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Virulence-dependent induction of Hsp70/Hsc70 in tomato by *Ralstonia solanacearum*

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Abstract – The selective induction of heat shock protein(s) (HSP) in human cells during bacterial, parasitic or viral infection and the accompanying cytoprotection initiated an interest in this conserved group of proteins – either as markers of disease outcome and severity or in novel therapeutic approaches. Knowledge concerning the induction and role of HSP in plant-pathogen interactions is however limited. The objective of this study was to investigate the expression of constitutive and inducible members of the 70-kDa HSP family (Hsp70/Hsc70) during compatible and incompatible interactions in tomato *Lycopersicon esculentum* L. cv. UC82B. Cell suspensions were co-cultured with virulent (biovar III) or avirulent (biovar II) strains of *Ralstonia solanacearum*, the causative agent of bacterial wilt, and samples harvested at specific time points for the analysis of pathogenesis-related protein-1 (PR-1) accumulation, phenylalanine ammonia-lyase (EC 4.3.1.5, PAL) activity, Hsp70/Hsc70 accumulation and cell survival. Besides inducing PR-1 (6–18 h, $P < 0.05$), biovar II caused a virulence-dependent induction of Hsp70/Hsc70 (24–48 h, $P < 0.05$) coinciding with the induction of PAL activity (24–48 h, $P < 0.05$) and maintenance of cell viability (48 h). It is proposed that, at least in tomato, Hsp70/Hsc70 is induced by avirulent strains of *R. solanacearum* as part of the defence response to chaperone newly synthesized defence proteins and to maintain cellular homeostasis essential for the execution of a defence response. © 2001 Éditions scientifiques et médicales Elsevier SAS

bacterial wilt / heat shock proteins / Hsp70 / *Lycopersicon esculentum* L. cv. UC82B / pathogenesis-related proteins / PR-1 / *Ralstonia solanacearum*

HR, hypersensitive response / HS, heat shock / HSE, heat shock element / HSF, heat shock factor / HSP, heat shock proteins / Hsp70/Hsc70, inducible or constitutive 70-kDa heat shock protein / PAL, phenylalanine ammonia-lyase / pcd, programmed cell death / PR-1, pathogenesis-related protein-1 / SA, salicylic acid / SAR, systemic acquired resistance

1. INTRODUCTION

In both plants and humans, there are complex interactions between the host and pathogen possibly modulated by heat shock/stress (HS) protein(s) (HSP) shared not only by plants and humans, but by pathogens as well [9, 12, 26]. HSP are conserved molecular chaperones in prokaryotes and eukaryotes [36], induced or increasingly expressed during exposure to elevated temperatures and a variety of cellular stresses such as oxidative stress (reactive oxygen species, ROS). HSP are classified according to their apparent molecular mass (Hsp100, Hsp90, Hsp70, Hsp60, small HSP (sHSP – most significantly induced in plants) and ubiquitin (8 kDa)), intracellular location (cytoplasm/nucleus, mitochondria, chloroplast, ER), main inducers (e.g. glucose-regulated protein(s): GRP) and their functions. One of the most intensely studied HSP is the Hsp70 family of heat shock proteins. Hsp70 is the most highly induced stress protein and was one of the first eukaryotic genes to be cloned [36]. Members of the Hsp70 family include the constitutive 73-kDa Hsc70 and the inducible 72-kDa Hsp70. All members of the Hsp70 family function as molecular chaperones in an ATP-dependent manner [20] and perform folding reactions mostly in co-operation with other chaperones [11]. Furthermore, they provide protection of newly synthesised polypeptides from premature aggregation

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and promote maturation or transport to target organelles for final packaging [4]. In eukaryotes, HSP expression is largely regulated at the transcriptional level through the binding of trimerized heat shock transcription factor (HSF) to the heat shock element (HSE) leading to *hsp* gene transcription [22].

The resistance response is characterised by the accumulation of defence proteins such as PR proteins and enzymes involved in the phenylpropanoid metabolism, e.g. phenylalanine ammonia-lyase (PAL). The localised response to avirulent pathogens includes the elicitation of cell death/hypersensitive response (HR) at the site of infection, proposed to be programmed cell death, while virulent pathogens cause necrotic cell death normally associated with disease, decreased metabolic activity and systemic infection [18].

