR1b1, Chitinase 3 (Chi3), Chitinase 9 (Chi9), Glucanase A (GluA), and Glucanase B (GluB) genes were retrieved from the tomato genome (assembly SL2.50) stored in the NCBI Reference Sequence Database. The upstream regions of tested PR genes were investigated for the existence of HSE-like sequences by manual annotation.

2.2.7. *Statistical analysis*

The Tukey-Kramer test was performed for experiments involving inoculation. For SA and SAG determination, significant differences between NT and HST were evaluated by a two-tailed t-test at each sampling time-point. Arcsine-transformed values were applied for statistical analysis when data were distorted from the normal distribution. All analyses were performed by Statistical Analysis System (SAS) University Edition software (version: university.cny.sas.com@sas: university-6p.2/6p.2.688de4662a09-1-1; SAS Institute Inc., North Carolina, USA).

2.3. Results and Discussion

2.3.1. Induction of disease resistance against Pst in HS-treated tomato leaves

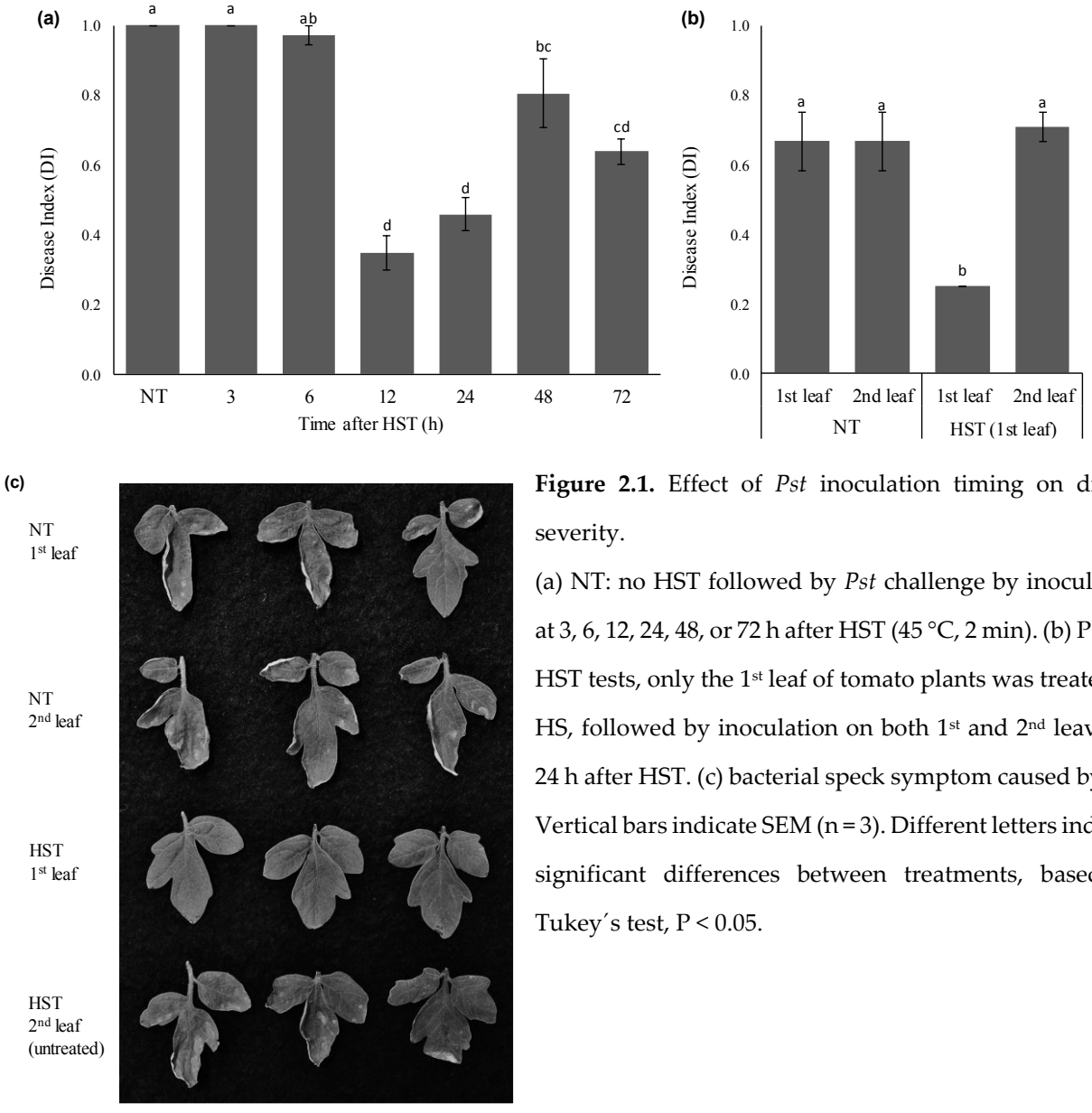

Figure 2.1. Effect of *Pst* inoculation timing on disease severity.

(a) NT: no HST followed by *Pst* challenge by inoculation at 3, 6, 12, 24, 48, or 72 h after HST (45 °C, 2 min). (b) Partial HST tests, only the 1st leaf of tomato plants was treated by HS, followed by inoculation on both 1st and 2nd leaves at 24 h after HST. (c) bacterial speck symptom caused by *Pst*. Vertical bars indicate SEM (n = 3). Different letters indicate significant differences between treatments, based on Tukey´s test, $P < 0.05$.

There was no significant difference in disease severity between NT (DI 1.00) and plants inoculated at 3 h (DI 1.00) and 6 h (DI 0.97) after HST. Disease severity was significantly reduced in plants inoculated at 12 and 24 h after HST to DI 0.35 and 0.46, respectively, relative to that of NT plants. Thereafter, disease severity increased to DI 0.81 at 48 h and decreased again to DI 0.64 at 72 h after HST (Figure 2.1a). A significant reduction in disease severity was observed in the 1st leaves but not in the 2nd leaves when only the 1st leaves were treated with HS (Figure 2.1b, c).

Disease severity was reduced in plants under HST compared to that of NT plants when inoculation was done 12 h after HST. However, the effect was not systemic. Induction of the defense response by HST was observed previously in melons (Widiastuti et al., 2011). HST at 50 °C for 20 s resulted in the maximum reduction of *Bc* symptoms in plants inoculated immediately after HST and 24 h after HST. On the other hand, when inoculation was performed more than 24 h after HST, plants were not protected against the pathogen. This result indicated that a similar mechanism mediates HSIR against *Bc* in melon plants and against *Pst* in tomato plants. On the other hand, HST reportedly activated systemic induction of the defense response, which was different from the result observed in tomatoes under the current experimental conditions (Widiastuti et al., 2013a). Hot water dipping of the first leaves (50 °C for 20 s) of melon seedlings significantly reduced *Bc* lesion diameter on detached untreated leaves. It is unclear whether the experimental conditions or the difference in plant species was the main reason for the failure to induce systemic resistance in tomatoes. Nevertheless, the reduction in *Pst* lesions suggested that the defense response against *Pst* in tomatoes was effectively induced by HST.

2.3.2. PR1a2, PR1b1, HsfA2, and HsfB1 expression profiles and SA accumulation in HS-treated tomato leaves

Gene expression analysis showed that *PR1a2* (Figure 2.2a) and *PR1b1* (Figure 2.2b) upregulation started at 12 h after HST and peaked at 24 h after HST, followed by a decline at 48 h after HST; whereas *PR1a2* and *PR1b1* gene expression were not detectable in the NT.

Furthermore, upregulation of *PR1a2* and *PR1b1* were not observed in the 2nd leaf, which was not treated in partial HS experiments (Figure 2.3a, b). On the other hand, *PR1a2*, but not *PR1b1,* was upregulated in the 2nd leaf at 24 h after partial inoculation (Figure 2.3c, d). Both *PR1a2* and *PR1b1* were upregulated at 72 h after HST in *Pst*-inoculated plants at 24 h after HST to a similar extent as HST-untreated *Pst*-inoculated plants, whereas plants subjected to HST only did not show induced gene expression (Figure 2.3e, f). The upregulation of both *HsfA2* and *HsfB1* peaked at 6 h after HST (Figure 2.4a, b). Although *HsfA2* was upregulated at 3 h after HST and peaked at 6 h after HST, expression declined beginning at 12 h after HST and until the end of the experimental period. The expression pattern of *HsfB1* was different from that of *HsfA2*, as it gradually decreased from 12 to 24 h after HST but still retained a higher expression level than that of the NT. SA accumulated significantly at 12 h after HST but then constantly decreased from 24 to 48 h after HST (Figure 2.5a). In turn, SAG began to accumulate significantly at 48 h after HST, when SA accumulation had already ceased (Figure 2.5b).

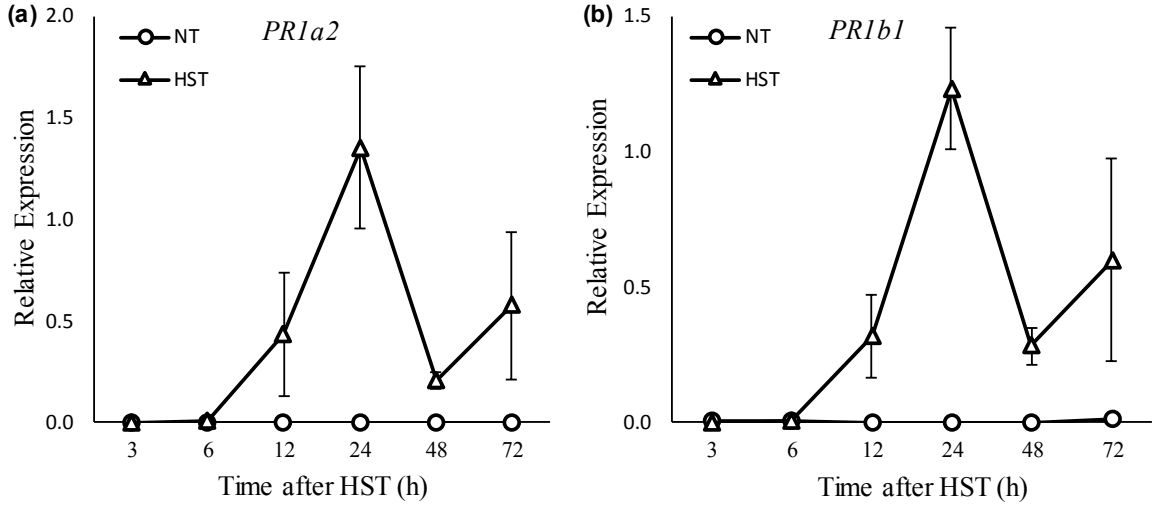

Figure 2.2. Changes in (a) *PR1a2,* and (b) *PR1b1* expression levels in the tomato cultivar Natsunokoma. Gene expression levels in the 1st leaf at 3, 6, 12, 24, 48, or 72 h after HST (45 °C, 2 min) were quantified by qPCR and normalized to *GAPDH* expression. Vertical bars indicate SEM (n = 4).

