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Protection of phenylpropanoid metabolism by prior heat treatment in *Lycopersicon esculentum* exposed to *Ralstonia solanacearum*

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Abstract – Heat shock inhibits pathogen-induced resistance mechanisms in incompatible plant hosts, leaving them vulnerable to pathogen attack. Prior exposure of organisms to non-lethal heat induces heat shock proteins and acquired thermotolerance to otherwise lethal high temperatures. The phenylpropanoid pathway is a target of heat-related inhibition but it is unknown whether thermotolerance protects this pathway or its key regulator, phenylalanine ammonia-lyase (EC 4.3.1.5, PAL). It was hypothesised that prior exposure to a heat shock pulse to induce the accumulation of heat shock proteins, specifically the 70-kDa heat shock protein (Hsp70 – inducible/Hsc70 – constitutive), would protect phenylpropanoid metabolism from heat-induced inhibition. The tomato, *Lycopersicon esculentum* L. cv. UC82B, transformed with PAL2-GUS, and *Ralstonia solanacearum*, biovar II, were used as incompatible host-pathogen model. A prior heat shock pulse induced significant accumulation of Hsp70/Hsc70 and enhanced cell viability. This protected the pathogen-activated phenylpropanoid pathway (PAL2-GUS activity, PAL enzyme activity, lignin deposition) from heat-induced inhibition and promoted cell survival after a subsequent prolonged heat shock. This study suggests phenylpropanoid metabolism as a target of Hsp70/Hsc70-related protection of the resistance response activated in tomato against avirulent strains of *Ralstonia solanacearum* from heat-induced inhibition during a concomitant heat shock.

Hsp70 / lignin / *Lycopersicon esculentum* / phenylalanine ammonia-lyase / phenylpropanoid metabolism / *Ralstonia solanacearum* / thermotolerance

cfu, colony forming units / GUS, β-glucuronidase / HS, heat shock / Hsc70, constitutive 70-kDa heat shock protein / HSP, heat shock protein(s) / Hsp70, 70-kDa heat shock protein (inducible) / MUG, 4-methyl-umbelliferyl β-D-glucuronide / PAL, phenylalanine ammonia-lyase / pcd, programmed cell death / RNP, ribonucleic protein / *Rs*, *Ralstonia solanacearum* / *Rs* II, *Ralstonia solanacearum* biovar II / SAC, specific absorption coefficient / sHsp, small heat shock protein(s)

1. INTRODUCTION

Heat shock activates a highly conserved, protective and adaptive response in all organisms. It is characterised by the induction or increased expression of heat shock (HS)/stress proteins (HSP) with a concomitant decrease in expression of normal household proteins [32]. HSP are classified into families according to their apparent molecular mass (Hsp100, Hsp90, Hsp70, Hsp60, sHsp – small HSP abundantly induced in plants, and ubiquitin – 8 kDa), localisation and function. HSP expression is regulated at the transcriptional level by the heat shock transcription factor (HSF) through binding to the heat shock element (HSE), a consensus sequence in the promoter region of all *hsp* genes. In unstressed cells, monomeric HSF is chaperoned by Hsp70, Hsp90 and other co-chaperones that prevent its activation. Upon stress, the demand for chaperones increases due to the elevated appearance of denatured proteins, and HSF is released to trimerise and initiate *hsp* gene transcription [22].

When exposed to non-lethal heat shock, a variety of organisms and cells acquire transient resistance to otherwise lethal hyperthermia. This phenomenon is termed thermotolerance. While the mechanism of

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thermotolerance is not well understood, several studies support a role for HSP in the development of thermotolerance [12, 18]. Proposed functions of HSP in thermotolerance, some of which were summarised by Nover [27], include: (a) protection by Hsp70 in the nuclear compartment of pre-mRNP processing, ribosome biosynthesis and removal of unwanted proteins or RNP aggregates; (b) preservation of untranslated mRNA in complex with sHsp and cytoskeletal elements – referred to as HS granules; (c) protection of the cytoskeleton by sHsp, Hsp70 and Hsp90; (d) membrane protection (plasma and intracellular); (e) protection of mitochondria [8] and chloroplasts by Hsp70, Hsp60 and sHsp [11]; and (f) removal of aberrant proteins by ubiquitin. One of the best characterised HSP families often linked with thermotolerance-related protection is the Hsp70 family [26], containing both constitutive (Hsc70) and inducible (Hsp70) members localised in the cytoplasm, nucleus (after stress), cell membrane, mitochondria and endoplasmic reticulum. Hsp70 plays a central role in the maintenance of cellular homeostasis through its molecular chaperoning of protein folding, transport, maturation and degradation; functions are performed largely in cooperation with other chaperones and in an ATP-dependent manner [16]. Hsp70 and ATP are partners in cell homeostasis [20] – Hsp70 is often induced as an adaptive and protective mechanism during ATP depletion to maintain cellular homeostasis. Mitochondria are selective targets for the protective effects of Hsp70 [29] and the major source of ATP, the check-point between life or death, whether apoptosis (moderate ATP depletion) or necrosis (severe ATP depletion) [17]. Consequently, overexpression of Hsp70 protects cells from death by both apoptosis and necrosis and plays a pivotal role in maintaining cell viability.