HSP are not generally considered to be part of the host defence protein repertoire, in contrast to the pathogen-related (PR) proteins, which are hallmarks of plant disease resistance. In fact, induction of PR proteins and HSP by their respective model inducers (pathogen infection and HS, respectively), often appears to be mutually exclusive. The accumulation of salicylic acid (SA), induction of PR proteins [19] and activity of PAL are temperature sensitive defence-related events and most likely account for the commonly observed disease susceptibility at elevated temperatures [29]. Despite reports that PR proteins are not induced by HS and HSP not induced by pathogen infection [7, 8, 31], there are exceptions to these generally accepted phenomena. HS induces two putative extracellular PR proteins in barley [29], a thaumatin-like protein in maize [10] and P4 chitinase in bean [24]. A member of the Hsp90 family, Grp94, is induced in barley during attempted infection by virulent or avirulent strains of powdery mildew fungus [29]. Aranda et al. [2] described the expression of two heat-inducible genes in pea coding for Hsp70 and polyubiquitin at the onset of pea-seed borne mosaic virus replication. Moreover, an evolutionary relationship has been proposed to exist between HSP and PR proteins. Potato PR-1 induced during infection with late-blight fungus (compatible or incompatible strains) exhibits a 51 % amino acid sequence identity to the 26-kDa HSP from soybean and certain PR protein genes contain HSE in their promotor regions, although inactive [31]. Both HSP and PR proteins are ubiquitous in plants and they are induced by a number of inducers common to both stress protein groups. For example, both HSP and PR proteins are induced in plants by ozone (oxidative stress) [8], wounding, heavy metals and UV radiation [7].

Bacterial wilt, caused by *Ralstonia solanacearum* (formerly *Pseudomonas solanacearum*), is an extensively studied plant-pathogen model [34, 38]. The molecular basis of the interaction between *R. solanacearum* and eukaryotic target cells involves the translocation of an effector protein, PopA1 (*Pseudomonas* out protein), into the host cell via the Hrp type III secretion system leading to infection in susceptible hosts (causing bacterial wilt) or the HR response in resistant hosts [3].

In this study, the accumulation of Hsp70/Hsc70 was investigated in *Lycopersicon esculentum* L. cv. UC82B tomato cell suspensions in response to infection with virulent (biovar III), and avirulent (biovar II) strains of *R. solanacearum* and compared to the induction of classical plant defence proteins (PR-1 and PAL) and host viability. Induction of PR-1 during an incompatible interaction was followed 16 h later by the induction of Hsp70/Hsc70 coinciding with increased PAL activity and sustained host cell viability. It is proposed that induction of Hsp70/Hsc70 by avirulent pathogens chaperones the production of defence proteins and contributes to cellular homeostasis.

2. RESULTS

2.1. Differential effects of *R. solanacearum* biovars II and III on tomato plant and cell viability

In order to confirm the differential virulence of biovars II and III as suitable models to investigate compatible and incompatible interactions, viability of both tomato plants and cell suspensions were studied after inoculation. Biovar III caused severe wilting in tomato plants 5 d after inoculation (*figure 1A*, 3), while those inoculated with biovar II (*figure 1A*, 2) or sterile distilled H_2O (*figure 1A*, 1) remained unaffected. Besides confirming the differential virulence of biovars II and III in planta, the influence of these strains on the viability of tomato cell suspensions was also investigated using the Alamar Blue cell viability assay. The percentage reduction of Alamar Blue as an indicator of viability of tomato cell populations after co-culturing with *R. solanacearum* biovar II or III is shown in *figure 1B*. Compared to normal tomato cells, cells cultured with virulent biovar III showed a significant decrease in their ability to reduce Alamar Blue (from 94 to 63 %) at 48 h. In contrast, viability of cells exposed to biovar II was not significantly different from control cells at 48 h.