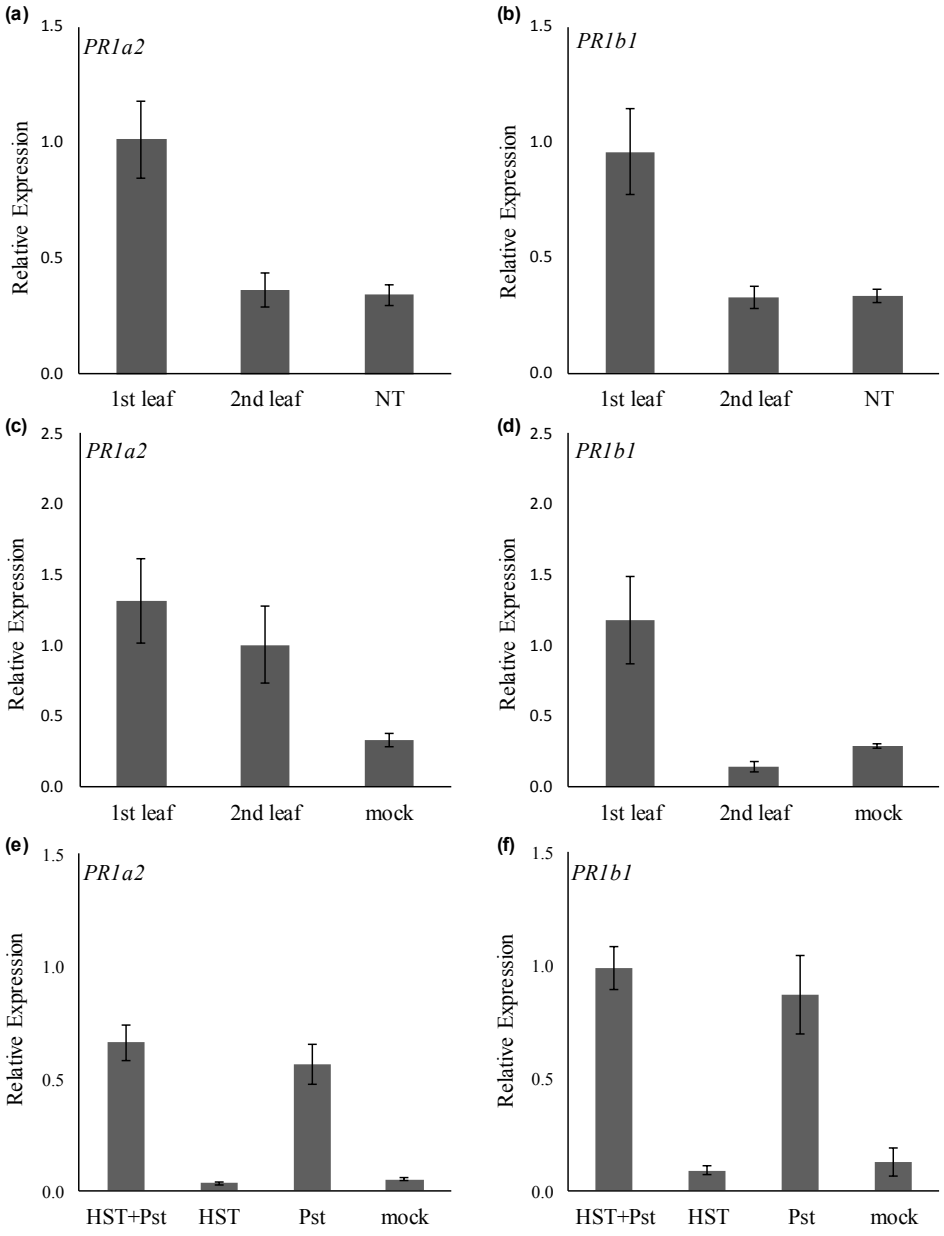

Figure 2.3. Relative expression levels of *PR1a2* and *PR1b1* in the leaves of the tomato cultivar Natsunokoma after partial treatment by HS, Pst inoculation or combined HS + Pst inoculation.

Only the 1st leaf was subjected to HST at 45 °C for 2 min (a-b) or dipped into *Pst* solution (c-d). Thereafter, total RNA from the 1st and 2nd leaf was extracted at 24 h after HST or *Pst* inoculation. In combined treatment (e-f), *Pst* was inoculated at 24 h after HST. Total RNA from the 1st leaf was extracted at 48 h after *Pst* inoculation, which was 72 h after HST. Gene-expression levels were quantified by qPCR and normalized to *GAPDH* expression. Vertical bars indicate SEM (n = 6).

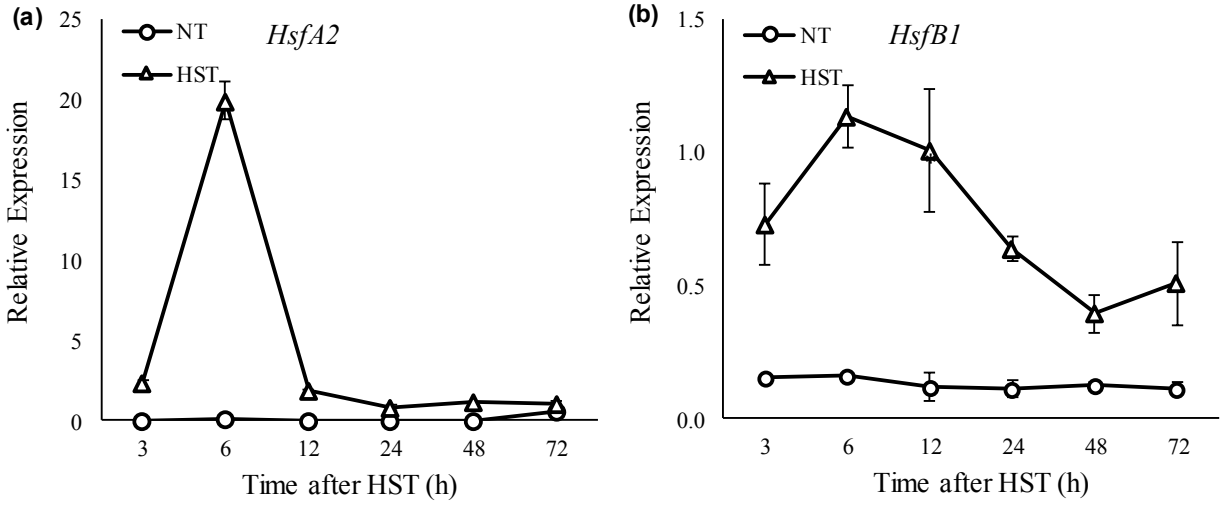

Figure 2.4. Changes in the relative expression level of (a) HsfA2 and (b) HsfB1.

Gene expression levels in the 1st leaf at 3, 6, 12, 24, 48, or 72 h after HST (45 °C, 2 min) were quantified by qPCR and normalized to *GAPDH* expression. Vertical bars indicate SEM (n = 4).

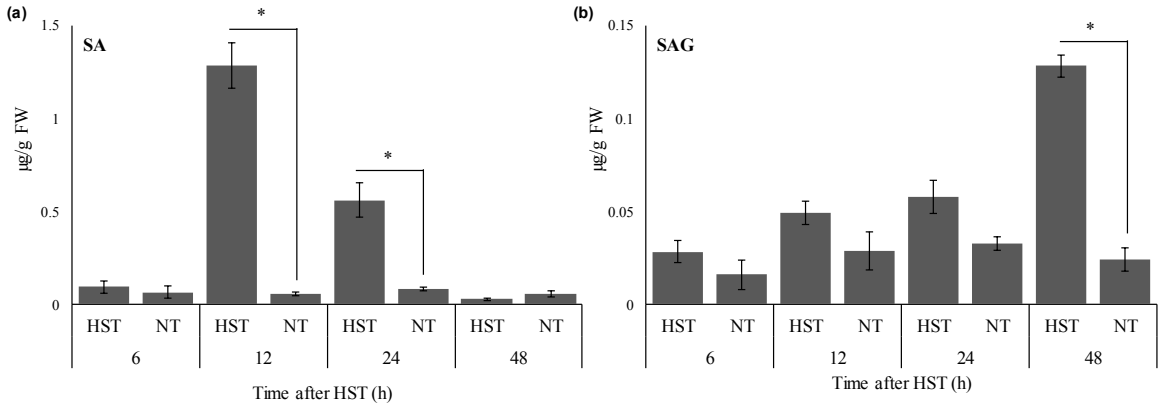

Figure 2.5. Accumulation of (a) SA and (b) SAG at 6, 12, 24 and 48 h after HST.

Vertical bars indicate SEM (n = 3). Significant differences between groups (indicated by asterisks) were obtained from performing two-tailed *t*-tests, $P < 0.05$.

Gene expression analysis showed that *PR1a2* (Figure 2.2a) and *PR1b1* (Figure 2.2b) upregulation started at 12 h after HST and peaked at 24 h after HST, followed by a decline at 48 h after HST; whereas *PR1a2* and *PR1b1* gene expression were not detectable in the NT. Furthermore, upregulation of *PR1a2* and *PR1b1* were not observed in the 2nd leaf, which was not treated in partial HS experiments (Figure 2.3a, b). On the other hand, *PR1a2*, but not *PR1b1,* was upregulated in the 2nd leaf at 24 h after partial inoculation (Figure 2.3c, d). Both *PR1a2* and *PR1b1* were upregulated at 72 h after HST in *Pst*-inoculated plants at 24 h after HST to a similar extent as HST-untreated *Pst*-inoculated plants, whereas plants subjected to HST only did not show induced gene expression (Figure 2.3e, f). The upregulation of both *HsfA2* and *HsfB1* peaked at 6 h after HST (Figure 2.4a, b). Although *HsfA2* was upregulated at 3 h after HST and peaked at 6 h after HST, expression declined beginning at 12 h after HST and until the end of the experimental period. The expression pattern of *HsfB1* was different from that of *HsfA2*, as it gradually decreased from 12 to 24 h after HST but still retained a higher expression level than that of the NT. SA accumulated significantly at 12 h after HST but then constantly decreased from 24 to 48 h after HST (Figure 2.5a). In turn, SAG began to accumulate significantly at 48 h after HST, when SA accumulation had already ceased (Figure 2.5b).

