Heat shock, with recovery, promotes protection of *Nicotiana tabacum* during subsequent exposure to *Ralstonia solanacearum*

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Received: 24 March 2013 / Revised: 3 June 2013 / Accepted: 18 June 2013 / Published online: 13 August 2013
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Abstract Host–pathogen interactions in plants are complex and potentially influenced by heat shock/stress (HS). Host HS proteins (HSPs) induced prior to bacterial exposure may facilitate the folding of newly synthesized defense proteins and promote incompatible host–pathogen interactions. We hypothesized that a non-lethal HS, with recovery, promotes protection of *Nicotiana tabacum* during subsequent exposure to avirulent soilborne necrotrophic pathogen *Ralstonia solanacearum*. The objective of this study included investigating the effects of HS with or without recovery on the outcome of bacterial exposure to a virulent and avirulent biovar of *R. solanacearum* in *N. tabacum* cell suspensions. This was assessed by quantifying host Hsp70/Hsc70 levels, mitochondrial electron (e^-) transport activity as a marker of viability, and phosphatidylserine externalization and DNA fragmentation as markers of apoptosis. Our findings support the hypothesis that HS, with recovery, promotes protection of *N. tabacum* during subsequent exposure to *R. solanacearum*, suggesting a role for Hsp70/Hsc70 in the observed protection of e^- transport, increased apoptosis, and DNA fragmentation.

Keywords Hsp70 · *Ralstonia solanacearum* · Programmed cell death · Heat shock · Plant–pathogen · *Nicotiana tabacum*

Introduction

Pathogen attack strategies are sophisticated, but plants have evolved ways to prevent infection, making disease-associated death the exception rather than the rule. Nonetheless, cell death is a feature of both compatible (susceptible) and incompatible

(resistant) plant–pathogen interactions (Dickman et al. 2001). Pattern recognition receptors (PRRs), located in the plant cell membrane, are the second line of defense in innate immunity, after preformed barriers, such as waxy cuticles and rigid cell walls. PRRs recognize common features of microbial pathogens, such as flagellin and lipopolysaccharides, generally referred to as microbial- or pathogen-associated molecular patterns (M/PAMPs) (Jones and Dangl 2006). M/PAMP recognition initiates signaling pathways resulting in M/PAMP-triggered immunity (M/PTI) (Coll et al. 2011), a slow basal resistance to pathogens. As counter-attack, pathogens produce effector molecules (avirulence (*avr*) gene products) that are pathogen species-, race-, and strain-specific. Effectors are delivered directly into the host cell to suppress M/PTI and promote virulence resulting in effector-triggered susceptibility (ETS) (Jones and Dangl 2006). In response, plants have a second, more rapid, and robust line of defense—effector-triggered immunity (ETI) (Jones and Dangl 2006). In ETI, effectors are recognized by plant resistance (*R*) gene products, located in the cytoplasm (Hammond-Kosack and Jones 1997). M/PTI and ETI initiate signaling pathways resulting in defense gene expression involved in cell wall strengthening, production of antimicrobial compounds (Morel and Dangl 1997; Coll et al. 2011), pathogenesis-related (PR) proteins, as well as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) synthesis (Pieterse et al. 2009). ETI is often accompanied by accumulation of reactive oxygen species (ROS), triggering the hypersensitive response (HR). HR is a rapid form of programmed cell death (PCD) associated with plant defense (Espinosa and Alfano 2004) and characterized, morphologically, by the formation of localized necrotic lesions around the site of pathogen entry (Király et al. 2008). HR isolates the pathogen, preventing dissemination and further damage while limiting pathogen access to water and nutrients (Coll et al. 2011). HR is considered a hallmark of ETI, while no macroscopic symptoms are associated with M/PTI (Göhre and

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Robatzek 2008). Accumulating evidence supports a continuum (Thomma et al. 2011) or overlap (Katagiri and Tsuda 2010) between M/PTI and ETI, speed and strength being the ultimate determinant of efficacy (Katagiri and Tsuda 2010; Henry et al. 2012). The outcome of the battle between pathogen and host depends on the balance between the ability of the pathogen to suppress the plant immune system and the ability of the plant to recognize the pathogen and activate effective defenses (Pel and Pieterse 2012).

Plant pathogens are generally divided into necrotrophs and biotrophs (Glazebrook 2005). Most toxin-producing pathogens are necrotrophs that destroy host cells to feed on their contents. Biotrophs, on the other hand, derive nutrients from living host tissues. Necrotrophic effectors, like biotrophic effectors, also induce host resistance responses and some hallmarks of PCD, such as HR (Alfano and Collmer 2004; Kulye et al. 2012; Liu et al. 2012), suggesting an overlap in the response elicited by necrotrophs and biotrophs. While biotrophs mainly suppress HR, virulent necrotrophs hijack host resistance responses (Grassmé et al. 2001; Hammond-Kosack and Rudd 2008) resulting in expanding necrotic lesions (Govrin and Levine 2000; Romanenko et al. 2002; Alfano and Collmer 2004) that play into the hand of the pathogen rather than protecting the host (Glazebrook 2005). These necrotic lesions are thought to mark a delayed HR resulting from suppressed effector recognition and a jeopardized defense response in the plant (Alfano and Collmer 2004).