The HS response has priority over other stress responses and prevents or reduces activation of these responses and associated protection when given in conjunction with HS. One such example is the pathogen-induced resistance response induced in plant cultivars that possess the genetic capacity to respond, in a gene-for-gene fashion, by activating several defence strategies. These strategies include an oxidative burst, salicylic acid accumulation, activation of the phenylpropanoid pathway and the hypersensitive response – localised programmed cell death (pcd) at the primary site of infection to restrict dissemination. Several components in this response are suppressed by HS, including phenylpropanoid metabolism [37] and the accumulation of salicylic acid [9], PR-proteins and phytoalexins. Phenylalanine ammonia-lyase (PAL), a key enzyme in the phenylpropanoid pathway, is not induced by HS, while wounding induces Hsp70, ubiquitin and PAL [30].

To our knowledge, the effect of thermotolerance on heat-induced inhibition of pathogen-activated resistance, in particular the phenylpropanoid pathway, has not been investigated. We hypothesised that thermotolerance, marked by prior accumulation of Hsp70/Hsc70, protects the resistance response from heat-induced inhibition during a concurrent exposure to heat and avirulent pathogens. This hypothesis was investigated in *Lycopersicon esculentum* by studying the effect of thermotolerance, induced by a prior HS pulse, on phenylpropanoid metabolism activated by exposure to an avirulent strain of *Ralstonia solanacearum* given simultaneously with a prolonged HS. This study indicated that thermotolerance associated with Hsp70/Hsc70 accumulation protects phenylpropanoid metabolism against heat-induced inhibition, in particular PAL2-GUS activity, PAL enzyme activity, to a certain extent lignin production and cell viability, during a second prolonged HS. In contrast, a prolonged HS without a prior HS pulse suppressed phenylpropanoid metabolism and promoted cell death.

2. RESULTS

2.1. Hsp70/Hsc70 accumulation and cell viability in response to different temperatures

Since not only heat-induced but also constitutively expressed isoforms of stress proteins are essential for the overall effect of thermotolerance [27], both constitutive and inducible isoforms of the Hsp70 family were investigated in this study. Various temperatures, duration of exposure and recovery were evaluated to obtain an appropriate heat pretreatment protocol that allows the significant accumulation of Hsp70/Hsc70. Furthermore, a HS protocol representing a severe HS that induces a HS response without significantly modulating cell viability had to be established. A schematic diagram of the final experimental protocol, as described in Methods (section 4.5) shows the symbols used in subsequent figures to depict the different treatments performed on PAL2-GUS transformed *Lycopersicon esculentum* (*figure 1*).

Hsp70/Hsc70 accumulation and cell viability obtained for the chosen temperature regimes are indicated in *figure 2*. Treatment with a heat pulse (40 °C, 15 min) 24 h beforehand resulted in a significant accumulation ($P < 0.05$) of Hsp70/Hsc70 at 0 h compared to cells that received no pretreatment (*figure 2A*,

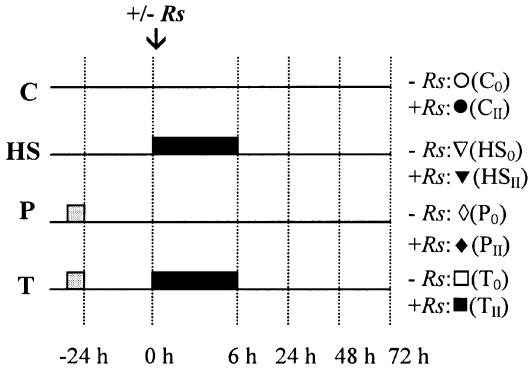

Figure 1. A schematic diagram illustrating the experimental protocol for treatment of tomato cell suspension cultures derived from PAL2-GUS transformed *Lycopersicon esculentum* L. Cells were incubated under different temperature regimes. C, Controlled condition (25 °C); HS, heat shocked from 0 to 6 h at 40 °C (black rectangle); P, treated with a heat pulse of 15 min at 40 °C, 24 h beforehand (–24 h) (grey square); T, made thermotolerant by a heat pulse of 15 min at 40 °C 24 h before being heat shocked from 0 to 6 h at 40 °C (grey square plus black rectangle). Each of these temperature regimes were done with (+, subscript II) or without (–, subscript 0) inoculation with avirulent, biovar II of *Ralstonia solanacearum* at time 0 h (arrow), coinciding with the beginning of the HS where applicable. Cells were harvested at 0, 6, 24, 48 and 72 h for analysis of various parameters. Symbols and abbreviations in brackets indicated to the right correspond to those used in line graphs (following figures) and the text, respectively.

B). Heat shock (40 °C, from 0 to 6 h) without any pretreatment led to a significant accumulation of Hsp70/Hsc70 24 h later ($P < 0.05$) reaching a peak at 48 h, while it decreased but remained significantly higher than levels in control cells at 72 h or HS_0 cells at 0 h. Hsp70/Hsc70 levels in thermotolerant cells did not fluctuate significantly from 0 to 72 h and were almost identical to HS_0 cells 24 h after heat shock. Hsp70/Hsc70 levels in T_0 cells returned to almost normal levels 48 and 72 h after HS, and were not significantly different from C_0 at these time points, while Hsp70/Hsc70 levels in HS_0 remained significantly elevated above control levels.