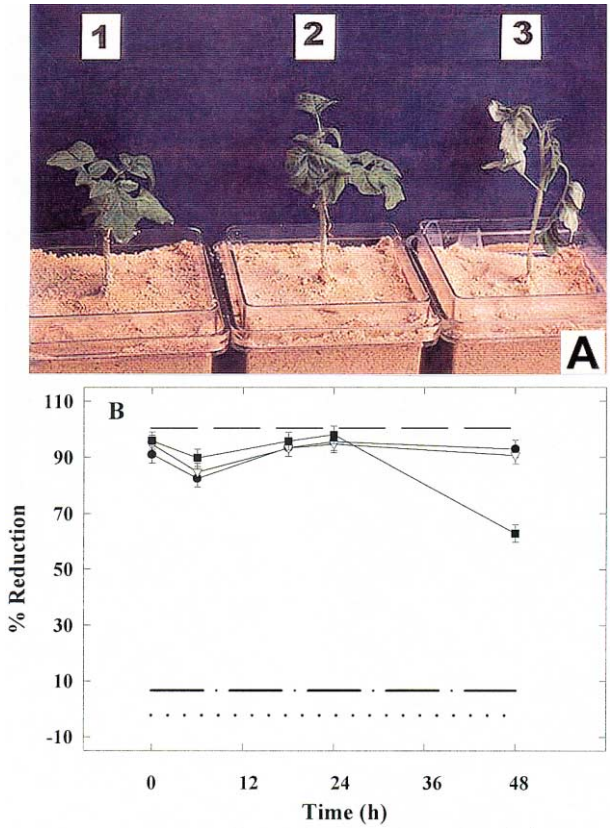

Figure 1. Virulence of biovars II and III of *Ralstonia solanacearum* in tomato, *Lycopersicon esculentum* L. cv. UC82B. **A**, The response of 9-week-old tomato plants 5 d after inoculation with *R. solanacearum* the avirulent biovar II (2) and virulent biovar III (3) at the stem base and in the leaf axils of the lower two leaves using approximately $1{\cdot}10^9$ cfu·mL^{-1}. Control plants (1) were inoculated with an equal volume of sterile distilled H_2O. Symptoms characteristic of bacterial wilt were evident in the plants inoculated with biovar III 5 d after inoculation. **B**, Viability of tomato cell suspensions over time detected by Alamar Blue reduction following exposure to biovar II or III of *R. solanacearum*. Cells were kept under control conditions (●), co-cultured with $1{\cdot}10^7$ cfu·mL^{-1} biovar II (▽) or III (■) of *R. solanacearum*. Cells were harvested at specific time points and viability assessed by the Alamar Blue cell survival assay based on the reduction of resazurin by viable cells. Percentage reduction of Alamar Blue was calculated from values obtained spectrophotometrically at 540 and 620 nm. Completely reduced and fully oxidised Alamar Blue are indicated by dashed (100 % reduction) and dotted lines (0 % reduction) respectively, while the dash-dot line represents Alamar Blue reduction by heat-killed tomato cells. Error bars represent the LSD at $P < 0.05$ ($n = 6$).

2.2. Differential effects of *R. solanacearum* biovars II and III on PAL enzyme activity and PR-1 accumulation

The activities of PAL, a key enzyme in the phenylpropanoid metabolism, activated during the resistance response [23] and PR-1, a classical defence protein normally induced by avirulent pathogens, were studied as additional markers of incompatibility in *L. esculentum* cell suspensions exposed to *R. solanacearum* biovars II and III. *Figure 2A* illustrates that cells exposed to biovar II showed a significant induction ($P < 0.05$) of PAL enzyme activity at 24 h, increasing further at 48 h compared to PAL activity under normal conditions. In contrast, cells exposed to biovar III showed no increase in PAL activity at the same time intervals and was in fact significantly suppressed at 24 h ($P < 0.05$). *Figure 2B* illustrates the fold induction of PR-1 in tomato cell suspensions exposed to biovar II or III of *R. solanacearum*, compared to control levels, over time as determined by western blot analysis (*figure 2C*) showing only up to 24 h. Compared to control levels, both biovar II (light grey bar) and biovar III (dark grey bar) induced a significant accumulation of PR-1 at 6 and 18 h, ($P < 0.01$) (*figure 2B*). Induction of PR-1 with the virulent biovar III at 6 h was however significantly higher ($P < 0.05$) than with the avirulent biovar II at that time. At 24 h, PR-1 levels in cells treated with either virulent or avirulent strains had returned to basal levels.

2.3. The use of human anti-Hsp70/Hsc70 to detect the tomato 70-kDa Hsp

An antibody directed specifically towards tomato Hsp70 was not commercially available. As a result the immuno-reactivity of anti-human Hsp70/Hsc70 (indicated by StressGen to detect plant Hsp70/Hsc70 in general) towards the heat-inducible tomato 70-kDa polypeptide was verified. The profile of polypeptides synthesised in response to heat shock in tomato cell suspensions was investigated using biometabolic labelling and the same samples were used to perform western blot analysis. *Figure 3A* shows the profiles of labelled polypeptides synthesised during a 4-h period under normal conditions (*figure 3A*, C) and following heat shock (40 °C for 15 min with 3 h recovery, *figure 3A*, HS). Heat shock showed the increased synthesis of polypeptides of approximately 70 and 90 kDa compared to synthesis under control conditions. Western blot analysis using the anti-human Hsp70/Hsc70 detected polypeptide(s) of 70 kDa in control samples (*figure 3B*, C) that increased approximately 2-fold following heat shock (*figure 3B*, HS), compared to control cells. No other non-specific binding occurred. As a result of the constitutive and inducible nature of this immunoreactive band, it is hereafter referred to as Hsp70/Hsc70.