Knowledge about plant–necrotroph interactions remains limited as most plant–pathogen studies focus on biotrophic pathogens. *Ralstonia solanacearum*, a Gram-negative, *necrotrophic* β -proteobacterium, causes bacterial wilt in diverse plant types (Smith et al. 1995). This soilborne pathogen enters through root wounds colonizing the water-transporting xylem tissue through which it spreads up into the plant (Jacobs et al. 2012). Like other necrotrophs, *R. solanacearum* takes its nutrients from dead or dying host cells and evolved mechanisms to expand necrotic lesions causing plant host cell death (Alfano and Collmer 1996). Having overcome host M/PTI, *R. solanacearum* secretes effector molecules such as *Pseudomonas* outer protein (based on its previous genus name) directly from the bacterial cytoplasm into the plant cytoplasm via the hypersensitive response and pathogenicity (Hrp) (Alfano and Collmer 1996) type III secretion system (TTSS) (Alfano and Collmer 2004), suppressing innate immunity (Espinosa and Alfano 2004) to finally induce necrotic cell death instigated from the necrotic lesion (Jacobs et al. 2012).

As yet there are no specific plant markers to discriminate between necrosis and HR. Therefore, studies are based on animal PCD—apoptosis. HR shares some apoptotic features (Heath 1998; Tada et al. 2001; Collazo et al. 2006; van Doorn 2011) including DNA fragmentation and phosphatidylserine (PS) externalization (Khurana et al. 2005). Yao et al. (2002) found that irrespective of the pathogen type (fungi, bacteria, or virus) or interaction (compatible or incompatible), apoptotic-like

features are a common outcome (Ryerson and Heath 1996; Wang et al. 1996; Tada et al. 2001; Yao et al. 2002). The necrotic lesion, initially coined by plant pathologists without reference to necrosis/apoptosis (Morel and Dangl 1997), is now linked to HR sharing apoptotic characteristics. However, the exact nature of the death leading to HR or necrosis remains poorly understood, particularly involving exposure to necrotrophs.

Besides biotic stress, cells also encounter abiotic stress such as increased temperature. This activates the heat shock/stress (HS) response in which a set of genes encoding heat shock proteins (HSPs) are rapidly induced (Vierling 1991), accompanied by decreased synthesis of most other proteins (Saltveit 1998). HSPs are generally classified according to their molecular weight (Vierling 1991), and the 70-kDa HSP family (HSP70) is the most highly induced stress protein family commonly associated with protection. The constitutively expressed cognate member (Hsc70), under non-stressed conditions, acts as a chaperone, regulating protein structure and function through translocation, folding, and assembly (Mathew and Morimoto 1998) of de novo synthesized proteins (Sung et al. 2001). The stress-inducible 70-kDa HSP family member Hsp70 is induced during exposure to high temperatures and other stresses. Hsp70 regulates the activity of many signal transduction proteins (Pratt and Toft 2003) and binds to damaged, misfolded, or aggregated polypeptides and either facilitates their repair by refolding and restoring biological functions or targets irreparably damaged polypeptides for ubiquitin–proteasome-dependent degradation (Mathew and Morimoto 1998; Sung et al. 2001). 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) (Morimoto 1998).

The response to combined or consecutive stresses in plants is distinct and cannot simply be inferred from the individual responses (Mittler 2006). We hypothesized that a non-lethal HS, with recovery, promotes protection of *Nicotiana tabacum* during subsequent exposure to avirulent *R. solanacearum*. We investigated the effects of HS with or without recovery on the outcome of exposure to the virulent and avirulent necrotroph *R. solanacearum* in *N. tabacum* cell suspensions. This was assessed by studying electron (e^-) transport as a marker of viability and PS externalization and DNA fragmentation as markers of apoptosis together with the quantification of Hsp70/Hsc70. Host defense protection was reflected by increased apoptosis and DNA fragmentation and protection of e^- transport, accompanied by Hsp70/Hsc70 induction. We propose that host HSP expression facilitates plant defense during necrotrophic bacterial exposure.

Materials and methods

The experimental model aimed to evaluate physiological responses in plant cells to the combination of heat and pathogen

stress, commonly inflicted upon plants in nature. None of the heat or pathogen treatments used in this study were intended to be lethal, as stress or defense responses had to be followed for at least 24 h posttreatment.

N. tabacum cell suspensions and protoplast preparation

N. tabacum L. cv. SamSun NN cell suspensions were initiated from sterile leaf and stem tissue and cultured in Murashige and Skoog (MS) medium (pH 5.8) supplemented with 2,4-dichlorophenoxyacetic acid (2 mg/l) on a 7-day subculture cycle at 23 °C with constant rotation (125 rpm). Protoplasts were prepared using a solution of cellulase (1 % w/v) and macerozyme (0.3 % w/v) from Yakult Honsha Co. Ltd, Tokyo, Japan, and Pectolyase Y-23 (0.05 % w/v, Seishin Pharmaceutical Co. Ltd, Tokyo, Japan), pH 5.8. Protoplasts were filtered and washed in protoplast wash fluid (PWF; MS medium, 0.27 M NaCl, pH 5.8), centrifuged, and re-suspended in MS medium supplemented with ascorbic acid (0.1 % w/v), sorbitol (6 mM), mannitol (6 mM), and glucose (0.35 M).