The effect of the different temperature regimes on tomato cell viability assessed by the Alamar Blue cell survival assay is shown in *figure 2C*. Viability of cells that received HS with or without a pulse heat treatment 24 h beforehand, or cells that only received the pulse heat treatment without subsequent HS, did not differ significantly from each other or from control cells at any time except at 6 h. Cells that received a prior heat pulse had a significantly greater ability to reduce Alamar Blue ($P < 0.001$) immediately following HS than those that received any of the other treatments. As a result, T_0 cells were referred to as thermotolerant.

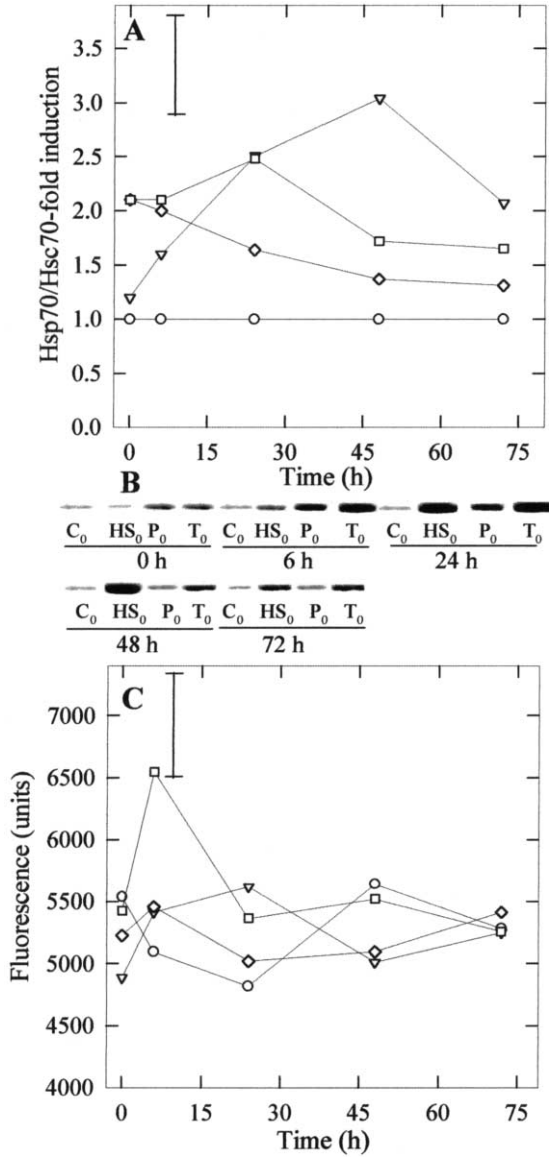

Figure 2. Time course of Hsp70/Hsc70 accumulation (**A** and **B**) and cell viability (**C**) of PAL2-GUS transformed tomato cells exposed to different temperature regimes without *R. solanacearum*, biovar II infection. Symbols and abbreviations for the different treatments correspond to those allocated in *figure 1*: open symbols, without infection; circles, control condition; triangles, heat shocked (40 °C, 0–6 h); diamonds, heat pulse (40 °C, 15 min, 24 h beforehand); rectangles, heat pulse plus heat shock. Accumulation of Hsp70/Hsc70 (verified as 70 kDa using molecular mass markers) was analysed by western blot analysis (**B**), quantified by densitometry (**A**) and expressed as *x*-fold induction relative to control values. Cell viability (**C**) was assessed by the Alamar Blue assay based largely upon mitochondrial electron transport activity. Fluorescence of reduced Alamar Blue was quantified by spectrofluorometry and the data expressed in arbitrary fluorescence units. Error bars (top left corner of figure boxes) indicate LSD ($P < 0.05$) ($n = 4$) calculated from the pooled variance including all treatments indicated in *figure 1*.

2.2. Thermotolerance protects against heat-related inhibition of PAL2-GUS activity

The first component evaluated in this study was the expression of β-glucuronidase activity under the regulation of the bean PAL2 promoter, hereafter referred to as PAL2-GUS activity. *Figure 3A* illustrates PAL2-GUS activity for the various treatments without subsequent inoculation with avirulent biovar II of *Ralstonia solanacearum* – C_0, HS_0, P_0 and T_0. No significant differences for PAL2-GUS activity were obtained at any of the different time points for these treatments. The effect of thermotolerance in tomato cells on concurrent heat shock and infection (T_{II} versus HS_{II}) on PAL2-GUS activity is shown in comparison to cells kept under normal temperature with or without infection (*figure 3B*). Cells kept under control conditions showed no significant change in PAL2-GUS activity in the absence of *Rs* II (C_0) throughout the 72-h incubation period. The specific concentration of *Rs* II used as inoculum in this study significantly induced PAL2-GUS activity ($P < 0.001$) in PAL2-GUS transformed tomato cells kept under normal temperature 48 h after inoculation (C_{II}) compared to control cells (C_0). In contrast, HS prevented this induction and in fact caused a significant suppression ($P < 0.001$) of normal PAL2-GUS activity (116 % reduction), almost significantly lower than the control activity (C_0). However, thermotolerance (T_{II}) alleviated the heat-induced reduction of PAL2-GUS activity at 48 h from a 116 % decrease (HS_{II}) to a 33 % decrease (T_{II}) of the activity induced by *Rs* II under normal temperature (C_{II}). In addition, thermotolerance supported a significant induction of PAL2-GUS activity in infected cells T_{II} by 24 h compared to C_0, in contrast to PAL2-GUS activity in C_{II} that was not significantly different from C_0 at this time. Comparable to the effect of thermotolerance, cells treated with a heat pulse and infected (P_{II}) showed significant induction of PAL2-GUS activity at 48 h compared to C_0 and HS_{II}.