Widiastuti et al. (2011, 2013a) showed that peroxidase and chitinase genes were upregulated at 12 h after HST in melon plants, whereas in cucumbers, the peroxidase gene was upregulated at 24 h after HST (Yoshino et al., 2011). The results in tomato leaves were consistent with those in melons and cucumbers, although experimental conditions differed. Transient expression profiles of *PR1a2* and *PR1b1* (Figure 2.2) corresponding with the appearance of induced resistance against *Pst* (Figure 2.1a) suggested that the expression of these genes was triggered as part of the HSIR pathway of plant response to heat stress. However, the expression was not systemic, thereby confirming the difference between partial-HST and *Pst* challenge. When plants were inoculated with Pst, systemic expression of PR1a2 was observed. This result indicated that the defense response triggered by HS was

different from that triggered by pathogen infection. Stout et al. (1999) observed that partial inoculation induced SAR in tomato plants. The failure of the systemic induction of disease resistance and PR gene expression showed that HST could not activate SAR after 24 h.

The expression pattern of *HsfA2* (Figure 2.4a) was different from that of *HsfB1* (Figure 2.4b). According to Hahn et al. (2011), the interaction of HsfB1 with Hsp70/90 under normal conditions maintains HsfB1 production at a low level because of rapid degradation, thus, explaining the low expression of *HsfB1* over time under NT, which was not observed for *HsfA2*. These results suggested the possibility that Hsfs might be activated prior to SA accumulation or PR gene expression in response to HS. Hence, Hsfs might be the triggering molecules that induce defense responses following HST in addition to SAR.

SA is involved in providing systemic immunity to pathogens (Kumar, 2014; Derksen et al., 2013; Park et al., 2007). Transient accumulation of SA was considered to cause the failure of systemic resistance. In the case of pathogen infection or chemical activator treatment, an elicitor derived from the pathogen or a chemical derivative often remains around the infected part and persistently acts as a signaling compound. In contrast, the duration of HSIR was shorter than that of SAR because no compound was applied to plants externally in conjunction with HST.

SA plays a different role in response to HS from the one it plays in the alleviation of the damaging effects of high temperature. Exogenous application of SA protects Arabidopsis against heat-induced oxidative damage (Larkindale and Knight, 2002), thus, reducing the adverse effects of heat stress on photosynthesis in wheat (Khan et al., 2013) and grapevines (Wang et al., 2010). Snyman and Cronjé (2008) reported that SA assisted in the binding of Hsfs to HSEs in the promoter regions of stress genes in tomato plants. They noted that SA alone had no significant effect on *HsfA1* and *HsfB1* expression, but potentiated the basal level of *HsfA1*, followed by accumulation of Hsps in heat-treated plants. Moreover, tomato *PR1b1* promoter activity was enhanced by SA (Tornero et al., 1997). It appears that SA content post-HS did not reach a SAR-inducing concentration; thus, it was unable to induce

systemic expression of PR genes, as well as systemic resistance against *Pst*. Further, it was not clear whether the appearance of local resistance after HST was induced by SA. However, if previous studies on the ability of SA to enhance Hsf-DNA binding are considered, the hypothesis of direct regulation of locally induced resistance by Hsfs gains strong support.

2.3.3. Identification of HSEs motifs in the upstream regions of target genes

Multiple possible HSE motifs were discovered in the upstream regions up to 2500 bp from the start codon of all tested PR genes. Four possible HSE motifs were found on *PR1a2* and *Chi9;* two motifs on *PR1b1*, *Chi3*, and *GluA*; and one motif on *GluB* (Figure 2.6a). To our knowledge, all tested genes possessed these motifs. At least two HSE motifs are in reverse orientation and perfectly match the requirements for the minimal Hsfs-binding motif 5′-nGAAn-3′ or 5′-nTTCn-3′ (Figure 2.6b).

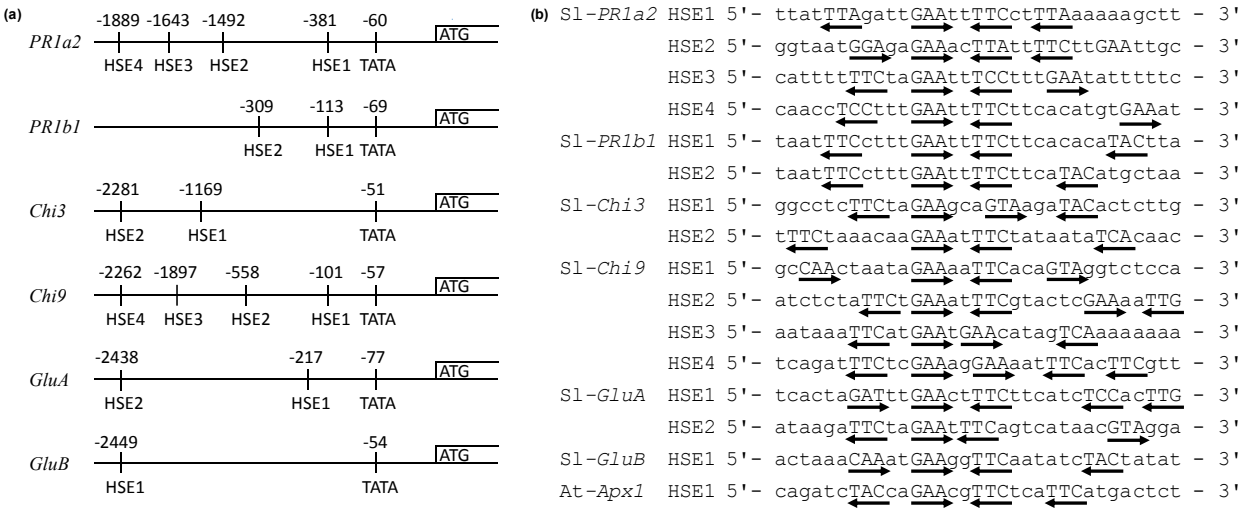

Figure 2.6. HSEs in the upstream area of PR genes.

(a) The location of possible HSEs in tomato PR genes. The numbers indicate the distance (in bp) from the proximal transcription start site (ATG). TATA represents the TATA box consensus sequence. (b) Comparison of the HSE from tomato PR genes (Sl-) and Arabidopsis apx1 (At-). Sequences matching the nGAAn, the basic 5 bp HSE motif are indicated in uppercase letters. Orientation of the nGAAn-like motifs are indicated by arrows.

The number of HSEs, as well as the distance from the start codon, varied among PR genes. Nevertheless, the expression profiles of PR genes did not change. Nover (1987) reported that heat shock inducible genes possessed a functional HSE at a distance of -40 to -400 from the start codon in eukaryotic cells, such as those of *Drosophila*, humans, *Xenopus laevis*, *Caenorhabditis elegans*, plants, and *Dictyostelium*. Our data showed that *PR1a2*, *PR1b1*, *Chi9*, and *GluA* fulfilled those criteria. Consistently, Hoffman and Corces (1986) demonstrated that cascades of HSE that extended up to -2100 must interact for optimum HS-induced Hsp28 activity in *Drosophila*. In tomatoes, PR1b1 with distinct HSE structure has been extensively studied as a major protein responsive to chilling temperature. Exposure of tomato fruits to chilling temperature (2 °C) followed by re-warming of the fruits to 20 °C led to a several-fold increase in PR1b1 content (Goyal et al., 2016). This result confirmed that PR1b1 was responsive to changes in temperature. Moreover, the interaction between tomato HsfB1 and the Arabidopsis *apx1* promoter confirmed that *apx1* HSE was functional as an Hsfs-binding site (Storozhenko et al., 1998). Therefore, it is possible that the existence of an HSE in the upstream area contributes to HS-induction of PR genes, although further experimentation is required to confirm that the corresponding HSE in PR genes is recognized by Hsfs.

2.3.4. The effect of Hsp90 and Hsfs inhibitor treatment to the induction of defense response

The optimum concentration of GDA required to induce *PR1b1* gene expression was 10 μM, whereas lower or higher concentrations failed to induce it (Figure 2.7). Upregulation of *PR1a2*, *PR1b1*, *Chi3*, *Chi9*, *GluA*, *GluB*, *HsfA2*, and *HsfB1* after GDA treatment, as well as HST, are shown in Figure 2.8a, b, c, d, e, f, g, and h, respectively. The most effective concentration of KB to inhibit HSR was 100 μM, and the expression level of *PR1b1* was reduced to 60.9% of the level induced by HST (Figure 2.9). Foliar spray of KB repeated at 6, 12, and 18 h after HST, successfully suppressed the upregulation of all tested PR genes. The expression level of *PR1a2, PR1b1, Chi3, Chi9, GluA,* and *GluB* decreased to 30.5%, 32.1%, 46.2%, 62.3%, 36.4%, and 54.2%, respectively (Figure 2.10a, b, c, d, e, and f), upon KB

treatment. Conversely, the expression levels of *HsfA2* and *HsfB1* were increased by 39.9% (Figure 2.10g) and 25.1% (Figure 2.10h), respectively. The effect of KB-induced inhibition of Hsfs on disease resistance against *Pst* was confirmed by challenge inoculation. HST alone caused a significant reduction in disease severity, whereas the results from HST combined with KB were similar to those of DMSO (Figure 2.11).

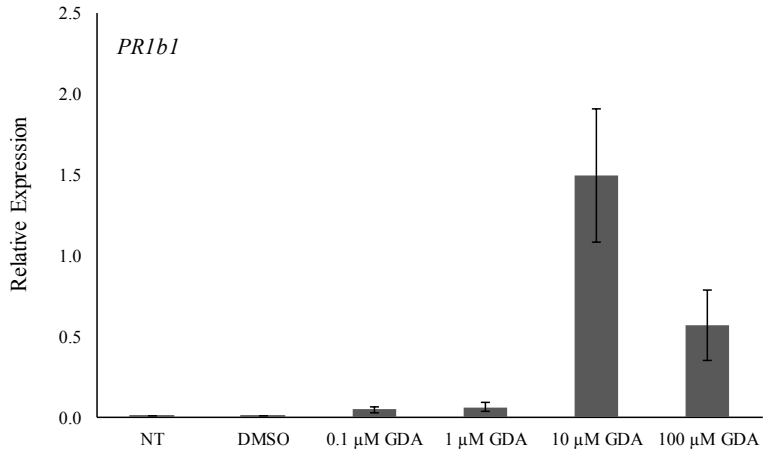

Figure 2.7. The relative expression level of *PR1b1* in the leaves of the tomato cultivar Natsunokoma at 24 h after GDA treatment.

Different GDA concentrations (0.1 μM, 1 μM, 10 μM, and 100 μM) and 0.1% (v/v) DMSO were applied by foliar spray. Gene expression levels were quantified by qPCR and normalized to *GAPDH* expression. Vertical bars indicate SEM (n = 4).

