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Hsp70 and Hsp90 expression in citrus and pepper plants in response to *Xanthomonas axonopodis* pv. *citri*

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Abstract Hsp70 and Hsp90 expression in response to high and low temperatures was studied in orange, the host plant of *Xanthomonas axonopodis* pv. *citri* and in a non-host resistant plant, pepper. As expected in both plants, the expression of these chaperones was induced at high temperatures while at cold temperatures the response was chaperone and plant-dependent. Expression of Hsp70 and Hsp90 was analysed during citrus canker and no changes in their levels could be observed whereas pepper plants infiltrated with the phytopathogen showed an increase in the levels of both chaperones. These results suggest that no changes in Hsp70 and Hsp90 expression are necessary during the disease while they are increased in non-host resistance.

Keywords Citrus canker · Non-host resistance

Abbreviations

hpi	hours post-infiltration
HR	hypersensitive response
Hsp70	Heat-shock proteins of 70 kDa
Hsp90	Heat-shock proteins of 90 kDa
Hsps	Heat-shock proteins

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Heat-shock proteins (Hsps) are polypeptides highly conserved in all living organisms and cellular compartments examined to date. They are induced by heat shock, this being their main task to assist refolding of partially unfolded or denatured proteins occurring under elevated temperatures (Rutherford 2003). Heat-shock proteins of 70 kDa (Hsp70) are involved in stabilizing heat-labile proteins and preventing them from becoming irreversibly denatured or damaged. In *Arabidopsis thaliana* the variation in Hsp70 expression has been shown to lead to pleiotropic consequences on growth, development and thermotolerance (Sung and Guy 2003). Heat-shock proteins of 90 kDa (Hsp90) have been shown to have functions in folding, assembling and transporting proteins and are also involved in the activation of component proteins in signal transduction. It has been proposed that Hsp90 buffers genetic variation in nature by maintaining mutant proteins in wild-type conformations. When this buffering is compromised by temperature, stress differences are exposed allowing selection to adjust developmental processes (Rutherford and Lindquist 1998; Queitsch et al. 2002; Salathia and Queitsch 2007). In addition, Hsp90 has been implicated in plant immunity as it has been suggested that it acts physically close to resistance (R) proteins (Hubert et al. 2003).

Previous studies in plant–pathogen interactions have showed that *A. thaliana* Hsp90 (*Hsp81.1*) expression was induced in the compatible and incompatible interactions with *Pseudomonas syringae* pv. *tomato* (Takahashi et al. 2003) and analyses of microarray databases of *A. thaliana* leaves subjected to *Phytophtora infestans* revealed that Hsp90 expression is also induced in this non-host resistance (Swindell et al. 2007). Furthermore, it has been demonstrated that Hsp70 and Hsp90 are essential components of the hypersensitive response (HR) defence mechanism. Using viral-induced gene silencing to decrease Hsp70 and Hsp90 levels in *Nicotiana benthamiana*, Kanzaki et al. (2003) have shown that resistance is dependent on these two chaperones. The non-host resistance to *Pseudomonas cichorii* was compromised and no HR was induced in Hsp70 or Hsp90-silenced *N. benthamiana* plants, whereas strong HR was induced in the control plant (Kanzaki et al. 2003). The current hypothesis, supported by the fact that resistance mediated by R genes is dependent on Hsp90 function (Lu et al. 2003; Liu et al. 2004; Kanzaki et al. 2003), proposes that in the absence of pathogens, R proteins are stabilized and functionally silenced by intramolecular interactions and that upon infection, R proteins acquire a destabilized, degradation-sensitive conformation that allows subsequent signalling (Sangster and Queitsch 2005). Since changes in Hsp70 and Hsp90 expression may be compromised in processes such as disease or non-host resistance during plant–pathogen interactions, we analysed their expression in previously untested plant–pathogen interactions.