R. solanacearum

The Gram-negative, necrotrophic β -proteobacteria, *R. solanacearum* race 3 biovar 2 (avirulent) and race 1 biovar 3 (virulent), were obtained from Reinette Gouws, Agricultural Research Council—Vegetable and Ornamental Plant Institute (Pretoria, South Africa). Cultures, plated on TZC Kelman's medium containing peptone (10 g/l), casamino acid (1 g/l), glycerol (5 ml/l) and solidified with agar (8 g/l), were incubated (48 h, 37 °C). Colonies were suspended in sterile distilled water, and the optical density of a sample was measured by spectrophotometry (620 nm). The stock suspension was adjusted to 10^9 colony-forming units (cfu)/ml for inoculation.

Experimental treatments

A schematic diagram of the experimental protocol is illustrated in Fig. 1. *N. tabacum* cell suspensions (5 ml) in the exponential growth phase were kept under control conditions (C); exposed to calcium ionophore A23187 (5 μ M (A5) or 10 μ M (A10), 24 h); exposed to heat shock (40 °C, 1 h) with recovery (r) (HSr, 49 h); exposed to different biovars of *R. solanacearum* (1×10^7 cfu/ml, avirulent (Bv2) or virulent (Bv3), 24 h); exposed to heat shock (40 °C, 1 h) with recovery (r, 25 h) prior to Bv2 or Bv3 exposure (HSr-Bv2, HSr-Bv3, respectively, 24 h); or exposed to Bv2 or Bv3 directly after HS (HS-Bv2, HS-Bv3, respectively, 24 h), without any recovery between HS and bacterial exposure. All cells were harvested 50 h after the start of the experiment (diamond). Excess unbound bacteria were washed off with MS medium before protoplast preparation or whole cell analysis.

Cell viability

Viability of cell suspensions was assessed using the alamarBlue® assay according to Byth et al. (2001b). The assay is based on the ability of the mitochondrial e^- transport chain to reduce alamarBlue® (resazurin) to the fluorescent molecule resorufin.

Annexin V binding and PI staining

After treatment of cells, protoplasts were prepared and maintained in PWF containing 3 mM CaCl_2 , as Annexin V is a calcium-dependent phospholipid-binding protein. The protoplasts were pelleted and re-suspended in 500 μ l binding buffer (Apoptest™-FITC kit, NeXins Research BV, Hoeven, The Netherlands). A 50- μ l aliquot of the suspension was incubated with fluorescein isothiocyanate (FITC)-conjugated Annexin V (2 μ l) and propidium iodide (PI, 5 μ l) in the dark on ice (1 h). After adding more binding buffer (200 μ l), the suspended protoplasts were analyzed for FITC and PI by flow cytometry as described below.

Protoplast fixation and permeabilization

Protoplasts isolated from treated cultures were pelleted (80 $\times$ g, 4 °C, 4 min), fixed (30 min, 4 °C, in the dark) in freshly prepared ice-cold paraformaldehyde (2 % in phosphate-buffered saline (PBS), pH 7.4), washed twice with PWF, and dehydrated (overnight, -20 °C) in cold ethylene glycol monomethyl ether. Protoplasts were washed twice with PWF and permeabilized (6 min, 4 °C) using Triton-X 100 (0.1 % v/v in PWF). Protoplasts were washed twice with PBS and centrifuged (80 $\times$ g, 4 °C, 4 min).

DNA fragmentation by TUNEL

DNA fragmentation was quantified using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; in situ Cell Death Detection Kit with fluorescein, Roche, Mannheim, Germany) generally used as a marker of apoptosis in plant tissues (Wang et al. 1996; O'Brien et al. 1997). For the TUNEL assay, sodium citrate (45 mM) was included in the PWF (pH 5.8) to facilitate access to nuclei during permeabilization. Fixed and permeabilized protoplasts were incubated (1 h, 37 °C) with a mixture of terminal deoxynucleotidyl transferase (TdT) and fluorescein-labeled dUTP (50 μ l). Protoplasts were washed with PBS, centrifuged (80 $\times$ g, 4 °C, 4 min), and re-suspended in PBS to be analyzed by flow cytometry as described below.

Hsp70/Hsc70 quantification

Hsp70/Hsc70 was quantified in fixed, permeabilized protoplasts, incubated with a mouse monoclonal primary antibody

Fig. 1 A schematic diagram of the experimental protocol used. *N. tabacum* cell suspensions were kept under control conditions (C); exposed to calcium ionophore A23187 (5 μ M (A5) or 10 μ M (A10), 24 h); exposed to heat shock (40 $^{\circ}$ C, 1 h) with recovery (r) (HSr, 49 h); exposed to different biovars of *R. solanacearum* (avirulent (Bv2) or virulent (Bv3), 24 h); exposed to heat shock (40 $^{\circ}$ C, 1 h) with recovery (r, 25) prior to Bv2 or Bv3 exposure (HSr-Bv2, HSr-Bv3, respectively, 24 h); or exposed to Bv2 or Bv3 directly after HS (HS-Bv2, HS-Bv3,

respectively, 24 h) without any recovery between HS and bacterial exposure. All cells were harvested 50 h after the start of the experiment (diamond). Cell suspensions were analyzed using the alamarBlue[®] assay to assess viability based on e^{-} transport, while protoplasts were analyzed by flow cytometry to assess death (apoptosis/necrosis) based on membrane changes (Annexin V/PI) and to quantify Hsp70/Hsc70 and DNA fragmentation (TUNEL). Protoplast preparation took approximately 4 h