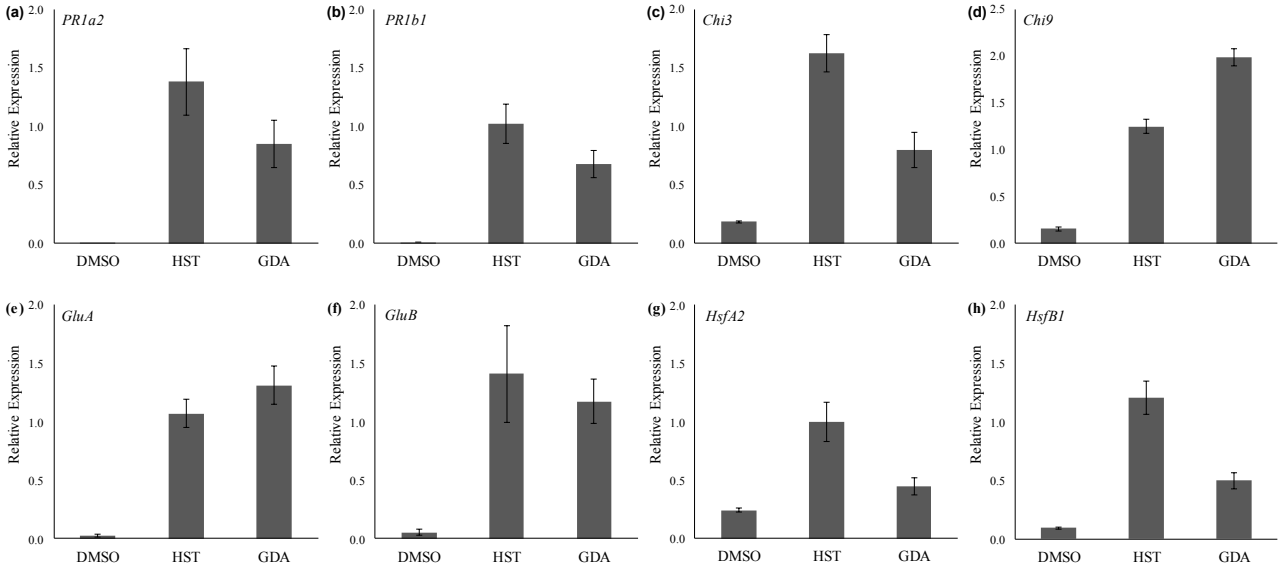

Figure 2.8. Relative expression level of PR (a-f) and Hsfs (g-h) genes in the leaves of the tomato cultivar Natsunokoma at 24 h after treatment with DMSO, HST, and GDA.

0.1% (v/v) DMSO and 10 μM GDA were manually applied by foliar spray. Gene expression levels were quantified by qPCR and normalized to *GAPDH* expression. Vertical bars indicate SEM (n = 4).

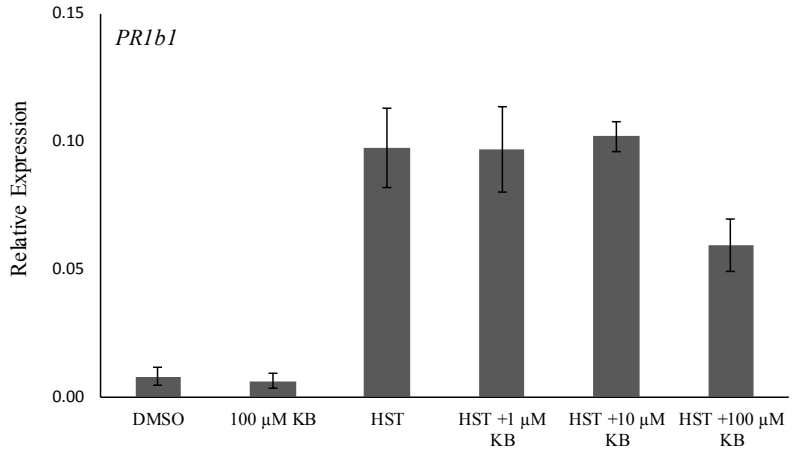

Figure 2.9. The relative expression level of *PR1b1* in the leaves of the tomato cultivar Natsunokoma at 24 h after HST combined with KB.

Different KB concentrations (1 μM, 10 μM, and 100 μM) were manually applied by foliar spray at 6 hours after HST. Gene expression levels were quantified by qPCR and normalized to *GAPDH* expression. Vertical bars indicate SEM (n = 4).

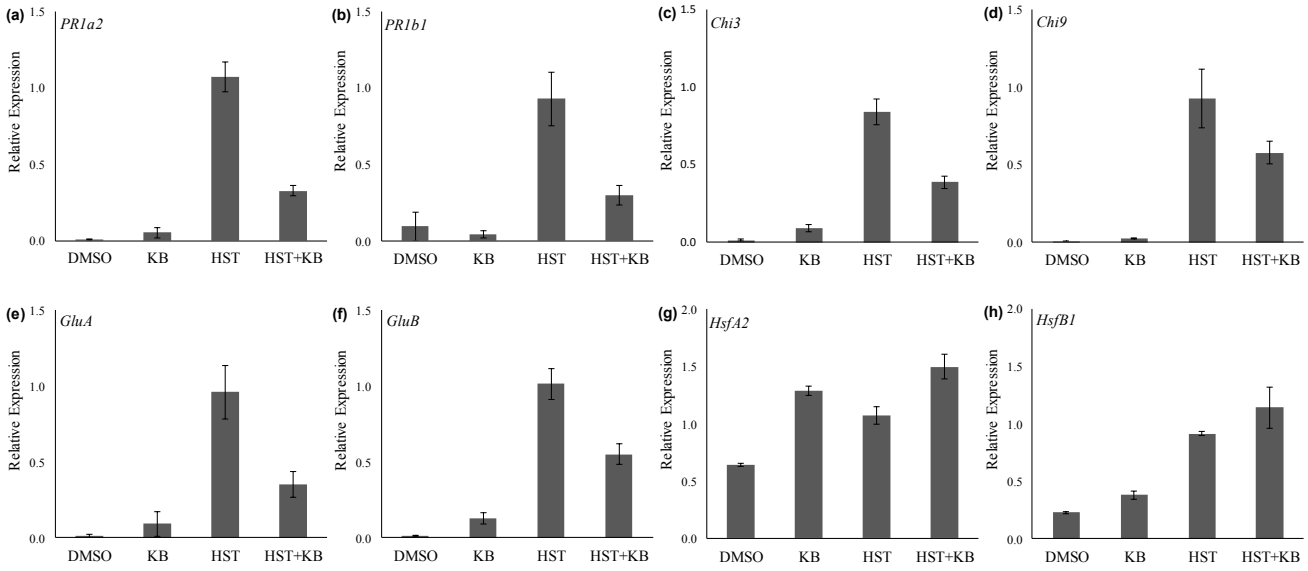

Figure 2.10. The relative expression level of PR (a-f) and Hsfs (g-h) genes in the leaves of the tomato cultivar Natsunokoma at 24 h after HST combined with KB.

100 μM of KB was manually applied by foliar spray at 6, 12, and 18 h after HST. Gene expression levels were quantified by qPCR and normalized to *GAPDH* expression. Vertical bars indicate SEM (n = 6).

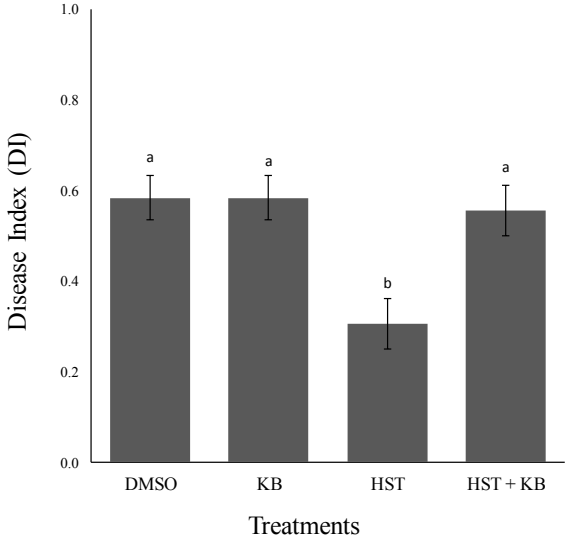

Figure 2.11. Disease severity upon inoculation of *Pst*. 1% DMSO, 100 μM KB, HST, and HST combined with 100 μM KB manually applied by foliar spray at 6, 12, and 18 h after HST.

Vertical bars indicate SEM (n = 3). Different letters indicate significant differences among treatments based on Tukey´s test, $P < 0.05$.

Induction of gene expression was lower in GDA-treated plants, compared to HST plants, except for *GluA* and *Chi9*. Under HS, HsfA1 is released from HSP90 complexes; thus, a high amount of free HsfA1 can bind the HSE to induce downstream gene transcription. On the other hand, the mode of action of GDA is to prevent Hsp90 binding the newly synthesized HsfA1 by substrate competition, due to the similarity of the Hsp90 GDA-binding pocket to the substrate-binding sites (Stebbins et al., 1997); thus, GDA cannot release HsfA1 already bound to Hsp90. Therefore, the amount of free HsfA1 is less than that under HST; consequently, downstream gene transcription is also lower.

GDA has been shown to enhance HsfB1 protein production. The amount of HsfB1 protein gradually increased from 3 to 9 h after incubation of tomato suspension-cell cultures in the presence of 1 μM GDA (Hahn et al., 2011). In addition, Hsp90-silenced tomatoes showed an increased level of HsfA2 protein compared to the leaves transformed with an empty vector (Moshe et al., 2016). Because Hsp90 regulates the activation of HsfA1 (Hahn et al., 2011), previous results of HsfB1 induction by GDA treatment (Hahn et al., 2011) and HsfA2 upregulation in Hsp90-silenced cells indicated that both HsfA2 and HsfB1 were regulated by HsfA1 (Moshe et al., 2016). Meanwhile, the application of Hsp90 inhibitor GDA induced the expression of HsfA2, HsfB1, and PR genes (Figure 2.8), which was found to have an HSE in the upstream gene area (Figure 2.6). These results suggested that the mechanism that regulates HSR via Hsfs might also operate in the regulation of HSIR. Moreover, data from an analysis of the time course followed by gene expression profiles showed that the induction of *HsfA2* and *HsfB1* preceded the expression of PR genes, which means that either HsfA2 or HsfB1 is likely responsible for the upregulation of PR genes after HST.

Upregulation of both *HsfA2* and *HsfB1* under the combined HST-KB treatment showed that KB did not affect the master regulator of HSR, HsfA1, which regulates the expression of *HsfA2* and *HsfB1*. On the other hand, the expression of PR genes, as well as the preventive effect of HS against *Pst* were suppressed under the same treatment. Because PR gene expression was detected following *HsfA2* and *HsfB1,* the suppression of PR genes is likely caused by KB-induced inhibition of HsfA2 or HsfB1.