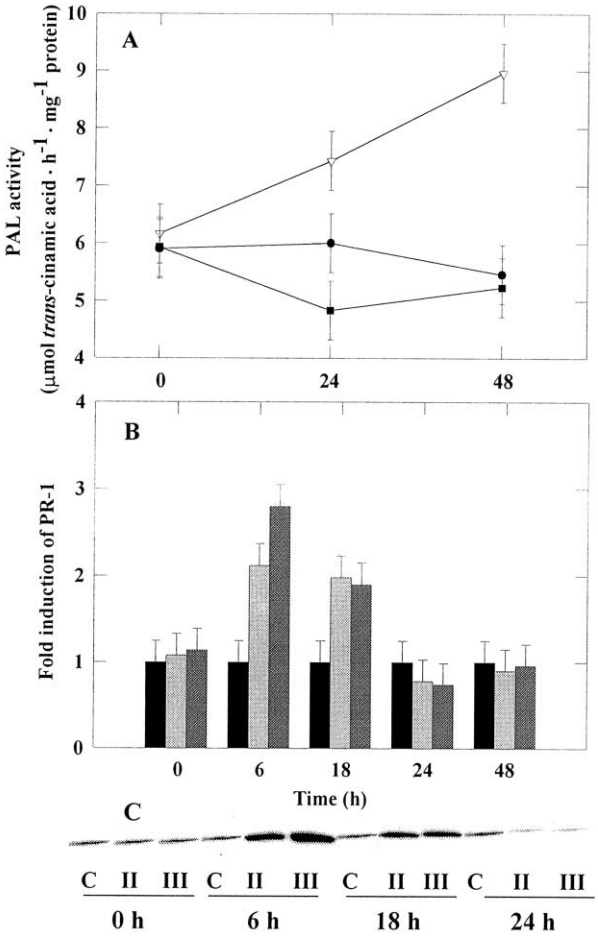

Figure 2. Differential effect of biovars II and III on phenylalanine ammonia-lyase (PAL) enzyme activity and PR-1 accumulation in tomato cell suspensions. **A**, PAL enzyme activity at specific time intervals after inoculation with sterile distilled water (●), biovar II (▽) or III (■) of *R. solanacearum*. Error bars indicate LSD ($P < 0.05$) ($n = 3$). **B**, The time course of PR-1 accumulation in tomato cell suspensions in response to biovars II and III of *R. solanacearum* as determined by western blot analysis (**C**) and densitometric quantification (**B**). Cells were kept under control conditions (black bars, or C), exposed to biovar II (light grey bars, or II), or to biovar III (dark grey bars, or III) of *R. solanacearum* for different time intervals. The bar chart (**B**) illustrates the mean fold induction of PR-1 ($n = 5$) relative to time controls. Error bars represent the LSD at $P < 0.05$. **C** illustrates a representative western blot showing the accumulation of a 15-kDa PR-1 band at selected time intervals.

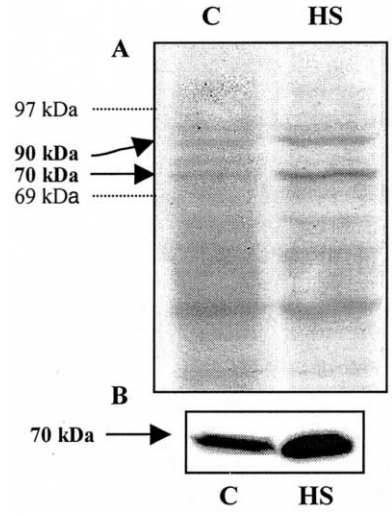

Figure 3. Cross reactivity between anti-human Hsp70/Hsc70 and the tomato Hsp70/Hsc70 homologue. **A**, An autoradiograph showing the profile of polypeptides synthesised during a 4-h time period under normal conditions (C) and following heat shock (HS, 40 °C, 15 min plus 3 h recovery). The positions of molecular mass standards are indicated to the left of the first lane by dotted lines and the arrows show the position of polypeptides of approximately 70 and 90 kDa. **B**, Representative western blot of samples identical to (**A**) illustrating increased accumulation of a polypeptide(s) of 70 kDa with immunoreactivity towards anti-human Hsp70/Hsc70 following heat shock.

