Effect of Extreme Temperatures on Powdery Mildew Development and Hsp70 Induction in Tomato and Wild *Solanum* spp.

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Abstract

Kubienová L., Sedlářová M., Wünschová A., Piterková J., Luhová L., Mieslerová B., Lebeda A., Navrátil M., Petřivalský M. (2013): **Effect of extreme temperatures on powdery mildew development and Hsp70 induction in tomato and wild** *Solanum* spp. Plant Protect. Sci., **49** (Special Issue): S41–S54.

Changes in *Hsp70* gene expression and protein level were studied in three *Solanum* spp. genotypes in response to short-term high and low temperatures and to infection by powdery mildew. Development of *Oidium neolycopersici* was compared on plant leaves and leaf discs with regard to the influence of temperature. Heat and especially cold pre-treatment of host tissues inhibited pathogenesis and decreased chlorophyll concentration. Exposure to heat increased Hsp70 (70 kDa heat shock proteins) content in all three genotypes of *Solanum* spp., whereas the infection induced the accumulation of Hsp70 only in susceptible *S. lycopersicum*. These results are in accordance with the suggested role of Hsp70 chaperons in plant responses to metabolic pathway disturbances triggered by pathogen challenge.

Keywords: heat shock proteins; *Oidium neolycopersici*; real-time PCR; Western blot

Abbreviations: GAPDH – glyceraldehyde-3-phosphate dehydrogenase; hpi – hours post inoculation; HR – hypersensitive response; Hsp – heat shock protein; HSR – heat stress response; qPCR – quantitative real-time polymerase chain reaction; SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Plants are exposed to a variety of abiotic stress factors, such as low and high temperatures, salinity, heavy metals, and UV radiation, and biotic stress factors, such as herbivores and pathogens (Vierling 1991; Smirnoff 1998; Pastori & Foyer 2002). Plant exposure to stress factors often results in accumulated irreversible damage to cell components like proteins, membrane lipids, and nucleic acids, and can finally result in the cell death. Specific groups of proteins are synthesised *de novo* and accumulated in response of plant cells to stress conditions. A large family of proteins known to play an important role during various stresses has been designated as "heat shock proteins" (Hsps), also named as "stress-induced proteins" or "stress proteins" (Lindquist & Crig 1988; Morimoto *et al*. 1994; Gupta *et al*. 2010). Initially, Hsps were identified as proteins strongly induced and accumulated by high temperature stress (Ritossa 1962). Their production increases in plants that experience an abrupt or gradual increase of temperature (Nakamoto & Hiyama 1999), although some Hsps are induced also by low temperatures (Neta-Sharir *et al*. 2005). Hsps are highly conserved among higher plants and produced during all ontogenetic phases (Vierling 1991). Five main

Supported by the Ministry of Education, Youth and Sports of the Czech Republic, Project No. MSM6198959215, and by the Czech Scientific Foundation, Grants No. GD522/08/H003 and No. GAP501/12/0590.

classes of Hsps have been distinguished in plants according to their approximate molecular weight: Hsp100 (Clp) family, Hsp90 family, Hsp70 (DnaK) family, Hsp60 and GroEL/GroES complex (chaperonins), and small heat-shock proteins (sHsps) of 15–30 kDa (Schlesinger 1990; Schöffl *et al*. 1998; Kotak *et al*. 2007; Wahid *et al*. 2007). Hsps of molecular weight ranging from 10 kDa to 200 kDa are known as chaperones and they form part of signal transduction pathways under heat and other stresses (Schöffl *et al*. 1998). Individual groups of Hsp were shown to co-operate in the protection of cell functions (Wang *et al*. 2004).

ATP-dependent Hsp70 chaperones, originally discovered in *Drosophila* as "puffing pattern" (Ritossa 1962, 1996), are known to be associated with numerous cellular roles like protein translation and translocation, protein folding or chaperoning, suppression of aggregation and re-activation of denatured proteins (Zhang *et al*. 2005; Hubert *et al*. 2009). Plant Hsp70s, together with their co-chaperones DnaJ/Hsp40 and GrpE, are localised in the cytosol, endoplasmic reticulum, mitochondria, chloroplasts, and peroxisomes (Vierling 1991; Boston *et al*. 1996; Bukau & Horwich 1998). The members of the 70-kDa Hsp family are most commonly associated with the cellular defence against high temperatures and their accumulation thus confers thermotolerance (Vierling 1991), protection against oxidative stress (Banzet *et al*. 1998), and inhibition of apoptosis through the prevention of DNA fragmentation (Cho & Choi 2009). A general protective role of Hsp70 was demonstrated in *Arabidopsis thaliana* mutants deficient in Hsp70 synthesis which exhibited higher sensitivity to the heat injury (Burke 2001). It was proposed that Hsps protect cells from the injury and facilitate recovery and survival after the return from stress to normal growth conditions (Morimoto & Santoro 1998), whereas Iba (2002) hypothesised that Hsp70 participates in ATP-dependent protein refolding or assembly/disassembly and thus prevents proteins from the heat denaturation.