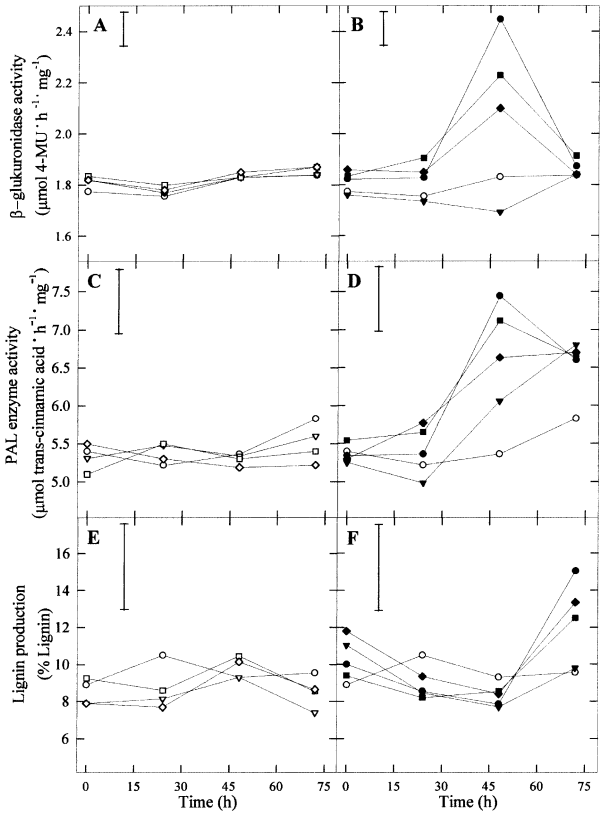

Figure 3. Time course of GUS activity under the regulation of PAL2 promoter (**A, B**), phenylalanine ammonia-lyase (EC 4.3.1.5, PAL) PAL enzyme activity (**C, D**) and lignin production (**E, F**) in PAL2-GUS transformed tomato cells exposed to different temperature regimes with (**B, D** and **F**) or without (**A, C** and **E**) inoculation with *R. solanacearum* biovar II at 0 h. Values for C_0 cells are indicated in **B, D** and **F** as reference. Symbols and abbreviations for the different treatments correspond to those allocated in *figure 1*: open symbols, without infection; filled symbols, with infection; circles, control condition; triangles, heat shocked (40 °C, 0–6 h); diamonds, heat pulse (40 °C, 15 min, 24 h beforehand); squares, heat pulse plus heat shock. Cells were harvested at 0, 6 (results not shown), 24, 48 and 72 h for analysis of different parameters. Error bars (top left corner of figure boxes) indicate LSD ($P < 0.05$) ($n = 4$) calculated from the pooled variance of all treatments indicated in *figure 1*.

2.3. Thermotolerance protects from heat-induced suppression of PAL enzyme activity

PAL enzyme activity was the second feature of the phenylpropanoid pathway studied to investigate possible protection from heat-induced inhibition by thermotolerance. The effect of the different treatments on PAL enzyme activity is shown in *figure 3C* and *D*. *Figure 3C* illustrates the various treatments without subsequent inoculation with avirulent biovar II of *Ralstonia solanacearum* – C_0, HS_0, P_0 and T_0. Similar to the results obtained for PAL2-GUS activity, no significant changes for PAL enzyme activity were observed at any of the different time points for these treatments. PAL enzyme activity was significantly induced at 48 h in cells infected with *Rs* II (C_{II}) compared to control cells that received no bacterial treatment (C_0) ($P < 0.05$) (*figure 3D*). This induction of PAL enzyme activity was significantly reduced by the 6-h HS (HS_{II}) ($P < 0.05$) after 48 h to 34 % of the induced activity in C_{II}. However, thermotolerance (T_{II}) protected against significant reduction of PAL enzyme activity ($P < 0.05$), allowing heat-induced suppression to only 84 % of the activity in C_{II}. Cells that received

a prior heat pulse showed increased PAL enzyme activity 48 h after infection (P_{II}) compared to C_0, not shown by HS_{II}.