(1:100 dilution in PBS, 37 $^{\circ}$ C, 1 h) against HeLa cells Hsc70 and Hsp70 (SPA 820, StressGen, Victoria, Canada), indicated by StressGen to detect plant Hsp70/Hsc70 and previously shown to be reactive against plant Hsp70/Hsc70 (Byth et al. 2001a). Protoplasts were washed with PBS and centrifuged (80 $\times$ g, 4 $^{\circ}$ C, 4 min). Primary antibody binding was detected with a goat anti-mouse IgG (H+L)-FITC secondary antibody (Immunotech, Marseille, France, 1:50 dilution with PBS, 1 h, 37 $^{\circ}$ C). After washing with PBS and centrifugation (80 $\times$ g, 4 $^{\circ}$ C, 4 min), the protoplasts were re-suspended in PBS to be analyzed by flow cytometry as described below.

Flow cytometry

An Epics Altra flow cytometer (Beckman-Coulter) with an air-cooled argon laser emitting light at 488 nm was used for acquisition and analysis of the data. Populations of unstained protoplasts (autofluorescent control) were identified from forward and side scatter characteristics on dot plot profiles and analyzed for fluorescence intensity using defined, fixed gates. Fluorescence intensity of at least 5,000 protoplasts was acquired per sample. FITC-, TUNEL-, and Hsp70/Hsc70-labeled protoplasts were analyzed with excitation at 488 nm and emission at 525 $\pm$ 10 nm using a band-pass filter. PI was analyzed with 488-nm excitation and 610 $\pm$ 10-nm emission.

Statistical analysis

Data were analyzed using IBM SPSS Statistics, version 20. ANOVA was followed by the Tukey or Dunnett T3 post hoc test based on whether the assumption of equal variance was met or not. Differences were considered significant at $p < 0.05$,

$p < 0.01$, or $p < 0.001$ and indicated in graphs or charts with one, two, and three symbols, respectively. Pearson correlation analysis was performed between different variables followed by hierarchical multiple regression (HMR) analysis. HMR was used to identify predictive models for necrosis and e^{-} transport. In each of the models, a categorical dummy variable, representing the specific treatment (HSr) to be controlled for, was entered at step 1 and the continuous variable was entered at step 2.

Results

Data for viability, DNA fragmentation, and Hsp70/Hsc70 are presented as normalized to the mean of the control being equal to 100 %.

HS with recovery protected e^{-} transport in *N. tabacum* cell suspensions compromised by exposure to avirulent *R. solanacearum*

Viability, as reflected by e^{-} transport, of *N. tabacum* cell suspensions was quantified using the alamarBlue[®] assay. Active mitochondrial e^{-} transport allows the reduction of alamarBlue[®] (resazurin) to the fluorescent molecule resorufin. Before normalization (divided by control $\times$ 100), the mean viability of the control was 89 %. A23187 at both 5 μ M (A5) and 10 μ M (A10) significantly decreased e^{-} transport compared to the control ($p < 0.001$). A10 showed the most notable decrease in e^{-} transport of all the treatments, almost significantly lower than A5 ($p < 0.057$). HSr did not affect e^{-} transport on its own, but protected from the significant decrease in e^{-}

transport induced by Bv2 ($p<0.01$) (Fig. 2, compare Bv2 and HSr-Bv2, $p<0.05$). The protected e^- transport shown by HSr was not seen with HS without recovery (compare HSr-Bv2 and HS-Bv2, $p<0.01$). Although Bv3 treatments did not significantly change e^- transport compared to the control, HSr-Bv3 significantly increased e^- transport ($p<0.01$) compared to Bv3 alone. The e^- transport for HS-Bv3 was not significantly different from that for HSr-Bv3 or Bv3, but differed significantly from that for HS-Bv2 ($p<0.01$). PS externalization in combination with PI nucleic acid staining was investigated next to differentiate death as either apoptosis or necrosis during loss of e^- transport.

HS with recovery enhanced apoptosis in *N. tabacum* protoplasts during subsequent exposure to *R. solanacearum*

The Annexin V/PI apoptosis assay was performed on protoplasts to allow flow cytometry quantification. Protoplasts remain viable, as measured using the alamarBlue® assay, up to 48 h after preparation (results not shown). Scatter plots represent the untreated control (C), apoptosis control (A5), and necrosis control (A10) (Fig. 3a) and distinguish the four Annexin V/PI quadrants representing normal (Annexin V⁻ PI⁻), early apoptosis (Annexin V⁺ PI⁻), late apoptosis (Annexin V⁺ PI⁺), and necrosis (Annexin V⁻ PI⁺). The apoptotic population, induced by A5, is clearly observed in the upper right quadrant (Annexin V⁺ PI⁺), while the