2.4. Differential effects of *R. solanacearum* biovars II and III on Hsp70/Hsc70 accumulation

Western blot analysis (accumulation) instead of biometabolic labelling (synthesis) was subsequently used as a more cost effective and specific method to evaluate Hsp70/Hsc70 expression in *L. esculentum*, in response to biovars II and III of *R. solanacearum*. *Figure 4* illustrates the fold induction compared to control levels of Hsp70/Hsc70 in cell suspensions exposed to biovar II or III, over a 48-h period. A representative western blot showing Hsp70/Hsc70 accumulation at 0, 24 and 48 h in tomato cell suspensions exposed to biovars II and III are indicated in *figure 4B*. Compared to control levels, biovar II (light grey bar) induced a significant accumulation of Hsp70/Hsc70 at 24 h ($P < 0.001$) that decreased but remained significantly elevated at 48 h ($P < 0.05$). No significant modulation of Hsp70/Hsc70 levels was observed with biovar III (dark grey bar) treatment at any time point. Positive control samples that received heat shock at every time point showed significant ($P < 0.05$) induction of Hsp70/Hsc70 throughout (results not shown).

2.5. Hsp70/Hsc70 accumulation relative to PAL enzyme activity, PR-1 levels and cell viability

The relation between the expression of Hsp70/Hsc70 and PR-1 accumulation, PAL enzyme activity and host cell viability was investigated performing correlation and regression analysis on data obtained for biovars II

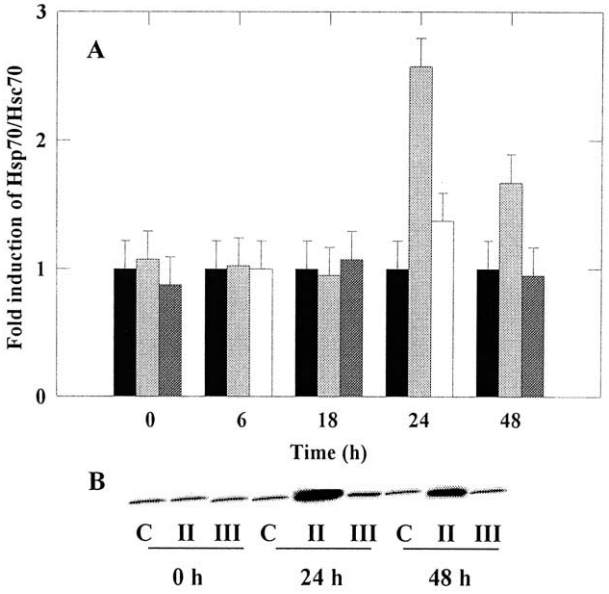

Figure 4. The time course of Hsp70/Hsc70 accumulation in tomato cell suspensions in response to virulent biovar III and avirulent biovar II of *R. solanacearum* as determined by densitometric quantification (**A**) of western blots (**B**). Cells were kept under control conditions (black bars, or C), exposed to biovar II (light grey bars, or II), or biovar III (dark grey bars, or III) of *R. solanacearum* for different time intervals. The bar chart illustrates the mean fold induction of Hsp70/Hsc70 levels ($n = 4$) (**A**) relative to time controls. Error bars represent the LSD at $P < 0.05$. A representative western blot (**B**) shows Hsp70/Hsc70 accumulation at selected time intervals.

and III exposure. A significant negative relation ($r = –0.43$) was shown between the accumulation of Hsp70/Hsc70 and PR-1 during exposure to biovar II ($P < 0.05$). A two-parameter exponential decay equation ($y = 2.96 \cdot e^{(-0.5808x)}$) most accurately described this relation. The values for R and R^2 were 0.51 and 0.25, respectively, while the standard error of the estimate was 0.77. Both coefficients were different from zero at $P = 0.0182$ and $P = 0.0884$, respectively. In contrast, no relation between the accumulation of Hsp70/Hsc70 and PR-1 was evident in cells exposed to biovar III.

3. DISCUSSION

Results reported in this study support the virulence-dependent induction of Hsp70/Hsc70 in *L. esculentum* L. cv. UC82B cell suspensions by avirulent biovar II of *R. solanacearum*. Induction of Hsp70/Hsc70 (24–48 h) followed the induction of PR-1 (6–18 h), coinciding with the onset of increased PAL enzyme activity (24–48 h) and was associated with sustained host cell viability (48 h) not observed in compatible interactions.