2.4. Thermotolerance protects from heat-induced suppression of lignin production

Lignin, an essential product of phenylpropanoid metabolism and particularly important in resistance to pathogen attack, was the final attribute of the phenylpropanoid pathway used to investigate the effects of thermotolerance on heat-induced inhibition of this resistance mechanism. The effect of the different treatments on the percentage production of lignin is shown in *figure 3E* and *F. Figure 3E* illustrates the various treatments without subsequent inoculation with avirulent biovar II of *Ralstonia solanacearum* – C_0, HS_0, P_0 and T_0. No significant changes in lignin production were observed at any of the different time points for these treatments (*figure 3E*). *Rs* II induced a significant increase in the production of lignin in tomato cells 72 h after inoculation (C_{II}, $P < 0.05$) as compared to control cells (C_0) (*figure 3F*). HS given in conjunction with *Rs* II (HS_{II}) significantly suppressed lignin production ($P < 0.05$) to almost normal levels (C_0) (*figure 3F*). On the other hand, treatment with a prior heat pulse (T_{II}) increased *Rs* II-induced lignin production at elevated temperature to almost 50 % of the lignin production induced at normal temperature (C_{II}). Cells treated with a heat pulse showed increased lignification in infected cells (P_{II}) at 48 h compared to C_0 ($P < 0.05$) and HS_{II} ($P < 0.3$).

2.5. Thermotolerance enhanced cell viability after concomitant heat stress and pathogen infection

Cell viability was investigated to evaluate the significance of thermotolerance-related protection of phenylpropanoid metabolism. The effect of the different treatments on Hsp70/Hsc70 accumulation and cell viability is shown in *figure 4A* and *B*, respectively. Infected cells that received a prior heat treatment (P_{II} and T_{II}) showed significant accumulation ($P < 0.05$) of Hsp70/Hsc70 at 0 h compared to infected cells that received the 6-h HS (HS_{II}) thereafter and cells that received no infection or heat pretreatment (C_0) (*figure 4A*). Heat shock without any pretreatment led to a significant accumulation of Hsp70/Hsc70 24 h later ($P < 0.05$), decreasing at 48–72 h. At 72 h, all inoculated samples (C_{II}, HS_{II}, P_{II}, T_{II}) had lower, yet not significant at $P < 0.05$, accumulated levels of Hsp70/Hsc70 compared to C_0.

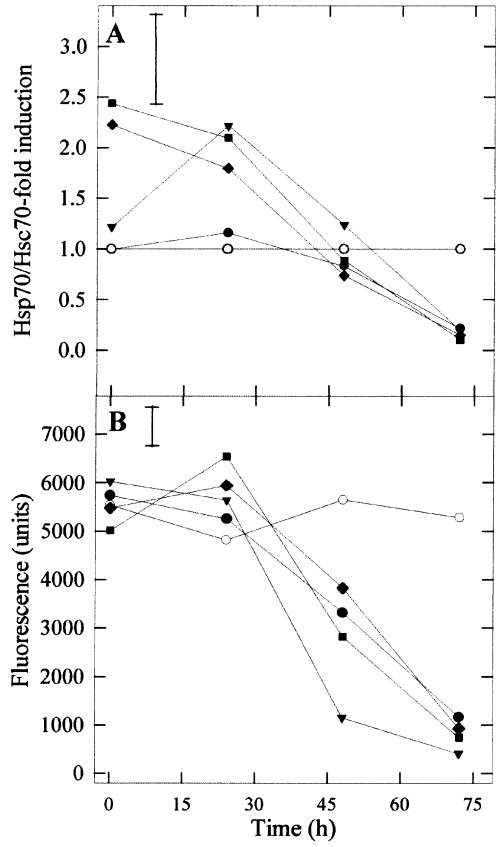

Figure 4. Time course of Hsp70/Hsc70 accumulation (**A**) and viability (**B**) of PAL2-GUS transformed tomato cells exposed to different temperature regimes with or without inoculation with *R. solanacearum*, biovar II at 0 h. Symbols and abbreviations for the different treatments correspond to those allocated in *figure 1*: open symbols, without infection; filled symbols, with infection; circles, control condition; triangles, heat shocked (40 °C, 0–6 h); diamonds, heat pulse (40 °C, 15 min, 24 h beforehand); squares, heat pulse plus heat shock. Cells were harvested at 0, 6 (results not shown), 24, 48 and 72 h for analysis. Accumulation of Hsp70/Hsc70 was analysed by western blot analysis (**A**) and expressed as *x*-fold induction relative to control values. Cell viability (**B**) was analysed by Alamar Blue and the fluorescence of reduced Alamar Blue quantified by spectrofluorometry and expressed in arbitrary fluorescence units. Error bars (top left corner of figure boxes) indicate LSD ($P < 0.05$) ($n = 4$) calculated from the pooled variance including all treatments indicated in *figure 1*.

Thermotolerance significantly enhanced cell viability of infected cells (T_{II}) compared to HS_{II} or C_{II} and even C_0 after 24 h. Cells treated with *Rs* II (C_{II}) showed a significant decrease ($P < 0.05$) in cell viability at 48 h (to ±58 % of C_0) that decreased further at 72 h to ±22 % of C_0 (*figure 4B*). The combined treatment of HS and *Rs* II infection (HS_{II}) decreased cell viability even more prominently ($P < 0.001$) than in cells that received only *Rs* II (C_{II}) at 48 h (to ±20 % of C_0) and at 72 h (±8 % of C_0). However, cell viability

of infected thermotolerant cells (T_{II}) and infected cells that received a heat pretreatment (P_{II}) was not significantly different from infected normal cells (C_{II}) at 48 h. Thus, thermotolerance (T_{II}) ensured a 2.4-fold increase in viability of infected cells after 48 h compared to HS_{II}. At 72 h, infected cells under all the conditions showed diminutive cell viability with no significant difference between C_{II}, HS_{II} or T_{II}.