Fig. 2 HSr protected e^- transport in *N. tabacum* cell suspensions compromised by exposure to avirulent *R. solanacearum*. Viability of *N. tabacum* cell suspensions, as reflected by e^- transport, was quantified using the alamarBlue® assay. The bar graph illustrates means with error bars of 95 % confidence intervals. Significant differences are indicated with one symbol ($p<0.05$), two symbols ($p<0.01$), or three symbols ($p<0.001$) for treatments compared to control (C, asterisk), HSr-Bv2/3 compared to the specific Bv alone (filled square), HS-Bv2/3 compared to HSr-Bv2/3 for the specific Bv (open diamond), and equivalent treatments (HS-Bv) compared between Bv2 and Bv3 (filled circle). Biological ($n=5$) and technical ($n=4$) replicates were performed

necrosis population, induced by A10, clustered protoplasts in the Annexin V⁻ PI⁺ quadrant (A10). In our hands, the percentage classical “early apoptosis” (Annexin V⁺ PI⁻) was low for all the treatments, with no significant differences between them (Fig. 3). Based on this and movement of the cell population from the bottom left quadrant to the top right quadrant (comparing C and A5, Fig. 3a) and the fact that PI can result in false-positive events (Rieger et al. 2010), we concluded that this movement likely better reflects apoptosis and is referred to as such from hereon.

Pie charts illustrate the percentage of cells in the four Annexin V/PI quadrants (Fig. 3b). A5 and A10 both had significantly lower and equal percentages of normal cells compared to the control ($p<0.01$). A5 and A10 differed from each other in terms of their mode of cell death; A5, the apoptotic control, showed a significantly higher percentage of apoptosis compared to the control ($p<0.001$) and A10 ($p<0.05$), while A10, the necrotic control, showed a significantly higher percentage of necrosis compared to the control ($p<0.05$) and A5 ($p<0.01$). Compared to the control, HSr significantly induced apoptosis ($p<0.001$), while it significantly decreased the percentage of normal cells ($p<0.01$). Bv2 significantly induced apoptosis compared to the control ($p<0.05$), while Bv3 significantly induced necrosis compared to Bv2 ($p<0.05$). Apoptosis was significantly enhanced when pathogen exposure occurred after HS with recovery (HSr-Bv2 and HSr-Bv3) compared to HSr alone ($p<0.01$) as well as compared to the control and Bv2/3 alone ($p<0.001$). Increased apoptosis was not achieved by HS without recovery (HS-Bv2/3), which rather resembled control cells albeit amidst pathogen exposure. In fact, the percentage normal cells in HS-Bv2/3 were very similar to those in the control but significantly increased compared to those in HSr-Bv2/3 ($p<0.001$). DNA fragmentation, marking later stages in apoptosis, was then assessed using the TUNEL assay.

HS with recovery induced DNA fragmentation in *N. tabacum* protoplasts during subsequent exposure to avirulent *R. solanacearum*

TUNEL is a flow cytometry-based method used to study 3'-OH DNA strand breaks characteristic in later stages of apoptosis and is believed to be a sound method to study PCD in plants. A5 showed no change in DNA fragmentation, unlike A10 that significantly increased DNA fragmentation compared to the control ($p<0.05$) and A5 ($p<0.05$) (Fig. 4). Neither HSr on its own nor Bv2/3 altered DNA fragmentation. However, DNA fragmentation increased significantly in the HSr-Bv2 ($p<0.01$) compared to the control, while in HSr-Bv3, DNA fragmentation was significantly decreased compared to HSr-Bv2 ($p<0.05$). DNA fragmentation also differed between strains when HS was given without recovery followed by *R. solanacearum* (HS-Bv). DNA fragmentation

Fig. 3 HSr enhanced apoptosis in *N. tabacum* protoplasts during subsequent exposure to *R. solanacearum*. Apoptosis of *N. tabacum* protoplasts was assessed by quantifying PS externalization and PI intercalation by flow cytometry. **a** Representative scatter plots of Annexin V/PI binding for C, A5, and A10 treatments, showing four quadrants: normal (Annexin V⁻ PI⁻), early apoptosis (Annexin V⁺ PI⁻), late apoptosis (Annexin V⁺ PI⁺), and necrosis (Annexin V⁻ PI⁺). **b** Pie charts summarize the mean percentages of the four Annexin V/PI quadrants after different treatments

stipulated in Fig. 1. Significant differences are indicated with *one symbol* ($p < 0.05$), *two symbols* ($p < 0.01$), or *three symbols* ($p < 0.001$) for treatments compared to control (C, *asterisk*), A10 compared to A5 (*hash*), HSr-Bv2/3 compared to HSr alone (*open inverted triangle*), HS-Bv2/3 and HSr-Bv2/3 compared to the specific Bv alone (*filled square*), HS-Bv2/3 compared to HSr-Bv2/3 for the specific Bv (*open diamond*), and Bv2 compared to Bv3 (*circle*). Biological ($n=2$) and technical ($n=3$) replicates were performed