2.3.5. *Concluding remarks*

A protective effect of HST against pathogen infection was observed in tomatoes at 12 and 24 h after HST. The accumulation of SA after HST showed that SAR was involved in HSIR. However, HST failed to induce systemic PR gene expression or systemic resistance against *Pst*. Local induction of the defense response and PR gene expression following HST demonstrated that a different mechanism was involved. Induced Hsfs gene expression preceded SA accumulation and PR gene upregulation. A schematic representation of our interpretation of the results reported herein is presented in Figure 2.12. We proposed that Hsfs-mediated HSIR is activated (Figure 2.12a) before SAR becomes activated (Figure 2.12b). This hypothesis was substantiated by the results of GDA-induced inhibition of Hsp90 and KB-induced inhibition of Hsfs. PR gene expression was upregulated after HS or GDA treatment, whereas KB suppressed HS-induced PR gene expression but not *HsfA2* and *HsfB1*. Overall, these results suggest that Hsfs were responsible for the activation of PR genes, which in turn led to the induction of resistance against *Pst* in tomato leaves.

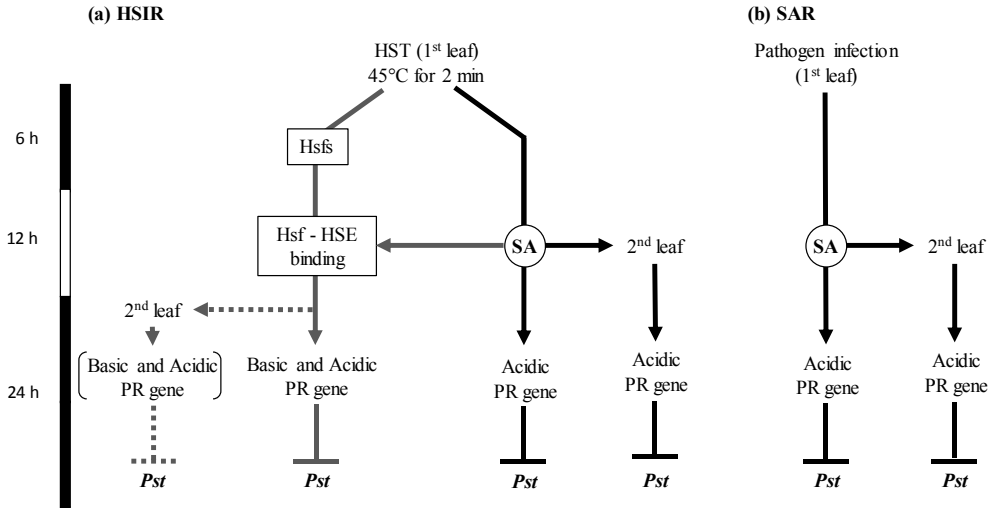

Figure 2.12. Comparison between HSIR (a) and SAR (b). Black lines indicate proved pathways and gray lines indicate proposed pathways as elucidated by the work reported herein.
During HSIR, Heat shock induced Hsfs gene expression, followed by SA accumulation, which in turn enhanced Hsfs binding to HSE, thereby activating non-systemic stimulation of basic and acidic PR gene expression. Disease severity in the 1st leaf was significantly reduced concomitantly with upregulation of PR gene expression when *Pst* was inoculated.

Further, if *Hsfs* were also induced by infection, they could be proposed as a universal trigger for the activation of a defense response. Finally, the possibility that Hsfs function as triggering molecules in HSIR provides new insights into the molecular mechanisms of the plant defense systems against pathogens, as well as the opportunity to develop new approaches for crop protection.

CHAPTER THREE

Prevention of powdery mildew disease in tomato nursery by an improved hot water spraying device

3.1. Introduction

During recent years, the number of nursery specialists has been increasing in Japan. The establishment of role division between nursery producer and farmers who produce crops after transplanting (hereafter, vegetable grower) has helped to reduce labor force and production cost for vegetable growers. Grafting is necessary for providing some desirable traits to nursery plants. The use of mechanical systems can accelerate the production of grafted nursery plants. Vegetable growers can systematically obtain grafted seedlings from nursery producer on schedule, as the cost of seedlings is comparable to that of raising seedlings on their own. Meanwhile, providing pathogen-free seedlings is an absolute requirement for nursery producers to prevent pathogens from spreading to areas where they are transported. There are two main reasons why nursery producers should reduce

chemical use. One is the emergence of chemical-resistant pathogens. In the case of tomato, chemical resistance has already been reported in some major pathogens such as powdery mildew, gray mold (Rodríguez et al., 2014), and leaf mold. Overapplication of fungicides that contain the same chemical component is considered a major cause of pathogen resistance. Another reason is that vegetable growers request nursery producers to minimize the use of chemicals on seedlings. As the upper limit of chemical application for every registered crop is determined by the Japanese Agricultural Chemical Regulations, vegetable growers are accountable for the number and amount of chemicals applied to their seedlings. Therefore, nursery producers are forced to balance pathogen-free seedling production with reduced chemical application.

Powdery mildew was not a serious disease in tomato because producers were applying fungicides against leaf mold until leaf mold-resistant varieties were released commercially in Japan. These fungicides are thought to be effective against powdery mildew as well. Recently, because most of the new tomato varieties are leaf mold-resistant, farmers have been tending to refrain from fungicide application. As a result, since 2000, powdery mildew has re-emerged as a highly infectious disease of tomato in Japan (Matsuda et al., 2001).

Induction of disease resistance by physical means is proposed as an alternative protection technique. In tomato, Sato et al. (2005) reported that heat shock treatment by hot water dipping induced resistance against gray mold, via increase in salicylic acid (SA) content and pathogenesis-related gene expression. A similar effect has been reported after hot water dipping of strawberry plant at 50 °C for 20 s (Widiastuti et al., 2013b; Sato et al., 2017); barley at 50 °C for 30–60 s (Schweizer et al., 1995); cucumber at 40 °C for 120 s (Yoshino et al., 2011); melon at 50 °C for 20 s (Widiastuti et al., 2011); and Arabidopsis at 45 °C for 120–180 s (Kusajima et al., 2012). Widiastuti et al. (2013b) suggested that heat shock-induced resistance (HSIR) has multiple signaling pathways involving systemic acquired resistance (SAR) that is mediated by SA. SAR is expected to confer broad-spectrum resistance against various pathogens in different crops. To clarify the activation of HSIR, some defense-related

genes were used as expression markers, such as pathogenesis-related protein 1 gene in Arabidopsis (Kusajima et al., 2012) as well as peroxidase, glucanase, and chitinase 1 genes in melon (Widiastuti et al., 2011). In such treatments, only a part of the plant is subjected to the shock condition, and HSIR is triggered systemically.

Hot water spraying has been suggested as a practical means of activating HSIR and directly inhibiting pathogen growth (Yamagishi et al., 2009; Ogawara et al., 2012; Yoshino et al., 2012; Sato et al., 2017). The development of a hot water sprayer prototype was initiated in the previous study, and it was proven to effectively protect cucumber against gray mold as well as induce the production of SA and the expression of peroxidase gene in the leaves (Yoshino et al., 2012). However, the implementation of sprayer prototype under field condition was laborious as the sprayer should be manually operated and was not designed to spray multiple seedlings simultaneously. In the present study, an improved design of hot water spraying device based on boom configuration was evaluated for the induction of disease resistance in tomato. Until now, the optimum condition of heat shock treatment has been clarified using a gray mold inoculation model experiment. The intervention of HSIR was confirmed by expression analysis of marker genes. Subsequently, a prototype of a hot water sprayer was developed and tested against powdery mildew in a tomato nursery.

3.2. Materials and Methods

3.2.1. Hot water treatment condition for inducing resistance against pathogen in tomatoes

Seedlings of tomato (*Solanum lycopersicum* L.) cultivars Momotaro and Natsunokoma were grown in pots with culture soil at room temperature with a 16:8 h (light: dark) photoperiod for 20 days. Natsunokoma has been used in a previous study for the initial investigation of HSIR (chapter two, Sato et al., 2005). On the contrary, Momotaro was used to confirm HSIR in different varieties. Two-leaf stage seedlings were used for the inoculation test. The aerial parts of seedlings were dipped upside down in water heated in a water bath to 50 °C for 20 s (HWD). As a positive control, a plant activator, 1,2-benzisothiazol-3(2H)-one-1,1-dioxide (BIT; 5 ml of 1 mg/ml, Wako Pure Chem Industries, Osaka, Japan) was sprayed on the aerial parts of seedlings at the same time as the HWD treatment. BIT has been known to activate SAR via an SA-mediated signaling pathway. Non-treatment (NT) was used as the negative control. Gray mold inoculum was prepared and inoculated at 24 h after HWD or BIT treatment according to Yoshino et al. (2011). Gray mold is caused by *Botrytis cinerea*, a polyphagous, saprophytic fungus. Gray mold was used as an inoculum because of the difficulties in handling powdery mildew fungus, which is an obligate parasite with limited occurrence. Contrarily, the inoculum of gray mold can be used any time, because it can be cultured on agar medium (Choquer, 2007). Furthermore, the HSIR of tomato was discovered using gray mold (Sato et al., 2005), and its mode of action has a close relationship with that of SAR against a broad spectrum of pathogens. The diameter of the disease lesions was measured 2 days after inoculation. Each treatment was applied to 5 plants and repeated 3 times.

3.2.2. Expression analysis of induced resistance marker genes

Seedlings of the tomato cultivar Momotaro with approximately the same leaf size were subjected to HWD, as described above. Relative changes in gene expression were measured by quantitative real-time PCR (qRT-PCR). Total RNA was extracted from each leaf disk at 3

days after treatment by using a commercial extraction kit (RNAiso Plus; Takara Bio Inc., Shiga, Japan), and mRNA was reverse transcribed. Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) with an anchored-oligo $(dT)_{18}$ primer was used according to the manufacturer's instructions. The synthesized first-strand cDNA was used as the template for quantitative real-time polymerase chain reaction (qPCR) on a Thermal Cycler Dice Real Time System (TP850; Takara Bio Inc.), according to the manufacturer's manual. Three genes encoding pathogenesis-related proteins: pathogenesis-related protein 1a (PR1a), basic intracellular β-1,3-glucanase (GluB), and basic intracellular chitinase (Chi9) were used as expression markers. The expression levels in each sample were normalized to the expression level of actin. The corresponding primer sequences were as follows: actin (U60480), forward 5′ -cctatgttggtgatgaagctcagtc-3′ and reverse 5′ -tgttcttcgggagcaacacgaa-3′ ; *GluB* (M80608), forward 5′ -tcttgccccatttcaagttc-3′ and reverse 5′ -tgcacgtgtatccctcaaaa-3′ ; *Chi9* (Z15140), forward 5′ -ctccaatggctcttccacat-3′ and reverse 5′ -gaaattgctgctttccttgc-3′ ; and *PR1a* (AJ011520), forward 5′ -acaagctcaaaactcccctca-3′ and reverse 5′ - tcaaaagccggttgattttcaaga-3′ . Three technical replicates of qRT-PCR were used for each biological replicate.