3. DISCUSSION

It is well documented that HS overrides the pathogen-induced resistance response in plants and most likely contributes to disease susceptibility and severity commonly observed at elevated temperatures [33]. Conversely, organisms survive an otherwise lethal heat stress when given a mild heat treatment prior to the lethal heat stress, a phenomenon known as acquired thermotolerance [7]. In this paper, we identified phenylpropanoid metabolism as a possible target of thermotolerance-related protection of disease resistance from heat-induced inhibition in tomato cell suspensions. Prior elevation of HSP, Hsp70/Hsc70 in particular, in tomato cell suspensions prevented its subsequent induction by HS given in combination with *Rs* II infection, allowing the activation of PAL2-GUS activity (24–48 h), PAL enzyme activity (48 h) and lignin production (72 h), while promoting cell viability (24–48 h).

A heat pulse (40 °C, 15 min) induced a significant accumulation of Hsp70/Hsc70 24 h later that buffered further induction of Hsp70/Hsc70 by a subsequent prolonged HS (40 °C, 6 h), while it facilitated the prompt recovery of the response (*figure 2A, B*). This confirms the negative autoregulatory role proposed for Hsp70 [3, 22, 28]. During stress, an increased demand for chaperoning to prevent protein aggregation and reverse protein unfolding [16] normally necessitates the dissociation of Hsp70 and other chaperones from the inert cytoplasmic monomeric HSF complex leading to HSF activation and *hsp* gene transcription [22]. Significant prior accumulation of Hsp70/Hsc70 in thermotolerant cells most likely ensures sufficient chaperoning capability during a subsequent HS and nullifies further *hsp* gene expression.

The prolonged HS protocol used in this study (40 °C, 6 h) did not influence cell viability, whereas a prior HS pulse (40 °C, 15 min) associated with the induction of Hsp70/Hsc70 conferred an immediate significant increase in viability following a prolonged HS compared to control cells or cells that received either a HS pulse or prolonged HS (*figure 2C*). This suggests that thermotolerant tomato cells can exceed normal metabolic activity after a subsequent HS, which may relate to their significant prior accumulation of Hsp70/Hsc70. Among the HSP, Hsp70 has been linked most frequently to protection and thermotolerance [36]. Lee and Shöffl [18] proposed a dual role for Hsp70 in plants: autoregulation of HSF activity and the heat shock response, and a protective role in thermotolerance. Mitochondrial membrane potential, essential for sustaining oxidative phosphorylation, has been identified as a specific target for Hsp70-related mitochondrial protection [2, 29]. Although the current study only addressed the accumulation of Hsp70/Hsc70, several other HSP induced by HS such as the sHSP, most prominently induced in plants, may also contribute to thermotolerance. Lee and Vierling [16] showed cooperation between the sHSP and Hsp70 in the renaturation of firefly luciferase in transformed *Arabidopsis thaliana*, the former by preventing protein aggregation in an ATP-independent manner and the latter by ATP-dependent folding. Downs and Heckathorn [8] reported that pre-induction of mitochondrial sHSP protects complex I and consequently electron transport from complex I to complex IV, as measured in submitochondrial vesicles during heat stress (48 °C, 7–15 min). Chou et al. [6] showed that normal ATP generation in isolated soybean mitochondria at elevated temperature is maintained only if the mitochondrial fractions were enriched in sHsp by HS preconditioning of the seedlings.

Results reported in the current study support Hsp70-related mitochondrial protection since the Alamar Blue viability assay, based to a large extent on mitochrondrial activity [1, 5], showed enhanced activity in thermotolerant cells after a prolonged HS. It is proposed that Hsp70/Hsc70, most likely in cooperation with other HSP, account for the potentiation of cell viability based upon enhanced mitochondrial activity as reflected by Alamar Blue reduction by thermotolerant cells following HS. However, direct measurements of mitochondrial activity are required to substantiate this suggestion.

Sustained viability of tomato cells after 6 h at 40 °C (*figure 2C*) confirmed that this HS protocol met the requirements for the current study, i.e. to be able to evaluate metabolism, in particular phenylpropanoid metabolism, without having HS-related detrimental effects on cell viability. Downs and Heckathorn [8] showed that severe HS conditions (48 °C, 7–15 min) could break down NADH:ubiquinone oxidoreductase (complex I) to a lower molecular mass resulting in

lower activity rates and a loss of oxidative phosphorylation during heat stress of submitochondrial vesicles from apple.

Rs II induced PAL2-GUS activity, PAL enzyme activity and lignin production in control cells, whereas HS (6 h at 40 °C) inhibited this induction (*figure 3B, D, F*). These results are in agreement with the well-known phenomenon that the heat stress response has priority over other stress responses [25] including the pathogen-induced resistance response [9]. Concerning the phenylpropanoid pathway, PAL enzyme is not induced under HS conditions [30], while induction of PAL enzyme by an elicitor from *Phytophtora megasperma* were found to be terminated upon HS [37]. Zacheo et al. [38] reported that heat treatment (34 °C, 6 d) reduced lignin levels of root-knot nematode (*Meloidogyne incognita*) infected and uninfected tomato roots that leads to heat-induced susceptibility. They proposed that heat-induced susceptibility is associated with a reduced hypersensitive reaction that correlated with decreased peroxidase activity and lignin levels.