Fig. 4 HSr induced DNA fragmentation in *N. tabacum* protoplasts during subsequent exposure to avirulent *R. solanacearum*. DNA fragmentation of *N. tabacum* was quantified by flow cytometry, staining fixed, permeabilized protoplasts with a mixture of TdT and fluorescein-labeled dUTP. The bar graph illustrates means with error bars of 95 % confidence intervals. Significant differences are indicated with one symbol ($p < 0.05$) or two symbols ($p < 0.01$) for treatments compared to control (C, asterisk), A10 compared to A5 (hash), HS-Bv2/3 and HSr-Bv2/3 compared to the specific Bv alone (filled square), HS-Bv2/3 compared to HSr-Bv2/3 for the specific Bv (open diamond), and equivalent treatments (HS-Bv or HSr-Bv) compared between Bv2 and Bv3 (filled circle). Biological ($n = 3$) and technical ($n = 3$) replicates were performed

for HS-Bv2 was not different from that for the control, while that for HS-Bv3 was significantly induced compared to that for HS-Bv2 ($p < 0.05$) with HS-Bv3 itself significantly induced compared to the control ($p < 0.05$), Bv3 ($p < 0.05$), and HSr-Bv3 ($p < 0.05$). Levels of Hsp70/Hsc70, implicated as either a pro-survival or a pro-apoptotic marker, were then studied.

HS with or without recovery induced Hsp70/Hsc70 in *N. tabacum* protoplasts during subsequent exposure to avirulent *R. solanacearum*

HSP70 is the most highly induced stress protein family and is most commonly associated with protection. We quantified intracellular Hsp70/Hsc70 by flow cytometry to evaluate its possible role in protection during apoptosis or necrosis during host–pathogen interactions. Hsp70/Hsc70 was not induced by the calcium ionophore (A5 or A10), HSr, or Bv2 alone. However, Bv3 alone significantly induced Hsp70/Hsc70 compared to the control ($p < 0.05$). All the treatments with *R. solanacearum* given in conjunction with HS, whether with or without recovery, showed significant increases in Hsp70/Hsc70 levels compared to the control (HSr-Bv2, $p < 0.001$; HS-Bv2, $p < 0.05$; HSr-Bv3, $p < 0.05$; and HS-Bv3, $p < 0.01$) (Fig. 5). Hsp70/Hsc70 levels were also significantly increased in HSr-Bv2 compared to Bv2 alone ($p < 0.01$).

Fig. 5 HSr or HS induced Hsp70/Hsc70 in *N. tabacum* protoplasts during subsequent exposure to avirulent *R. solanacearum*. Hsp70/Hsc70 of *N. tabacum* was quantified by flow cytometry, staining fixed, permeabilized protoplasts, with primary Hsp70/Hsc70 antibody and FITC-labeled secondary antibody. The bar graph illustrates means with error bars of 95 % confidence intervals. Significant differences are indicated with one symbol ($p < 0.05$), two symbols ($p < 0.01$), or three symbols ($p < 0.001$) for treatments compared to control (C, asterisk) and HS-Bv2/3 and HSr-Bv2/3 compared to the specific Bv alone (filled square). Biological ($n = 3$) and technical ($n = 3$) replicates were performed

Hierarchical multiple regression models predict necrosis and e^- transport

As the outcome of a host–pathogen interaction is either host death (necrosis) or survival (e^- transport), we aimed to propose models predicting these outcomes (dependent variables), controlling for the influence of HSr. HMR was the method of choice as it determines which independent variable, entered in a stepwise manner, contributes the most to predict the dependent variable, over and above HSr. This is in contrast to standard multiple regression that cannot distinguish the individual contributions of the independent variables. We confirmed no violation of normality or multicollinearity of variables while ensuring moderate to strong correlation between the dependent and independent variables, excluding independent variables showing intercorrelation. Besides giving the overall R^2 value, HMR allows the evaluation of the change in R^2 (ΔR^2) to estimate how much predictive power was added to the model by the addition of another variable.

HMR was first conducted to determine if Hsp70/Hsc70 contributed to the prediction of necrosis over and above that accounted for by HSr. HSr was entered in step 1, and Hsp70/Hsc70 was entered in step 2. Results indicated that HSr explained 23.5 % of the variance in necrosis, $F(1,26) = 8.009$, $p = 0.009$. After entry of Hsp70/Hsc70, the total variance explained by the model as a whole was 53.7 %, $F(2,25) = 14.511$, $p = 0.000$. Hsp70/Hsc70 explained an additional 30 % of the variance in necrosis, $\Delta R^2 = 0.302$,

$F(1,25)=16.300$, $p=0.000$, over and above the variance accounted for by HSr. The regression equation for the final model using the unstandardized beta coefficients was as follows: Necrosis = $-5.488 \text{ HSr} - 0.039 \text{ Hsp70/Hsc70} + 17.728$.

We next used HMR to determine if DNA fragmentation contributed to the prediction of e^- transport over and above that accounted for by HSr. HSr was entered in step 1, and DNA fragmentation was entered at step 2. Results indicated that HSr explained 6.9 % of the variance in e^- transport, $F(1,22)=1.642$, $p=0.213$. After entry of DNA fragmentation, the total variance explained by the model as a whole was 27.9 %, $F(2,21)=4.062$, $p=0.032$. DNA fragmentation explained an additional 21 % of the variance in e^- transport, $\Delta R^2=0.209$, $F(1,21)=6.101$, $p=0.022$, over and above the variance accounted for by HSr. The regression equation for the final model with the unstandardized beta coefficients was as follows: e^- transport = $5.756 \text{ HSr} + 0.063 \text{ DNA fragmentation} + 88.625$.