The phytopathogen *Xanthomonas axonopodis* pv. *citri* is the bacterium responsible for citrus canker, a disease that appears as raised necrotic corky lesions on leaves, stems, and fruits that reduce fruit quality and quantity (Brunings and Gabriel 2003). Since no resistance to this pathogen has been found either in citrus plants or in other members of the *Rutaceae* family and as *X. axonopodis* pv. *citri* induces non-host resistance with a HR in cotton, tobacco, tomato, bean, and pepper (Dunger et al. 2005), we used pepper as an example of non-host resistance to the bacterium. In that context we wondered if the non-host resistance with a HR to *X. axonopodis* pv. *citri* compromises Hsp70 and Hsp90 expression changes involved in signalling mechanisms of the defence response upon pathogen recognition. Accordingly, Hsp70 and Hsp90 expression in citrus and pepper plants under different temperatures as well as in citrus canker and in the resistance of non-host plant pepper to *X. axonopodis* pv. *citri* was analysed.

First, we analysed the expression of Hsp70 in orange and pepper in high and low temperatures. Orange (*Citrus sinensis* cv. valencia) and pepper (*Capsicum annuum* cv. grossum) plants grown in a greenhouse at 23–25°C with a photoperiod of 18 h were submitted to 37°C and 47°C for heat shock stress (Fig. 1a,b) and to 5°C and 15°C for cold shock stress (Fig. 1c,d). After treatments for 30, 60, 120 and 240 min protein extracts were prepared for each sample in 50 mM Hepes–KOH buffer pH 7.5, 330 mM sorbitol, 5 mM sodium ascorbate, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM MnCl2 and 0.33 mM PMSF in a ratio 1:2 (*w*/*v*) and analysed for total protein content (Sedmak and Grossberg 1977), using bovine serum albumin as the standard. A 30 μg aliquot of total proteins was run in 10% SDS polyacrylamide gels (Laemmli 1970) and transferred to nitrocellulose membranes. Western blotting was performed using a monoclonal human anti-Hsp70 (StressGen, Canada) that also reacts against plant cytosolic Hsp70s and then an anti-antibody conjugated to alkaline phosphatase. Hsp70 bands were visualised by an enhanced chemifluorescence detection system and band quantification was performed using Gel-Pro Analyzer Software 3.1 (Media Cybernetics; Fig. 1). In both plants, the heat stress treatment provoked an increase in Hsp70 expression of at least two-fold evident at 120 min of heat exposure and maintained at 240 min. In the treatment at 47°C Hsp70 expression increased more than three-fold in orange and six-fold in pepper at 240 min, consistent with the higher temperature of exposure. Differences in Hsp70 expression levels between species have been previously observed in other studies. A comparative study of Hsp70 expression levels in two leguminous plants showed that Chilean algarrobo has higher levels of induction at elevated temperatures than soybean (Ortiz and Cardemil 2001), while an exhaustive expression analysis of all the members of the Hsp70 family in spinach and tomato showed that the level of induction of Hsp70 of each member is variable in each specie (Li et al. 1999). In addition, the differences in Hsp70 expression levels observed in orange and pepper may be due to the fact that orange is a subtropical species with an optimal growth