3.2.3. Development of a hot water sprayer for tomato seedlings

A hot water sprayer prototype was designed (Figure. 3.1A-D) as follows. We set a cart (b) towed with a winch (n) through a wire rope (m) at 0.5 m/min (o) on a curtain rail (a). A cold-water hose (d) and a hot-water hose (e) were hung on the rail (g). A propane gas boiler (f) was used for heating water. The two water hoses were connected to a mixing valve (h) to maintain a temperature of 57 °C ± 2 °C, measured using a checking thermometer (j). The spray water amount was set at 3.5 L/min discharged through 15 nozzles via the main valve (k) monitored using a pressure gauge (i). Nozzles (p) were placed at every 10 cm in a boom (l).

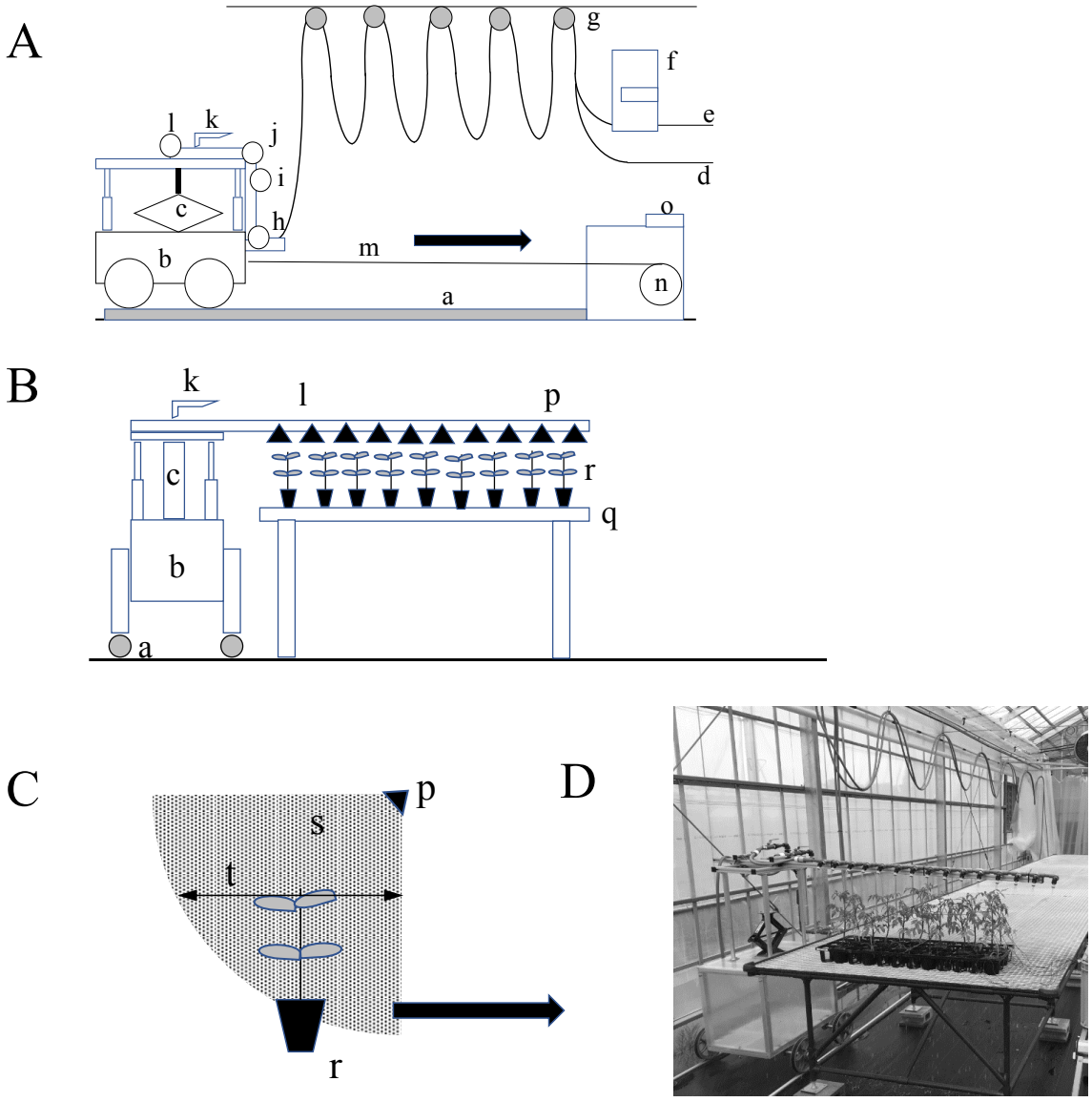

Figure 3.1. Schematic diagram and complete view of the developed hot water sprayer.

A: plane of the structure, B: back elevation, C: nozzle angle, D: complete view

a: rail, b: cart, c: height-adjusting pantograph, d: cold-water hose, e: hot-water hose, f: propane gas boiler, g: curtain rail, h: mixing valve, i: pressure gauge, j: thermometer, k: main valve, l: boom, m: wire rope, n: winch, o: speed controller, p: nozzle, q: bench, r: tomato seedling, s: spray area, t: effective duration for heating the seedlings, which should be more than 20 s + heating time.

For effective spraying, flat, wide-angle non-mist chip nozzles (N-KAL-15R; Yamaho Industry Co., Ltd., Wakayama, Japan) were installed. The specifications of these nozzles are as follows: nozzle hole diameter, 1.5 mm; average particle diameter, 670 μm at 0.2 MPa;

ejection amount, 0.99 L/min at 0.1 MPa; and zone of dispersion, 90°, based on the manufacturer's catalog. Tomato seedlings (r) were placed on the nursery bench (q). The nozzle height was adjusted to 5 cm above the tallest seedlings (c). To ensure uniform duration of exposure to high temperature at a certain point on the leaves, the flat nozzle was set along the moving direction, perpendicular to the ground (Figure. 3.1C). The leaf temperature was recorded using a thermocouple (wire diameter: 0.3 mm) connected to a data logger (GL-200A; Graphtec Corporation, Yokohama, Japan). The sensing parts of the thermocouple were attached behind the leaves by surgical tape.

3.2.4. Practical effect of hot water spray (HWS)

The performance of the sprayer for protection against powdery mildew in tomato was evaluated by inoculation test. Powdery mildew disease occurs throughout nursery areas in Japan. Two-leaf-stage seedlings of the cultivar Rinka 409 were used for the HWS experiment in the greenhouse as this variety spread rapidly in five years during this study period. We did not find any difference in tolerance against powdery mildew between Momotaro, Natsunokoma, and Rinka 409 varieties in the preliminary test. The HWS treatment was carried out as described above, repeated every week in a greenhouse. The conidia of powdery mildew were collected from infected leaves of tomato maintained in the greenhouse. Conidial suspension (2×10^4 conidia/ml) was sprayed on whole plants until run-off, immediately after the first HWS. Non-treated seedlings were used as negative control (NT). The score was determined 27 days after the first treatment by scoring the lesion area as follows: 0, healthy leaf; 1, less than 5%; 2, 5% or more; 3, 25% or more; and 4, more than 50%. The disease index was calculated as follows: disease index = $[\Sigma (n \times v)/4 \times Z] \times 100\%$, where n is the lesion score class, v is the number of samples in the score class, and Z is the total number of samples. The experiment was repeated thrice, with all leaves from five plants per replicate.

3.3. Results and Discussion

3.3.1. Hot water treatment for inducing resistance against gray mold pathogen in tomatoes

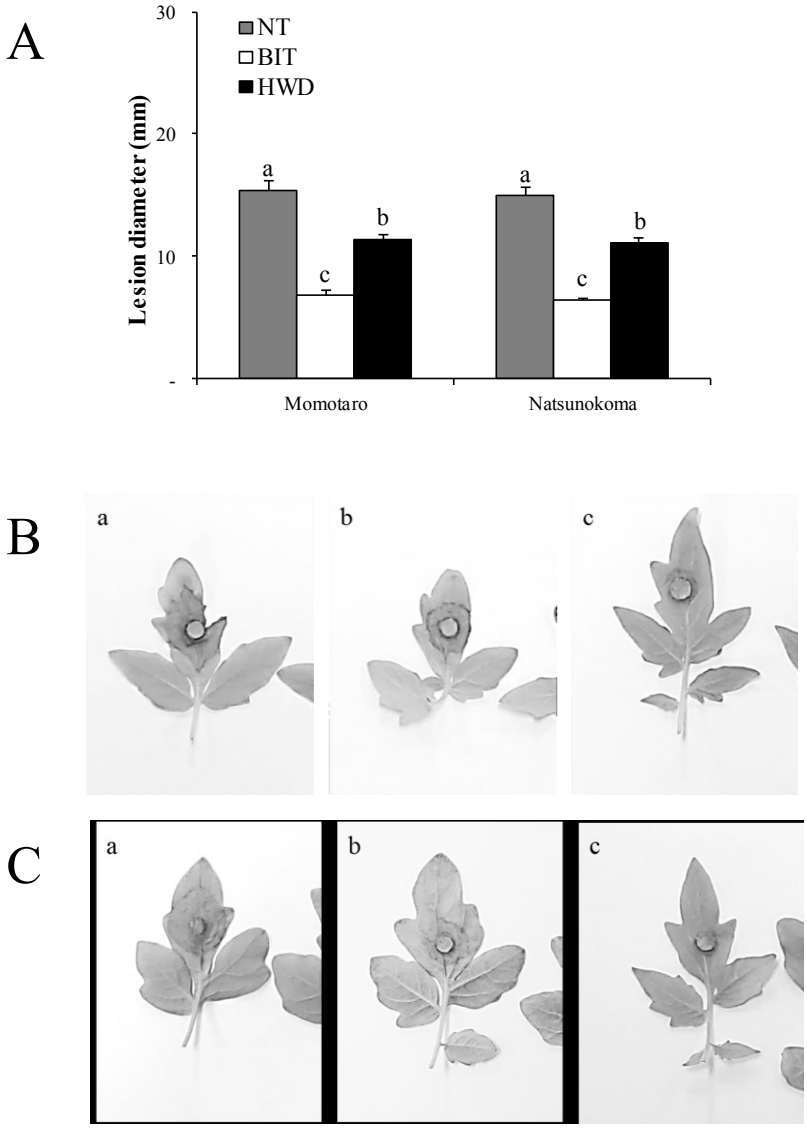

Figure 3.2. Effect of hot water dipping and BIT treatment on inducing resistance against gray mold in two tomato cultivars, Momotaro and Natsunokoma.

A: Gray mold lesion diameter; B: photograph of lesions on Momotaro; C: photograph of lesions on Natsunokoma. a: NT, b: HWD, c: BIT.