Discussion

This study supports the hypothesis that a non-lethal HS, with recovery, promotes protection of *N. tabacum* during subsequent exposure to avirulent, necrotrophic pathogen, *R. solanacearum*. This host defense protection is accompanied by Hsp70/Hsc70 induction and reflected by increased apoptosis, DNA fragmentation, and protection of e^- transport.

HSr promotes protection of *N. tabacum* during subsequent exposure to avirulent *R. solanacearum*. HSr alone had no effect on e^- transport, but cells exposed to avirulent *R. solanacearum* after HSr (Fig. 2) appeared to have been primed to rapidly respond for a stronger and more effective plant defense response. Pre-treatment of parsley cells with various abiotic stresses has been shown to prime cells for a stronger defense response to low doses of MAMPs (Conrath et al. 2002). JA- and ET-dependent signaling pathways are also associated with priming for enhanced defense, and as necrotrophs are sensitive to JA and ET (Pieterse et al. 2009), this priming may be essential to protect the defense response during exposure to *R. solanacearum*. The protection by HSr is supported by the two apoptotic markers (PS externalization as measured by Annexin V/PI, Fig. 3, and DNA fragmentation as measured by TUNEL, Fig. 4). Apoptosis for HSr-Bv2 was similar to that for A5, the apoptotic control, and significantly higher than Bv2 alone (Fig. 3b). The avirulent pathogen activated the HR response, in line with the literature (Espinosa and Alfano 2004), and was enhanced by the HSr-facilitated priming. This would be advantageous to the host, eliminating necrotrophs via an enhanced apoptotic defense response. The induction of DNA fragmentation with the HSr-Bv2 (Fig. 4) confirms the occurrence of apoptosis. The question then begs what helps to enhance host apoptotic protection during this priming. The answer may lie with the induction of Hsp70/Hsc70 seen with

HSr-Bv2 (Fig. 5). A few studies support Hsp70 induction along with apoptosis (Ran et al. 2004; Zheng et al. 2004). The results support the protective role proposed for Hsp70/Hsc70 in an incompatible plant-pathogen interaction, protecting the mitochondria and supporting cell survival (Byth et al. 2001a) or at least death in a programmed manner (PCD). HSr alone did not induce Hsp70/Hsc70 compared to the control (Fig. 5), as HS generally does. This moderate HS likely primed Hsp70 transcription through the binding of trimerized HSF to the HSE preparing for Hsp70 protein translation (Morimoto 1998) prior to the secondary stress imposed by Bv2. Overexpression of HSF1 has been found to enhance Fas-induced apoptosis in HeLa cells (Xia et al. 2000). The protective effect of an increase in HSPs reduces cell death via chaperoning proteins and the folding, assembly, and intracellular translocation of newly synthesized proteins (Sung et al. 2001) as well as protects cells from stress-induced damage by refolding partially denatured proteins (Morimoto 1998). The heat-induced "pathotolerance" may relate to acquired thermotolerance (Vierling 1991), supporting a common or convergent response to heat and pathogen stress.

HS, alone, does not promote protection of *N. tabacum* during subsequent exposure to avirulent *R. solanacearum*. HS-Bv2 did not protect e^- transport and was significantly less than the control and similar to Bv2 (Fig. 2). This lack of protection by HS is supported by the Annexin V/PI and TUNEL apoptotic markers. HS-Bv2 promoted more normal cells at the expense of apoptotic and necrotic cell death (Fig. 3b). At first glance, this might be considered beneficial since suppressing HR-like PCD has been proposed to improve resistance to necrotrophs (Khurana et al. 2005). Though difficult to interpret, we propose that this lack of PCD reflects a delayed HR resulting from suppressed effector recognition and defense response in the plant (Alfano and Collmer 2004) resulting in necrotic death (Fig. 2). The host would benefit more from rapid, apoptotic elimination of the bacteria, as seen for HSr-Bv2, than preventing cell death all together. Suppression of host-PCD has been proposed as one of the strategies bacterial pathogens use for successful invasion (Kang et al. 2004). HS-Bv2 showed no induction of DNA fragmentation and was similar to Bv2. HS-Bv2 also induced Hsp70/Hsc70 (Fig. 5), but accompanied by significantly less apoptosis than HSr-Bv2 (Fig. 3b). Several groups have shown that Hsp70 is induced along with inhibition of apoptosis (Mailhos et al. 1993; Samali and Orrenius 1998; Mosser et al. 2000). This is supported in plants by Cronjé et al. (2004) where HS in combination with salicylic acid enhanced Hsp70/Hsc70, jeopardizing apoptosis. The rapid induction of HSPs is accompanied by decreased synthesis of most other proteins (Saltveit 1998), which is detrimental during incompatible interactions relying on defense protein synthesis. This reveals a hierarchical response to stress where the HS response has priority over a pathogen response (Schweizer et al. 1995). Resistance to HR-type necrosis was overcome in TMV-infected tobacco kept at high temperature (30 °C), as seen by decreased ROS, SA, and

PR proteins, leading to eventual virus replication and spread (Király et al. 2008). This highlights the importance of recovery after HS, prior to pathogen exposure, allowing chaperone production without jeopardizing defense protein production.