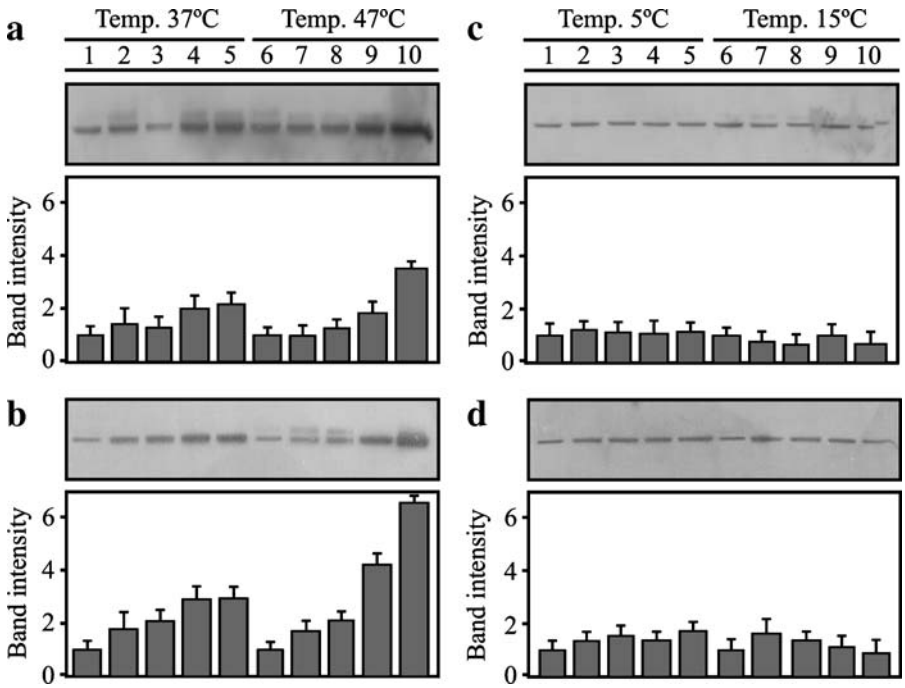

Fig. 1 Expression analyses of Hsp70 in orange and pepper plants in response to high and low temperatures. Western-blot analysis for cytosolic Hsp70 in orange (**a**, **c**) and pepper plants (**b**, **d**) after 0–4 h incubation at different temperatures: 37°C, 47°C (**a**, **b**) and 5°C, 15°C (**c**, **d**). Thirty micrograms of total protein were separated by SDS-PAGE, electrotransferred and incubated with antibodies directed against Hsp70. *Lanes 1* and *6*, 0 min; *2* and *7*, 30 min; *3* and *8*, 60 min; *4* and *9*, 120 min; *5* and *10*, 240 min. The relative amounts of protein were determined from the band intensity in the gel blot and are represented in the graphics under each blot. The experiments were repeated three times with similar results. In each case *bars* represent means of the three experiments and *error bars* represent standard deviation

temperature between 25°C and 30°C while pepper is between 20°C and 25°C. Moreover, orange plants with temperatures above 35°C only show reduction in fruit size whilst pepper undergoes flower falling. Thus, we suggest that high temperature is more detrimental for pepper than for orange plants and accordingly, pepper leaves might require more Hsp70 expression than orange to deal with protein instability caused by thermal stress. However, the cold stress treatment did not produce changes in the levels of Hsp70 expression either at 15°C or at 5°C for the two plants studied (Fig. 1c,d). These results are in agreement with those previously observed for spinach. Ten Hsp70 family members were examined and all of them exhibited increased RNA levels after 1 h of heat shock. However, the response to low temperature was variable. Though, in general, most of the family members were induced, others were transiently up-regulated, while others showed sustained up-regulation at a low temperature (Li and Guy 1998; Li et al. 1999). Another report on the expression of the Hsp70 family of *A. thaliana* gives a complete analysis for 11 Hsp70s. The overall response of the Hsp70s evaluated showed induction by heat shock treatment while the expression profiles in response to low temperature treatment, although induced, were more diverse than those evoked by heat shock treatment (Sung et al. 2001). However our results in orange and pepper showed no Hsp70 induction at low temperatures. This may be attributed to the fact that we could not differentiate between cytosolic Hsp70 family members and this may be obscuring what happens with individual members, even though the overall response did not show any change among Hsp70 expression in the time-course for both low temperatures studied.