In contrast to controls and treatment with the avirulent strain of *R. solanacearum* (biovar II), the virulent strain (biovar III) caused bacterial wilt in young tomato plants 5 d post-inoculation, and a 40 % decrease in viability of tomato cell suspensions 24 to 48 h after initiating co-cultivation (*figure 1*). Virulent races of *R. solanacearum* such as biovar III are known to cause wilting in many susceptible solanaceous host species, in particular the crop plant *L. esculentum* in tropical and warm climates [28].

The early induction of PAL enzyme activity by *R. solanacearum* biovar II observed in tomato cell suspension cultures at 24 to 48 h (*figure 2A*) typifies the resistance response to avirulent pathogens, while the reduction in PAL activity caused by biovar III (24 h) is in agreement with susceptibility [23]. Both virulent and avirulent biovars of *R. solanacearum* induced PR-1 accumulation in tomato cells after 6 to 18 h co-culturing, biovar III even more prominently than biovar II. Although PR protein induction is generally believed to accompany only incompatible interactions [18, 33], a few exceptions have been reported. In some instances PR-1 induction was also reported to be more prominently induced by virulent pathogens, e.g. the late-blight fungus in potato [31]. van Kan et al. [32] observed that the maximum level of mRNA for certain PR proteins is reached much faster in the incompatible interactions, but to similar levels, in the compatible interaction between tomato and the fugal pathogen *Cladosporium fulvum*. Based upon the above-described responses of whole plants and cell suspensions to pathogen interactions, the proposed host-pathogen model was used as a paradigm of compatible and incompatible plant-pathogen interactions using cell suspensions as the plant host model. Evidence exists that supports the use of cell suspensions as in vitro models to replace whole plants as experimental model. Godiard et al. [13] confirmed that the characteristics observed in tobacco leaves with respect to mRNA levels after inoculation with either the compatible or incompatible isolates of *Pseudomonas solanacearum*, were also clearly observed in tobacco cell suspensions with slight differences in timing and extent of mRNA induction. The latter should be taken into account when extrapolating between these models.

Biometabolic labelling following heat shock (*figure 3A*, HS) showed significant synthesis of polypeptides of approximately 70 and 90 kDa compared to the

control (*figure 3A*, C). A mouse monoclonal antibody directed against human Hsp70/Hsc70 detected and confirmed that the 70-kDa band in heat shocked *L. esculentum* cell suspensions was a protein corresponding to Hsp70/Hsc70. This antibody is known to detect proteins of 72 and 73 kDa, corresponding to the apparent molecular mass of Hsp70/Hsc70 in samples from human, mouse, chicken, frog and plant (StressGen Biotechnologies Corp). Immunoreactivity of this antibody to the tomato heat-inducible 70-kDa polypeptide(s) supported its use in subsequent analysis.

Avirulent *R. solanacearum* induced accumulation of Hsp70/Hsc70 in tomato cells from 24 to 48 h (*figure 4A*). This finding is in agreement with a role proposed for protein chaperones in plant-pathogen interactions. Marivet et al. [21] observed the induction of the chaperone cyclophilin (peptidyl-prolyl *cis-trans* isomerase) by alpha mosaic virus infection and signalling compounds associated with resistance, including ethephon and SA. Several well-known inducers of HSP such as ion fluxes, cytoskeletal changes, lipid peroxidation and generation of ROS [14, 16] are also events or components characteristic of the resistance response.

What are the possible function(s) of Hsp70/Hsc70 induced during the defence response, and could they influence the outcome of infection attempts by *R. solanacearum*? Hsc70 and Hsp90 have been shown to regulate certain signal transduction pathways in the eukaryotic cytosol and they could have a similar role in plants during resistance signalling [11]. HSP play an essential role in the formation and function of the cytoskeleton [17], which plays a central co-ordinating role in plant defence [14], and could participate in polarisation of the cytoskeleton to sites of attempted pathogen intrusion. As previously proposed, chaperones could assist newly synthesised defence proteins, for example enzymes involved in phenylpropanoid metabolism and PR proteins, in folding, transport or membrane translocation to subcellular organelles or the extracellular milieu [21, 37]. Results from our laboratory suggested that prior induction of Hsp70/Hsc70 by exposure to non-lethal heat shock protects specific components in the phenylpropanoid metabolism from heat-related inhibition (unpublished). The observed overlap in PAL and Hsp70/Hsc70 induction (*figures 2A, 4A*) may support this proposal and a similar mechanism may contribute to resistance against powdery mildew infection of barley induced by prior heat treatment [29].