Induction of lignin in C_{II} compared to C_0 was significant only at $P < 0.3$ after 72 h. Bacterial overgrowth occurred in cell suspensions at 72 h reducing viability significantly and most likely lignin production (*figure 4B*). A more appropriate protocol for studying late events in host-pathogen interaction should allow only a brief bacterial exposure and subsequent removal to avoid bacterial overgrowth of host cells.

Thermotolerance protects cells against heat-induced inhibition of PAL2-GUS activity (24–48 h), PAL enzyme activity (48 h) and lignin production (72 h) activated by exposure to avirulent *Ralstonia solanacearum* during a resistance response (*figure 3B, D, F*). The consecutive nature and time scale of events in the phenylpropanoid pathway observed conforms to the sequence and timing of events described for the resistance response [31]. However, protection of PAL2-GUS activity occurred at 24–48 h, PAL enzyme activity at 48 h and lignin production at 72 h, a time interval (24–72 h) during which Hsp70/Hsc70 levels were not significantly higher in T_0/T_{II} cells than in HS_0 cells. This may argue against a direct role for Hsp70/Hsc70 in the protection of the phenylpropanoid metabolism. However, the prompt down-regulation of the HS-response and a return to normal household functions in thermotolerant cells (from 48 h onwards, *figure 2A*), may maintain the status quo, allowing induction of phenylpropanoid metabolism, if and when required. A direct role for HSP in the protection of the phenylpropanoid metabolism cannot be ruled out, since induction of other HSP may overlap with the protection period and protection of early events in the phenylpropanoid pathway by elevated levels of Hsp70/Hsc70 in particular could have a delayed effect. On the other hand, elevated levels of HSP also mark irreversible damage to proteins and could therefore reflect cellular injury rather than a protective capacity that might be the case for Hsp70/Hsc70 levels in HS_0 at 48 h. Nonetheless, without prior accumulation of Hsp70/Hsc70, no protection from heat-induced inhibition of the phenylpropanoid pathway was conferred.

Thermotolerance enhanced cell viability of tomato cells 24 to 48 h after combined exposure to HS and *R. solanacearum* infection compared to cells that received HS and infection but no prior HS pulse (*figure 4B*). The improved cell viability may pertain to defence-related effects/products of the phenylpropanoid metabolism (except perhaps for lignin – induced only after 72 h), since it coincided with protection of PAL2-GUS and PAL enzyme activity at 48 h

Hsp70 has been proposed as a chaperone of life and death due to its protection of mitochondrial membrane polarity [35] and ATP production – key determinants in the choice between cell survival and cell death or death by apoptosis or necrosis [17]. Protection from cell death in thermotolerant cells (24–48 h) may reflect Hsp70/Hsc70-related mitochondrial protection and maintenance of ATP levels. Hsp70/Hsc70-related maintenance of ATP levels, supporting cell survival or at least death by apoptosis rather than necrosis is of particular importance in light of the role of the HR (a form of pcd), in disease resistance.

In conclusion, this study suggests that thermotolerance protects from heat-induced inhibition of phenylpropanoid metabolism during a resistance response. This protection coincided with prior accumulation of Hsp70/Hsc70, induced by a HS pulse, and supported a more efficient response towards a combined heat stress and pathogen infection. The primed response involved enhanced viability based upon mitochondrial electron transport activity, prompt recovery of the HS response and decreased cell death compared to normal cells infected during HS. Manipulation of Hsp70 levels, for example, using inhibitors of gene expression, protein-specific antibodies or gene technology (transformation and anti-sense RNA) could be used to pinpoint the contribution of Hsp70/Hsc70 in the protection of the phenylpropanoid pathway in future investigations. Thermotolerance-related protection of the phenylpropanoid metabolism and the possible role of Hsp70/Hsc70 could find application in the development of novel approaches in plant protection.

4. METHODS

All reagents used were purchased from Sigma Chemical Company (St Louis, MO, USA) unless otherwise specified.

4.1. Tomato transformation and cell suspension cultures

Lycopersicon esculentum L., cultivar UC82B (Hygrotech, Pretoria, South Africa) was transformed with the binary vector pBin19 containing a *gus* gene driven by a *Phaseolus vulgaris* L. phenylalanine ammonia-lyase (EC 4.3.1.5, PAL) promoter (accession number M11939) kindly provided by Christopher J. Lamb (Plant Biology Laboratory, Salk Institute for Biological Studies, USA) [19]. Binary vector constructs were transferred into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating and *A. tumefaciens*-mediated transformation was performed according to Frary and Earle [10]. Cell suspension cultures were developed from transgenic plant material and cultured in Murashige and Skoog medium (MS) [23], (Highveld Biologicals, Kelvin, South Africa) containing vitamins and supplemented with casein (2 $g \cdot L^{-1}$), kinetin (0.25 $mg \cdot L^{-1}$) and 2,4-dichlorophenoxy acetic acid (2 $mg \cdot L^{-1}$) (pH 5.8). All cell suspensions used were in the exponential growth phase and of equal density (OD_{600} = 1.2).