There seems to be no protection of *N. tabacum* by either HSR or HS following exposure to the virulent *R. solanacearum*. The trend of protected e^- transport was observed comparing HSR-Bv3 to Bv3 alone (Fig. 2), but as none of the Bv3 treatments differed from the control (Fig. 2), this protection seems minor. This probably reflects a delayed or absent recognition of the virulent pathogen effectors by the host (Jones and Dangl 2006), which will lead to ETS. The Annexin V/PI results for all Bv3 treatments showed a similar trend to those of the Bv2 treatments, although all the Bv3 treatments showed more necrosis (Fig. 3b). The increased apoptosis in HSR-Bv3 is probably due to the priming by HSR as mentioned for Bv2. This increased apoptosis, though, is not advantageous during virulent necrotrophic interactions that hijack host apoptotic

pathways (Grassmé et al. 2001; Hammond-Kosack and Rudd 2008) to colonize the host and cause necrosis (Govrin and Levine 2000; Romanenko et al. 2002; Alfano and Collmer 2004). The increased fraction of normal cells in HS-Bv3 may appear to be beneficial during a virulent necrotrophic interaction, similar to HS-Bv2, during which suppression of HR-like PCD or apoptosis is proposed to improve resistance (Khurana et al. 2005). This resistance would only be temporary because during the HS-Bv3 the HS response has priority over pathogen responses (Schweizer et al. 1995). A heat-related delay in the defense response makes the host vulnerable to infection and death. HS-Bv3 also induced DNA fragmentation (Fig. 4) which we surmise is due specifically to a synergy between HS and Bv3 possibly indicating damage as no protective PCD was launched. All Bv3 treatments induced Hsp70/Hsc70 (Fig. 5), accompanied by higher necrosis than their Bv2 counterparts (Fig. 3b), confirming HSPs' role as a biomarker of protein damage (Sung et al. 2001). Although there is no

Fig. 6 A diagram illustrating the proposed protection conferred by heat shock with recovery (HSr) in *N. tabacum* during subsequent exposure to avirulent *R. solanacearum*. In incompatible host–pathogen interactions, microbial- or pathogen-associated molecular patterns (M/PAMPs) are recognized by pattern recognition receptors (PRR) in the plant cell membrane, activating signaling pathways that lead to defense gene expression, i.e., M/PAMP-triggered immunity (M/PTI). As counter-attack, *R. solanacearum* transfers effector molecules directly into the host cytoplasm via the Hrp type III secretion system (TTSS) where they are recognized by host R-gene products, inducing effector-triggered immunity (ETI), associated with the hypersensitive response (HR). If

both M/PTI and ETI immunity fail, by PRR overlooking M/PAMP or by effector-triggered susceptibility (ETS), necrosis is the outcome. Heat shock with recovery before microbial/pathogen exposure likely primes heat shock factor (HSF) trimerization and DNA binding. Pathogen exposure following HSR induces the heat stress/shock protein Hsp70/Hsc70 (gray-filled circles). Hsp70/Hsc70 may promote host defenses through folding newly synthesized defense proteins, strengthening the cell wall, protecting e^- transport, and promoting apoptosis as reflected by PS externalization and DNA fragmentation. Hsp70/Hsc70 may reduce necrosis by preventing protein denaturation or removing denatured proteins in the event of necrosis

significant protection during the subsequent virulent exposure, HSr did significantly increase apoptosis and decrease necrosis compared to Bv3 alone, supporting an attempted PCD protection.

The HMR models predicting necrosis (death) and e^- transport (survival) support the overall finding of the study that HSr promotes protection of *N. tabacum* during subsequent exposure to *R. solanacearum*, by preventing necrosis and protecting e^- transport while inducing PCD. The first model predicts the prevention of necrosis by HSr which is strengthened when accompanied by Hsp70/Hsc70, while in the second model, HSr strengthened the prediction of e^- transport when accompanied by DNA fragmentation. DNA fragmentation and Hsp70/Hsc70 are both more likely markers of protection and apoptotic cell death, than markers of damage and necrosis in this scenario. This is supported by reports where Hsp70 is induced along with apoptosis (Ran et al. 2004; Zheng et al. 2004). These models do not exclude the possibility of other proteins induced by HSr prior to pathogen exposure, such as small HSPs that could play a role in supporting defense in *Nicotiana* plants as suggested in Maimbo et al. (2007). These regression models are based on cell suspension studies, and field trials would have to be done to verify their worth in the field.

Conclusion

In conclusion, these results support a protective role induced after a period of recovery, likely involving Hsp70/Hsc70, which was most notable in the defense response activated in *N. tabacum* by the avirulent biovar of the necrotroph, *R. solanacearum* (summarized in Fig. 6). Induction of Hsp70/Hsc70 in this scenario was accompanied by protection of e^- transport and increased apoptosis as measured by PS externalization and DNA fragmentation. This phenomenon has implications for defense responses to avirulent bacteria, which is overruled by concomitant HS (Hazen and Bushnell 1983; Malamy et al. 1992). This study aimed to illustrate on a cellular level what happens on a daily basis in nature during plant exposure to a combination of temperature and pathogen stress and emphasizes the importance of studying a combination of stresses where the eventual outcome is more than the sum of the individual stresses.

Acknowledgments This work was supported by the National Research Foundation, South Africa, through a grant awarded to Liza Bornman.

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