A significant negative correlation ($r = -0.43$, $P < 0.05$) exists between Hsp70/Hsc70 and PR-1 accumulation in cell suspensions exposed to biovar II. An exponential decay regression model revealed that PR-1 levels decreased exponentially with increasing accumulation of Hsp70/Hsc70, in agreement with their mutual exclusive regulation outlined in the Introduction, which suggests a role for Hsp70/Hsc70 at a time-point when PR-1 accumulation has already subsided (compare *figures 2B* and *4A*). Whether this reciprocal regulation is coincidental or interrelated remains to be determined. It is possible that Hsp70/Hsc70 may also facilitate the folding and export of the extracellular, endogenous endoproteinases involved in the degradation of PR proteins and the regulation of their biological function [27]. In vertebrates, Hsp70 accumulation is an event that has been referred to as Dr Jeckyll and Mr Hyde [25] or a two-edged sword [5]. Overproduction of Hsp70 protects an organism by shifting cells from necrotic death towards programmed cell death (pcd), a characteristic of HR. The same mode of action of Hsp70 could be detrimental when pathogen-infected cells are shifted from pcd to survival, providing the pathogen with the opportunity for systemic infection [35]. It is tempting to speculate that plants have through evolution selected to exclude HSP induction as part of their early defence response to avoid interference with the initiation of cell death during the hypersensitive reaction. This decreases the likelihood that Hsp70/Hsc70 is involved in early events of defence. Whether the rise in Hsp70/Hsc70 levels is an epiphenomenon or an essential component of resistance could form the subject of future studies.

The almost normal viability of biovar II-treated cells at 48 h (*figure 1B*) coincided with and was preceded by the significant elevation in Hsp70/Hsc70 levels (*figure 4*), compared to 64 % viability of biovar III-treated cells lacking a significant induction of Hsp70/Hsc70. This observation is in agreement with the well-established protective role of Hsp70. While the role of HR in determining plant resistance is debatable [15], Hsp70/Hsc70 could protect mitochondria and supports cell survival or at least the death of infected cells in a programmed manner, which requires active plant metabolism.

In conclusion, these results support a role for the 70-kDa HSP family in the defence response activated in tomato by an avirulent strain of *R. solanacearum*. Induction of Hsp70/Hsc70 followed PR-1 accumulation and coincided with increased PAL enzyme activity and prolonged cell survival. The particular molecular targets of Hsp70/Hsc70 protection in this incompatible interaction remains unclear and awaits further investigation. Whether this phenomenon is of general signifi

cance or unique to the utilized model system remains to be determined. The use of isogenic strains of *R. solanacearum* could further identify whether or not it is dependent on the presence or absence of *avr* genes.

4. METHODS

4.1. Reagents

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

4.2. Plant material

Seeds of the tomato, *Lycopersicon esculentum* L. cv. UC82B (Hygrotech, Pretoria, South Africa), were germinated under controlled conditions (25 °C, 16 h light and 8 h dark cycle) on Gamborg's B-5 medium with minimal organics (3.18 $g \cdot L^{-1}$) (Highveld Biologicals, Kelvin, South Africa), supplemented with sucrose (10 $g \cdot L^{-1}$) and solidified with bacto-agar (8 $g \cdot L^{-1}$) (Difco Laboratories, Detroit, MI).

4.3. Cell suspensions

Callus was initiated by transferring leaf and stem cuttings of 2-week-old plants to Murashige and Skoog media (Highveld Biologicals, Kelvin, South Africa), pH 5.8, containing vitamins and supplemented with casein enzyme hydrolysate (2 $g \cdot L^{-1}$), kinetin (0.25 $mg \cdot L^{-1}$) and 2,4-dichlorophenoxy acetic acid (2 $mg \cdot L^{-1}$) (hereafter referred to as MS media) and solidified with bacto-agar (8 $g \cdot L^{-1}$). Callus was transferred to fresh medium every fourth week. Cell suspension cultures were prepared by transferring callus, subcultured at least four times, to liquid MS medium, shaking at 97 rpm in the dark at 25 °C. All cell suspensions used were in the exponential growth phase with an optical density of 1.5 at 600 nm.

4.4. Bacteria

Ralstonia solanacearum biovar II (avirulent) and biovar III (virulent) strains, were obtained from the Vegetable and Ornamental Plant Institute (VOPI), Agricultural Research Council (ARC), Roodeplaat, South Africa, on tetrazolium chloride plates. Colonies were suspended in sterile distilled water and the concentration of bacteria estimated spectrophotometrically (optical density of 0.3 at 620 nm is approximately $1 \cdot 10^9$ $cfu \cdot mL^{-1}$).