Expression analyses of Hsp90 in citrus and pepper plants were carried out at the higher (47°C; Fig. 2a,b) and the lower (5°C; Fig. 2c,d) temperatures. In this case, as we could not express Hsp90 (*hsp81.1*) from an *A. thaliana* cDNA clone in *Escherichia coli* successfully (as previously reported for the barley variant, Takahashi et al. 2003) to produce antibodies, we performed an RT-PCR assay. To this end we used

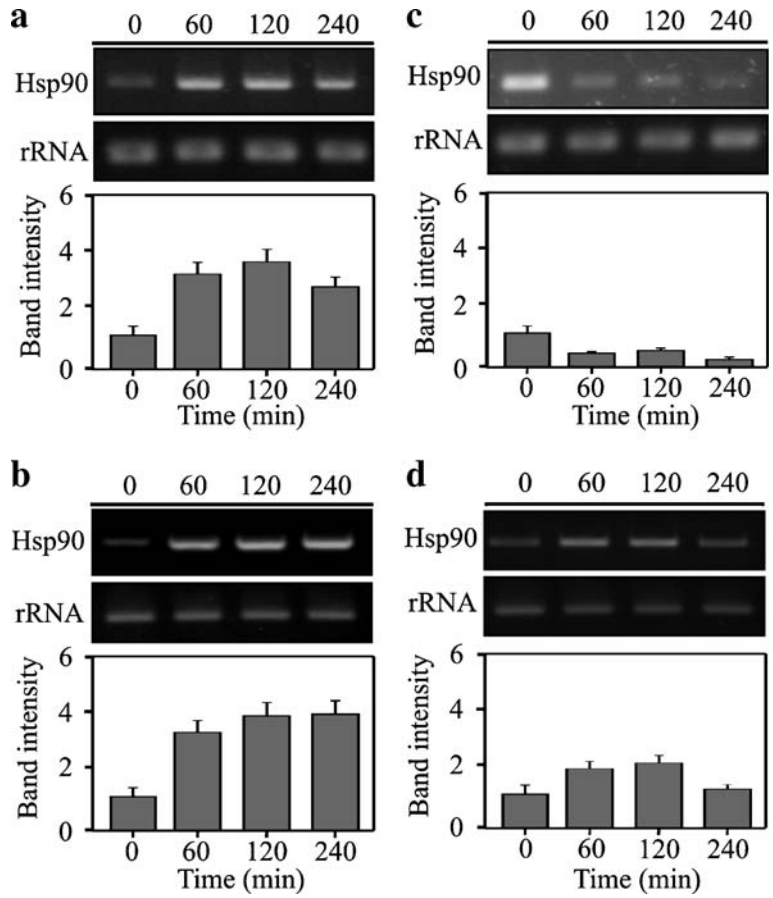

Fig. 2 Expression analyses of Hsp90 in orange and pepper plants in response to high and low temperatures. Orange (**a**, **c**) and pepper (**b**, **d**) plants were subjected to two different thermal treatments: 47°C (**a**, **c**) and 5°C (**b**, **d**). As control, samples were subjected to RT-PCR using 18S rRNA primers. The graphics below show band intensity relative to 0 h. The experiments were repeated three times with similar results. In each case *bars* represent means of the three experiments and *error bars* represent standard deviation

oligonucleotides designed on the conserved regions of cytosolic plant Hsp90 sequences available in order to analyse cytosolic Hsp90 expression in orange and pepper (Hsp90up: 5′-GACAATTTGGTGTTGGTT TCTA-3′ and Hsp90down: 5′-AGCTCTTCACAG TTGTCCAT-3′). After treatments at 47°C and 5°C RNA samples from leaves were prepared at 0, 60, 120 and 240 min using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Samples were then subjected to reverse transcription using the M-MLV Reverse Transcriptase (Promega) and PCR reaction (25 cycles of 94°C (1 min), 58°C (1 min) and 72°C (2 min)) followed by incubation at 72°C for 5 min. PCR products were electrophoresed in a 1% agarose gel and photographed with FOTO/Analyst®. Investigator Eclipse® (Fotodyne) and Gel-Pro Analyzer Software 3.1 (Media Cybernetics) were used to measure the intensity of each band. The heat stress treatment induced an increase in the Hsp90 expression after the first 60 min of temperature stress for both plants, showing values up to four-fold at 120 min for orange and at 240 min for pepper (Fig. 2a,b). In contrast, the cold stress treatment in orange plants produced a reduction in the levels of Hsp90 of almost three times at 60 min that was sustained for the 240 min period studied (Fig. 2c) and a two-fold increase in Hsp90 expression in pepper plants at 60 min, that returned to control values at 240 min. Several studies on Hsp90 expression including maize, rice, and *A. thaliana* showed induced accumulation of Hsp90 mRNA in response to heat (Marrs et al. 1993; Liu et al. 2006; Milioni and Hatzopoulos 1997). However studies on Hsp90 expression in response to cold stress are variable