4.2. Viability assay

Viability of the cell suspensions at different intervals following the various treatments was assessed using the Alamar Blue cell survival assay [5]. The Alamar Blue assay incorporates a redox indicator that changes colour or fluoresces in response to metabolic activity based largely upon mitochondrial electron transport activity. Reduced Alamar Blue was quantified through fluorescence and the data expressed in arbitrary fluorescence units.

4.3. Western blot analysis

Cell samples (0.1 g) were prepared in 0.25 mL 0.1 M Tris buffer (pH 6.8) containing, 20 % (w/v) glycerol, 4 % (w/v) SDS, 2 % (v/v) β-mercaptoethanol and 0.001 % (w/v) bromophenol blue. Protein concentrations were determined using the solid-phase method according to Sheffield et al. [34]. Samples corresponding to equal protein concentrations were resolved by SDS-PAGE according to Laemmli [15]. Western blot analysis was done using a mouse monoclonal antibody against Hsp70/Hsc70 (Stress-Gen, Victoria, Canada) as primary antibody and goat anti-mouse IgG conjugated to horse-radish peroxidase as secondary antibody (Pierce, Rockford, IL, USA). The secondary antibody was detected by chemiluminescence (Pierce). The detected bands were verified as 70 kDa by using low molecular weight markers (Boehringer Mannheim, SA). Densitometric analysis was done using Uvpgrp32 software (UVP, San Gabriel, CA, USA) and GeneTools (SynGene, Cambridge, UK) [21].

4.4. Pathogen

Biovar II of *Ralstonia solanacearum* (*Rs* II), an avirulent strain of the causative agent of bacterial wilt in tomato, was obtained from the Vegetable and Ornamental Plant Institute, Agricultural Research Council, Roodeplaat, South Africa. Avirulence of *Rs* II was verified in comparison to virulent biovar III cultures by plating on TZC (tetrazolium chloride) Kelmans medium [14] containing peptone (10 $g \cdot L^{-1}$), casamino acid (1 $g \cdot L^{-1}$), glycerol (5 $mL \cdot L^{-1}$), agar (8 $g \cdot L^{-1}$) and incubating for 48 h at 30 °C. Colonies were suspended in sterile distilled water and the concentration of bacteria estimated spectrophotometrically (OD_{620} = 0.3 is approximately $1 \cdot 10^9$ colony forming units $(cfu) \cdot mL^{-1}$).

4.5. Treatment of cell suspensions

Tomato cell suspension cultures derived from PAL2-GUS transformed *Lycopersicon esculentum* L. were seeded in 50-mL Erlenmeyer flasks (40 $mL \cdot flask^{-1}$) while in exponential growth phase and exposed to various treatments outlined in *figure 1*. Cells were incubated under different temperature regimes: control condition (C, 25 °C), heat shocked from 0 to 6 h at 40 °C (HS), treated with a heat pulse of 15 min at 40 °C, 24 h beforehand (–24 h) (P), or made thermotolerant by a heat pulse of 15 min at 40 °C, 24 h before being heat shocked from 0 to 6 h at 40 °C (T). Cells exposed to each of these temperature regimes were infected and co-cultured with *Rs* II ($5 \cdot 10^6$ $cfu \cdot mL^{-1}$ cell suspension; C_{II}, HS_{II}, P_{II}, T_{II}) or with an equivalent volume of sterile distilled H_2O (C_0, HS_0, P_0, T_0) at time 0 h, coinciding with the beginning of the HS where applicable. The cells were incubated on a rotary shaker in the dark at 22 °C for 72 h. Aliquots of cells (5 mL) were harvested aseptically at 0, 6, 24, 48 and 72 h for the analysis of PAL2-GUS activity, PAL enzyme activity, lignin production, Hsp70/Hsc70 accumulation and cell survival.

4.6. Fluorometric MUG assay

The fluorometric assay of Jefferson et al. [13] was used for the analysis of β-glucuronidase (GUS) gene expression in transgenic cells using 4-methyl-umbelliferyl glucuronide (MUG) as the substrate. Protein content of the extracts was determined using the Bio-Rad microassay [4] and bovine serum albumin as standard. GUS activity was expressed as μmol 4-MU·h^{-1}·mg^{-1} protein.

4.7. PAL enzyme assay

PAL enzyme (EC 4.3.1.5) activity was analysed in cell-free extracts according to Nagarantha et al. [24] based upon the spectrophotometric analysis of the production of *trans*-cinnamic acid. Protein content of the extracts was determined using the Bio-Rad microassay [4] and bovine serum albumin as standard. Enzyme activity was expressed as μmol *trans*-cinnamic acid·h^{-1}·mg^{-1} protein.

4.8. Lignin determination

The lignin content of cells was analysed according to Sasaki et al. [31] and percentage lignin was calculated using the following equation:

$$\%\ \text{lignin content} = \frac{OD_{280} \times 100}{SAC \times \text{sample concentration}\ (g \cdot L^{-1})}$$

where specific absorption coefficient (SAC) is 20 g^{-1}·cm^{-1} for lignin.

4.9. Statistical analysis

Analysis of variance was done using CoStat Software (CoHort Software, Berkley, CA, 1990). Differences in mean values were considered significant if the least significant difference (LSD), calculated from the pooled variance, was exceeded.

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