4.5. Treatments

4.5.1. Whole plants

Three-week-old seedlings were transferred to moist sterile sand and cultivated at 25 °C, in 16 h light, 8 h dark cycle until use. After 9 weeks, the tomato plants were stem inoculated with approximately $1 \cdot 10^9$ cfu biovar II or III of *R. solanacearum* suspended in 50 µL sterile distilled H_2O. Control plants were inoculated with an equal volume of sterile distilled H_2O.

4.5.2. Cell suspensions

Tomato cell suspensions were seeded in 24-well plates (1 mL per well). Cultures were inoculated with $1 \cdot 10^7$ cfu biovar II or III of *R. solanacearum* or an equivalent volume of sterile distilled H_2O and incubated for specific time intervals on a rotary shaker in the dark at 22 °C. Heat shock, the positive control for Hsp70/Hsc70 induction, was administered at 40 °C for 15 min in a circulating water bath followed by 3 h recovery at 22 °C before harvesting.

4.6. Cell viability

Cell viability was assessed using the Alamar Blue cell survival assay adapted for tomato cells [6]. Positive (100 % reduced) and negative controls (0 % reduced) were included in every assay. The Alamar Blue assay, a specific viability assay designed to measure quantitatively the proliferation of various cell lines including human, bacteria and fungi and recently plants [6], is based on the reduction of rasazurin by viable cells in response to metabolic activity based largely upon mitochondrial electron transport activity [1], an activity proposed to remain intact during programmed cell death [20].

4.7. PAL enzyme assay

PAL enzyme activity (EC 4.3.1.5) was analysed in cell-free extracts according to a modified method by Nagarantha et al. [23]. Cells (0.1 g) were homogenised in 1 mL 0.1 mM sodium borate buffer (pH 8.8) containing 3 mM β-mercaptoethanol. The homogenate was centrifuged at 15 000 × *g* for 20 min at 4 °C and the supernatant was used directly for the enzyme assay. The protein content of the extracts was determined using the Bio-Rad microassay with bovine serum albumin as standard. PAL enzyme activity was determined spectrophotometrically by measuring the production of *trans*-cinnamic acid from L-phenylalanine.

4.8. Biometabolic labelling

Cells were labelled for 4 h with a mixture of [^{35}S]-methionine and [^{35}S]-cysteine, 2.8 $\mu Bq \cdot mL^{-1}$ suspension (Amersham Pharmacia Biotech, Buckingham

shire, England), with or without heat shock (40 °C for 15 min) and 3 h recovery.

4.9. Gel electrophoresis

Cell samples were rinsed under vacuum filtration with fresh MS medium and homogenised in 0.1 M Tris/HCl buffer (pH 6.8) containing 4 % sodium dodecylsulphate (SDS), 10 % β-mercaptoethanol and 0.001 % bromophenol blue, boiled for 10 min. Equal amounts of protein in supernatants as determined by the solid phase method of Sheffield et al. [30] were resolved by 15 % SDS-PAGE gels (30 % acrylamide/0.8 % bisacrylamide).

4.10. Western blot analysis

A mouse monoclonal antibody directed against the human constitutive (Hsc70) and inducible (Hsp70) isoforms of the 70-kDa Hsp family (Hsp70/Hsc70; SPA 820, Stress-Gen, Vancouver, Canada) (1:5 000 dilution) and a mouse monoclonal antibody against acidic tobacco PR-1 (1:1 000 dilution), kindly provided by D.F. Klessig (Waksman Institute, Piscataway, NJ) were used as primary antibodies. Bound primary antibody was detected using a goat anti-mouse IgG conjugated to horse-radish peroxidase (1:20 000 dilution) as secondary antibody (Pierce, Rockford, IL). Peroxidase-labelled secondary antibody was detected by chemiluminescence (Pierce, Rockford, IL).

4.11. Data analysis

Immunoreactive polypeptide bands were quantified by image analysis using Uvpgr32 (UVP, San Gabriel, CA) and Gene Tools (SynGene, Cambridge, UK) and data was expressed as fold induction relative to the corresponding time control.

4.12. Statistical analysis

Analyses of variance and comparison of means were performed as complete randomised blocks using CoStat Software (CoHort Software, Berkeley, CA, 1990). Differences in mean values were considered significant if the LSD, calculated from the pooled variance, was exceeded. Correlation and regression analysis was done using SigmaPlot® 4.0 for Windows 95, NT & 3.1 (SPSS Inc, Chicago, IL, 1997).

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