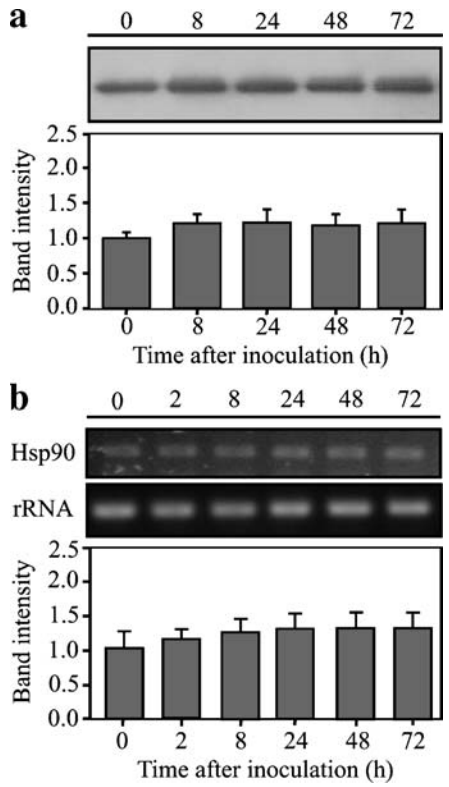

Fig. 3 Expression of Hsp70 and Hsp90 in host plants in citrus canker. *Xanthomonas axonopodis* pv. *citri* was inoculated in *C. sinensis* plants. Leaves were detached from the plant at different times post-inoculation for protein and RNA extraction. (**a**) Equal amounts (30 μg) of protein were separated by SDS-PAGE, transferred onto nitrocellulose and incubated with antibodies directed against Hsp70. (**b**) RT-PCR were carried out with 1 μg of total RNA using Hsp90-specific primers. As control, samples were subjected to RT-PCR using 18S rRNA primers. Graphics below show band intensity relative to 0 h. The experiments were repeated three times with similar results. In each case *bars* represent means of the three experiments and *error bars* represent standard deviation

and depend on the plant and tissue evaluated (Krishna et al. 1995). In this context, and as we did not observe an increase in Hsp90 mRNA expression at cold temperature, we could not rule out the possibility that at control temperatures, the Hsp90 expression level is enough to cope with the requirements of this chaperone at low temperatures at initial periods of the cold stress, probably increasing later in the acclimation process.

Once we established that Hsp70 and Hsp90 expression responds to temperature increase, we evaluated their expression in orange leaves during citrus canker. We cultured *X. axonopodis* pv. *citri* in SB broth to OD600 of 1, harvested by centrifugation, and resuspended in 10 mM $MgCl_2$ at a concentration 107 cfu ml^{-1} as previously described (Dunger et al. 2007). Bacterial suspension was infiltrated into orange leaves with needleless syringes. At 2, 8, 24, 48 and 72 h post-infiltration (hpi), pulverised leaves were frozen and protein extracts or RNA samples were prepared. At 72 hpi canker symptoms began to appear and one week after infiltration typical canker lesions on citrus leaves were observed. For proteins extracts, samples were prepared and subjected to SDS-PAGE and Western blotting, while RNA samples were subjected to RT-PCR as described above. Hsp70 and Hsp90 expression levels were similar regardless of the time after inoculation at which the leaves were harvested (Fig. 3a,b). These results indicate that during the compatible interaction pro-