Different letters in each treatment indicate significant differences by Tukey's honestly significant difference test (n = 3, $P < 0.05$).

The diameter of the lesions was as follows: 11.4 mm in HWD, 6.8 mm in BIT, and 15.4 mm in NT for Momotaro; and 11.1 mm in HWD, 6.5 mm in BIT, and 15.0 mm in NT for Natsunokoma (Figure. 3.2). Compared to NT, HWD at 50 °C for 20 s caused a significant reduction in the severity of infection in both varieties, although induced resistance in HWD was weaker than that in BIT. In the previous experiment (chapter two), we choose to use HWD at 45 °C for 2 minutes following the previous report of HSIR in tomatoes by HWD with the same treatment conditions (Sato et al., 2005). However, 2 minutes duration is not practical for field application because of the long duration of the overall treatment, which increases the cost of heating. Therefore, HWD treatment conditions at 50 °C for 20 s is preferable. Resistance was induced against gray mold under the same condition (50 °C for 20 s) as in melons (Widiastuti et al., 2011) and strawberries (Widiastuti et al., 2013b). Thus, the condition of hot water treatment (50 °C for 20 s) obtained in the present study is suggested as a common condition for inducing resistance in various crops.

3.3.2. Expression analysis of induced resistance marker genes after HWD

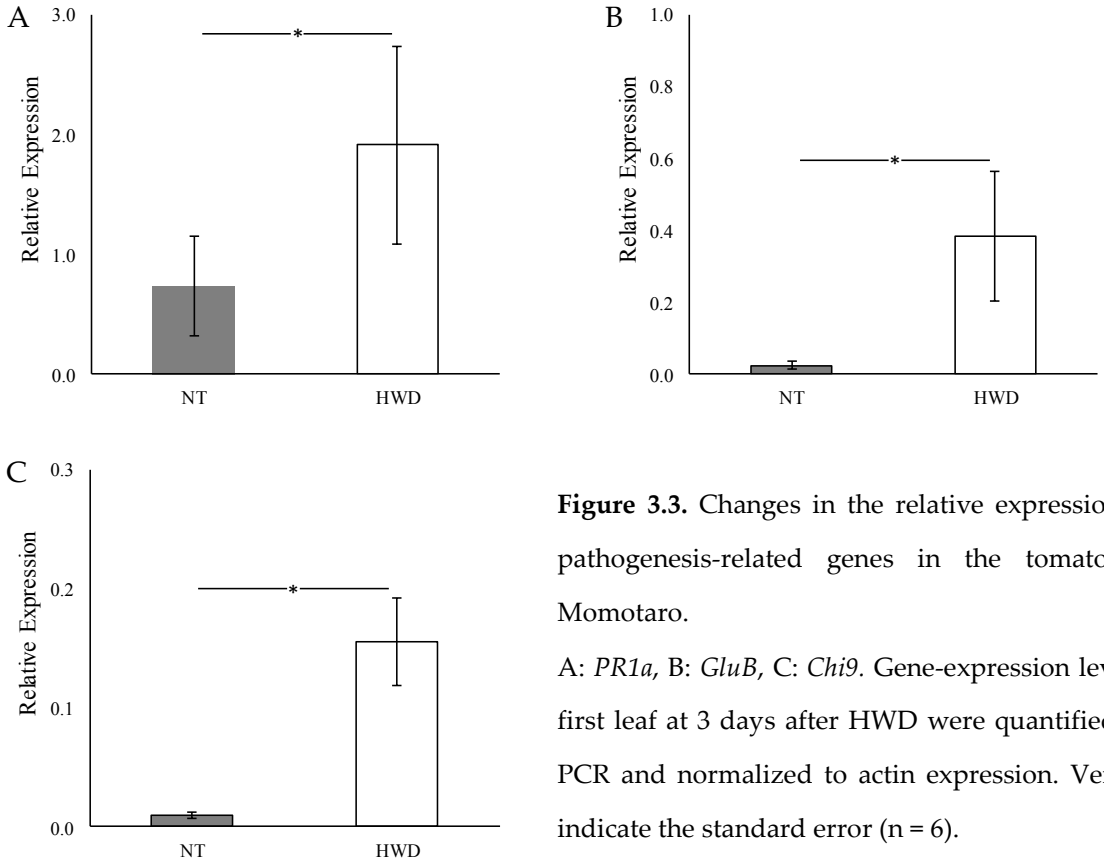

Figure 3.3. Changes in the relative expression level of pathogenesis-related genes in the tomato cultivar Momotaro.

A: *PR1a*, B: *GluB*, C: *Chi9*. Gene-expression levels in the first leaf at 3 days after HWD were quantified by qRT-PCR and normalized to actin expression. Vertical bars indicate the standard error (n = 6).

Tomato *PR1a* gene expression was upregulated during the invasion of root-knot nematode and treatment with exogenous SA (Lavrova et al., 2017), whereas *GluB* and *Chi9* expression in tomato leaves was induced after treatment with methyl jasmonate and ethylene, as well as wounding (Wu and Bradford, 2003). Therefore, in the present study, *PR1a*, *GluB*, and *Chi*9 were used as marker genes of HSIR in fungal infections of tomatoes. The expression of all tested genes increased 3 days after HWD (Figure. 3.3). This suggested that HWD activated the plant defense system in tomatoes in the same manner as that of HSIR, which was first reported in cucumbers by Stermer and Hammerschmidt (1987). Also, a previous study with melons showed that HSIR has a non-specific mechanism in plant stress responses, as it upregulates many defense-related genes in plants and functions against common pathogens (Widiastuti et al., 2011). Based on these results, the HS treatment at 50 °C for 20 s was sufficient to induce a defense response in the untreated parts. Also, treatment at a temperature below 50 °C is still capable of inducing resistance (Yoshino et al., 2011). Therefore, to treat all leaves with an even temperature condition is not necessary, hot water spraying from the upper part of plants can be used as a practical means for inducing resistance.

3.3.3. Development of hot water sprayer

The development of a prototype hot water sprayer was initiated in a previous study and has been proven effective in protecting cucumbers from gray mold and strawberries from powdery mildew (Yoshino et al., 2012; Sato et al., 2017). However, the prototype was developed for experimental use in a single plant; therefore, multiple plants could not be treated simultaneously. For practical application of hot water in a greenhouse, a novel hot water sprayer was designed in the present study (Figure. 3.1D). In this model, multiple nozzles were installed in a straight line, perpendicular to the ground in the forward direction, to cover multiple seedlings at the same time (Figure. 3.1B, C). The distance between each nozzle was set at 10 cm, which was equivalent to or shorter than the distance between seedlings grown in plastic pots with a 9 cm diameter. Thus, one or more leaves

could be treated with hot water. The sprayer was placed on a rail and rolled up using an electric winch that was installed at the end of the nursery bench. To attain the optimum conditions (50 °C for 20 s), a temperature higher than 50 °C is required because of heat loss due to vaporization. Moreover, heating time must be added to a 20 s duration of the target leaf temperature. In other words, at least one part of the seedling must fall under the moving spray area of hot water during HWS+20 s (Figure. 3.1C, t).

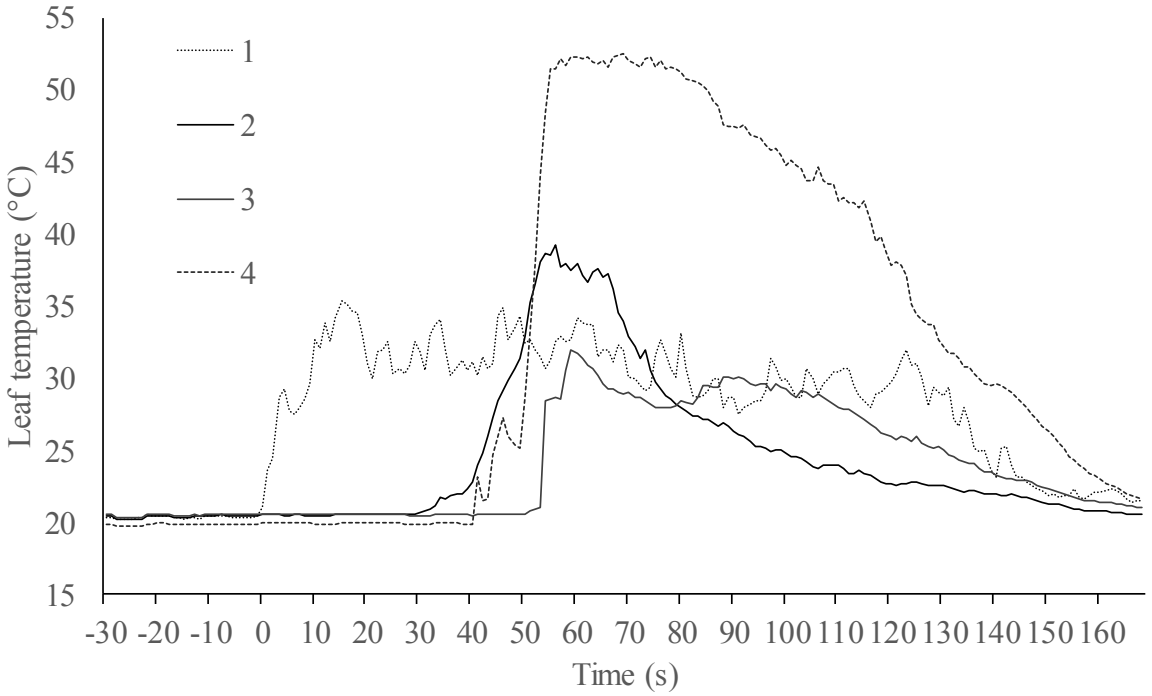

Figure 3.4. Changes in leaf temperature during hot water spraying.

Spraying condition: applied temperature, 57 °C ± 2 °C; amount of water, 3.5 L/min. Leaf temperature was measured in four randomly selected leaves (1–4). Hot water spraying was started at 0 s on the earliest emerging parts of tomato seedlings.

As a result of the preliminary test, the speed of the sprayer's horizontal movement was set at 50 cm/min. Changes in leaf temperature are shown in Figure. 3.4. Because the sprayer was moving on the rail and the sensing parts of the thermocouple were attached randomly on any leaf of a seedling, there was a time lag among the sensing points. The only leaf no. 4, which was in-line with the nozzle, exceeded the required condition for inducing resistance. No damage was observed in the treated parts after treatment, although longer duration (< 20 s) and higher temperature (50 °C) conditions were recorded. If the spray temperature

was higher than 50 °C, the temperature recorded may be higher than the expected internal temperature (50 °C) of the leaf.