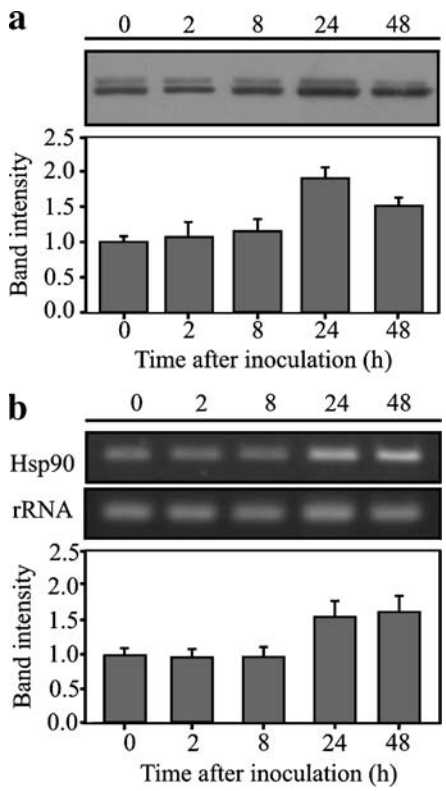

Fig. 4 Expression of Hsp70 and Hsp90 in non-host resistance. *Xanthomonas axonopodis* pv. *citri* were inoculated in pepper plants. Leaves were detached from the plant at different times post-inoculation for protein and RNA extraction. (**a**) Western-blot with antibodies against Hsp70. (**b**) RT-PCR with Hsp90-specific primers. The graphic under each blot represents the band intensity relative to 0 h. The experiments were repeated three times with similar results. In each case *bars* represent means of the three experiments and *error bars* represent standard deviation

duced between orange and *X. axonopodis* pv. *citri* no changes in the expression of these molecular chaperones take place, suggesting that if any member of the Hsp70 or Hsp90 families has a function in disease development, changes in their overall expression are not involved in the response.

To test the expression of Hsp70 and Hsp90 during HR we infiltrated *X. axonopodis* pv. *citri* in pepper leaves. At 2, 8, 24 and 48 hpi, protein extracts were obtained as described above and samples subjected to SDS-PAGE and Western blotting using antiHsp70 antibodies. Hsp70 expression displayed an induction of about two-fold at 24 hpi and then decreased slightly at 48 hpi (Fig. 4a). Hsp90 expression was analysed by RT-PCR and after bacterial inoculation on pepper leaves, samples were taken at 2, 8, 24 and 48 hpi as described above for orange leaves. An augment of 50% in Hsp90 expression was observed after 24 hpi and maintained until 48 hpi (Fig. 4b). These results are in agreement with the increase observed for the Hsp70 expression (Fig. 4a) suggesting that both chaperones might be coordinated in the non-host resistance with HR. Moreover, in the non-host interaction between *A. thaliana* and *P. infestans*, Hsp70 and Hsp90 expression were induced almost to the same extent both increasing about 70% compared to non-challenged controls (Swindell et al. 2007). In view of these results and our results we might suggest that similar levels of Hsp70 and Hsp90 expression are required in non-host plant–pathogen interactions.

In summary, in addition to corroborating the induction of Hsp70 and Hsp90 expression during heat shock in orange and pepper plants, our results demonstrate that changes in the expression of these chaperones are taking place in plant–pathogen interactions. Although no changes in Hsp70 and Hsp90 expression were observed in the pathogenic process in citrus plants, and even if the increase in the expression of heat shock proteins was less than in high temperature treatments, an induced expression for Hsps in the non-host resistance of pepper to *X. axonopodis* pv. *citri* was found, suggesting an involvement of Hsp70 and Hsp90 in the non-host resistance with a HR, probably in the regulation of protein stability and the signalling process.

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