3.3.4. Practical effect of HWS

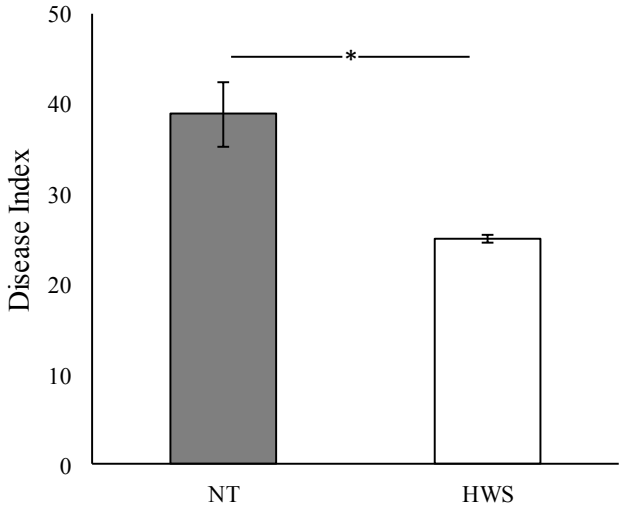

Figure 3.5. Effect of hot water spraying against powdery mildew.

Asterisk indicates a significant difference according to a two-tailed unpaired *t*-test (n = 3, $P < 0.05$). Vertical bars indicate standard error.

The severity of powdery mildew in HWS was significantly lower than that in NT seedlings (Figure. 3.5). The results of tomato HWS confirmed that partial achievement of optimum conditions in the whole plant succeeded in preventing powdery mildew. The same effect has been reported in strawberries under the same conditions (Sato et al., 2017). Meanwhile, hot water should be sprayed once a week (total, thrice until disease observation) because the duration of HSIR has been found to be less than 1 week in both melons (Widiastuti et al., 2013b) and strawberries (Widiastuti et al., 2013a). It is possible that the powdery mildew spores remained after HWS, and therefore, HWS treatment was repeated owing to the short duration of the HWS effect. The results obtained from the present study suggested that HSIR and the direct effect of hot water contributed to an integrated effect of inducing plant resistance and impairing pathogen growth, as observed in strawberries (Yamagishi et al., 2009).

3.3.5. Concluding remarks

The present study had a few limitations. For practical use and further development of the hot water sprayer in tomato nurseries, reducing propane gas consumption from the boiler used for HWS should be addressed. Also, the exhaust gas from the boiler could be used for carbon dioxide enrichment in a greenhouse for increasing yield, thereby increasing the cost efficiency of the HWS treatment.

Furthermore, gray mold was used for identifying the optimum conditions of hot water treatment in this study. However, gray mold disease often propagates under high humid conditions in a greenhouse. The greenhouse environment should, therefore, be managed carefully to not experience an increase in humidity after HWS.

The limitations notwithstanding, the present study highlights that HWS could be an effective technique to prevent powdery mildew by only hot water in a tomato nursery and ensure a reduction in the frequency of chemical application. Further studies are required to investigate the efficacy of this technique to prevent mildew in other crop plants.

CHAPTER FOUR

General Discussion

4.1. Consistency of basic and applied experiment

4.1.1. Heat shock treatment

Different conditions of HST were used between the basic experiment (chapter two) and applied experiment (chapter three). HWD at 45 °C for 2 minutes was performed for investigating the molecular mechanism of HSIR against *Pseudomonas syringae* pv. *tomato (Pst),* whereas HWD at 50 °C for 20 s was used against gray mold, and hot water spraying (HWS) at 50 °C for 20 s was carried out for the practical application of HSIR against powdery mildew. HWD at 45 °C for 2 minutes has been optimized in a previous experiment on HST for inducing resistance against pathogens in tomatoes (Sato et al., 2005). Also, previous studies of molecular investigation of HSR in tomatoes were generally performed using a

temperature range of 40 to 45 °C (Treuter et al., 1993; Mishra et al., 2002; Hahn et al., 2011). Thus, we performed HST at 45 °C for 2 minutes to maintain reproducibility of induced resistance against pathogens, as well as a similar molecular response to HSR that we used as a reference to investigate the molecular mechanism of HSIR. Meanwhile, for the practical application, 2 minutes duration of HWS is not suitable because of the long duration of the overall treatment, which increases the water heating cost. Processing time tends to be shorter when the temperature is higher. Furthermore, HWS at 50 °C for 20 s has been used in a previous study for the development of a prototype of hot water sprayer device (Yoshino et al., 2012), and it was proven to effectively protect cucumbers against gray mold. Therefore, 50 °C for 20 s was used for practical HWS against powdery mildew, referring to the HST condition in the previous treatment.

HWS using the improved sprayer device successfully induced disease resistance against powdery mildew infection in tomato seedlings, despite that the temperature attained by treated leaves were uneven and generally less than the target temperature. Similar results have been reported strawberries, HWD of the upper leaves at 50 ℃ for 20 s lowered the occurrence and growth rate of powdery mildew (Sato et al., 2017). They also mentioned that HSIR was triggered, albeit only in the limited part of the plant that attained the expected temperature. The systemic induction of disease resistance that is similar to systemic acquired resistance was suggested in the mode of action (Widiastuti et al., 2013b). However, data on the partial HST in chapter two showed that the effect of HSIR in tomatoes against *Pst* was not systemic; thus, the suggested the mode of action of HSIR was different than SAR. In a previous report, HWD at 40 °C for 2 minutes enhanced cucumber resistance against gray mold, as well as peroxidase gene expression. Also, Sato et al. (2005) reported HWD at 45 °C for 2 minutes induced resistance in tomatoes against the same pathogen. Based on these results, the reason the resistance against powdery mildew was induced although not all leaves reached the target temperature (50 °C for 20 s) may be the efficacy of HWD to induce plant defense response at lower temperature conditions.

4.1.2. Pathogen species

To gain insight into the molecular mechanism underlying HSIR in tomatoes, *Pseudomonas syringae* pv. *tomato* (*Pst*) was used as a model pathogen. *Pst* is the causal agent of bacterial speck of tomatoes. This pathogen has gained a high level of scientific interest because it is an easily cultured gram-negative bacterial pathogen, that is amenable to a wide range of molecular genetics and cell biology techniques, facilitating the experimental identification and manipulation of putative pathogenicity and virulence factors. Moreover, tomatoes are similarly amenable to transformation and genetic analysis, facilitating the isolation and characterization of plant genes involved in host responses. The necrotic and chlorotic symptoms produced by *Pst* are quite distinctive. Bacteria enter the intercellular spaces of leaves through natural openings, such as stomata, and multiply endophytically and asymptomatically prior to symptom development. The necrotic and chlorotic symptoms produced by *Pst* are quite distinctive and can be observed within three days after inoculation in tomato leaves. Therefore, it is suitable to obtain basic data of gene expression during plant-pathogen interactions in a relatively short time in comparison to *Bc* or powdery mildew.

The performance of an improved sprayer device for the practical application of HSIR was tested against a fungal pathogen because the primary target pathogen in the field is powdery mildew fungi, which was among the highly infectious diseases that have been reported to spread in Japan since 2000 (Matsuda et al., 2001). However, gray mold was used as an inoculum in the preliminary experiment for confirming the efficacy of HST for inducing resistance in different tomato varieties because of the difficulties in handling powdery mildew fungi. Because of its obligate parasite characteristic, obtaining powdery mildew culture for the experiment was difficult. Moreover, disease evaluation of powdery mildew infection can only be done qualitatively by observing the degree of disease severity.

4.2. Remaining problem and solutions

4.2.1. Molecular mechanism of HSIR

The present study suggested that Hsfs are involved in the regulation of HSIR. However, experiment by inhibitor treatment was not enough to provide solid evidence of protein-DNA binding interaction. Moreover, although KB showed a positive effect in suppressing the regulation of PR genes, as well as defense response against *Pst* after HST, it is still unknown which type of Hsfs is inhibited by KB. Clearly, further Hsfs-DNA binding assays are needed to understand the effectiveness of KB in preventing the Hsfs-HSEs association. In vitro analysis of the interaction between tomato Hsfs and the PR gene promoters would provide strong evidence that the PR gene HSEs represent a functional Hsfs-binding site.

Furthermore, Hsfs gene knock-down or HSE knock-out mutants are required to determine their role in the regulation of PR genes after HST. If the PR gene promoters with a mutated HSE lose inducibility and even become repressed under the heat-shock treatment. The specific Hsf that is responsible for the induction of the defense response could be confirmed. Further, once the responsible Hsf that plays a role in the regulation of HSIR has been isolated. An Hsf overexpressed mutant can be constructed to create a new line of resistant plants against a broad spectrum of pathogens.

4.2.2. Practical application of HSIR

The regulation of HSIR via Hsfs opens new possibilities for the screening of chemicals, which could induce the expression of Hsfs and activate plant defense responses, such as geldanamycin (GDA) or its derivative 17AAG, which enhance Hsfs gene expression by inhibition of Hsp90. An alternative to GDA or 17AAG may be available at lower cost for practical use. Similar compounds that prevent the attachment of Hsp90 to Hsfs or enhances the binding of Hsfs to the HSE could also induce plant resistance. Thus, any compound that acts as an Hsp90 inhibitor or Hsfs binding enhancer may be combined with HST to amplify

the effect. For example, the possibility of combining HST with plant activators, such as 1,2-benzisothiazole-3 (2H)-one 1,1-dioxide (BIT), could be examined, as BIT induces the accumulation of salicylic acid, which is known to enhance the binding of Hsfs to the HSEs. Applying BIT will increase the production of PR genes, which is then followed by enhanced disease susceptibility.

HST at 50 °C has generally been sufficient for inducing plant resistance against pathogens. In a previous study, HS treatment at 50 °C has been shown to induce defense responses in various crops. HWD of strawberry plants at 50 °C for 20 s (Widiastuti et al., 2013b; Sato et al., 2017); barley at 50 °C for 30–60 s (Schweizer et al., 1995); cucumbers at 40 °C for 120 s (Yoshino et al., 2011); melons at 50 °C for 20 s (Widiastuti et al., 2011); and barley at 50 °C for 30-60 s reduced subsequent infection by the pathogen. However, it was also noted that the induced resistance after HST usually could not achieve complete elimination of the disease. In addition to hot water treatment, cultural practices could be integrated to minimize conditions suitable for infection and spread of powdery mildew in greenhouse tomato nurseries. Development of powdery mildew is influenced by several environmental factors, including temperature, relative humidity (RH), and light level (Jacob et al., 2008; Guzman-Plazola et al., 2003). Unfortunately, greenhouses usually provide optimum levels for all of these conditions. Favorable environmental conditions that contribute to the development of tomato powdery mildew include moderate temperatures of between 15 to 25 °C, relatively low light levels, and intermediate RH levels of 50–70% (Jacob et al., 2008). Maintaining adequate plant spacing to reduce RH within the plant canopy; properly timed venting and heating to control RH level; as well as regular greenhouse cleaning of weeds, which might be potential hosts, could increase the likelihood of preventing powdery mildew infection.

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