In plants, multiple Hsp70 proteins have been identified in different species so far (Vierling 1991). The *Arabidopsis* genome contains at least 18 genes encoding members of the Hsp70 family (Lin *et al*. 2001; Sung *et al*. 2001) and at least 12 Hsp70 members have been found in the spinach genome (Guy & Li 1998). The role of Hsp70/Hsc70 (70-kDa heat-shock cognate) in responses to temperature stress was investigated in several plant models (Swindell *et al*. 2007; Zhang *et al*. 2009; Li *et al*. 2011). The cold tolerance of plant species seems to be influenced by the climate in the area of the plant origin. For example, several subtropical plant species like *Solanum* spp. are chill-tolerant and survive low temperatures between 2°C and 15°C, however they are freezing sensitive. High altitude accessions of *S. habrochaites* harbour the superior tolerance to low temperatures (Venema *et al.* 2005). The symptoms of low temperature injuries include the cessation of growth, wilting, chlorosis, and necrosis. The induction of Hsp70s in heat-exposed plants is mediated by heat shock transcription factors (HSFs) localised in the cytoplasm and corresponding heat shock elements (HSEs) in the promoters (Kregel 2002). Most plant Hsp70s show strong and rapid accumulation following 30-min to 2-h exposure to 37–45°C (Li *et al*. 1999; Sung *et al*. 2001). Increased levels of several plant Hsp70s are also induced by cold shock (Li *et al*. 1999). In tomato and spinach, cold-inducible Hsp70s show two induction peaks in 12 and 48 h at 5°C (Guy & Li 1998).

To study the mechanism and the importance of Hsp70 induction by abiotic and biotic stresses, we used the model pathosystem of *Solanum* spp.–*Oidium neolycopersici*. Three *Solanum* spp. genotypes with a different level of resistance to powdery mildew were used, i.e. susceptible *S. lycopersicum* cv. Amateur, moderately resistant *S. chmielewskii*, and highly resistant *S. habrochaites* (Mieslerová *et al*. 2004). Both accessions of *S. chmielewskii* and *S. habrochaites* expressed a typical hypersensitive response, characterized by the death of attacked cells, after plant inoculation with *O. neolycopersici* (Mlíčková *et al.* 2004). These genotypes also differ in their tolerance to low temperatures: the production of viable pollen was higher in *S. habrochaites*, while it moderately decreased in *S. chmielewskii*. *S. lycopersicum,* cultivated tomato accession, expressed poor viability of pollen below 10°C and is considered as cold-sensitive (Fernandez-Muñoz *et al.* 1995).

We evaluated the correlation of the phenotypic expression of host-pathogen interactions with the degree of Hsp70 expression under conditions of cold/heat stress. We were interested not only in the level of Hsp accumulation, but also in the timing of Hsps mRNA expression and protein formation. Our experimental set-up intended to simulate environmental conditions when the influence of abiotic and biotic stress factors on plants is often

combined. Our findings show that different ecological origin and powdery mildew resistance of *Solanum* spp. genotypes are linked to different Hsp70 levels under stress conditions.

MATERIAL AND METHODS

Plant material. Three genotypes of *Solanum* spp. with different degree of resistance to the tomato powdery mildew (*Oidium neolycopersici*) were used: highly susceptible *Solanum lycopersicum* L. cv. Amateur, moderately resistant *S. chmielewskii* (C.M. Rick, E. Kesicki, J.F. Forbes & M. Holle) Spooner, Andreson and Jansen (LA 2663), and highly resistant *S. habrochaites* S. Knapp and D.M. Spooner f. *glabratum* (LA 2128) (Mieslerová *et al*. 2004). Seeds of *S. lycopersicum* cv. Amateur were provided by the Research Institute of Crop Production, Gene Bank Division, Olomouc (Czech Republic). Seeds of *S. habrochaites* and *S. chmielewskii* were obtained from Tomato Genetics Resource Center, University of California, Davis (USA). Seeds were sown on moistened Perlite (Agroperlite, Nový Jičín, Czech Republic) and 1-week old seedlings were transferred into a garden soil-peat mixture (2:1, v/v) in plastic pots (7 cm in diameter). Plants were grown in a growth chamber at 20/18°C and 12/12 h day/night photoperiod (light intensity 100 µmol/m^2/s) for 8 weeks.

Pathogen isolate. *Oidium neolycopersici* Kiss (isolate C-2) from the collection of the Department of Botany, Palacky University in Olomouc, included in the Czech National Collection of Microorganisms (collection No. UPOC-FUN-127) was used for the experiments (Piterková *et al.* 2009). The pathogen was maintained and multiplied on plants of susceptible *S. lycopersicum* cv. Amateur aged 8–10 weeks, grown under plastic covers in a growth chamber at 20/18°C, 12/12 h day/night photoperiod and light intensity of 100 µmol/m^2/s.

Procedures of temperature stress application. Plants aged approximately 8 weeks were transferred to a cultivation box (SANYO E&E Europe BV, Etten-Leur, the Netherlands) with 12/12 h photoperiod (light intensity of 100 µmol/m^2/s) and constant temperature of 4, 10, 25 or 40.5°C, respectively. Control plants were placed in a growth chamber with 12/12 h photoperiod (light intensity of 100 µmol/m^2/s) and day/night temperature of 20/18°C. All experiments were initiated at 9 a.m. to maintain the circadian rhythm and to avoid the influence of day/time changes on the plant metabolism. Leaves from control and treated plants of each genotype were collected separately 1, 4, and 24 h after the transfer to cultivation boxes, immediately frozen in liquid nitrogen and stored at –80°C.

Inoculation, incubation and sample collection. Freshly sporulating mycelium of *O. neolycopersici* (C-2), approx. 8 days after subcultivation, was used for the inoculation of plants. The 4th true leaves of plants or leaf discs cut off the leaves (12 mm in diameter) were inoculated by a surface contact (dusting/tapping) with mildew-covered leaves. Both control and inoculated plants were incubated in a growth chamber at 20/18°C and 12/12 h day/night photoperiod (Lebeda & Mieslerová 2010). Leaves were collected at 4, 8, 24, and 72 h post-inoculation (hpi), immediately frozen in liquid nitrogen and stored at –80°C until studied. The preparation of the material for microscopic assessment was performed as described elsewhere (e.g. Mieslerová *et al*. 2004).

All experimental procedures were repeated as three independent biological replicates, from growing of plants to data collection. In each individual experiment, leaves from three corresponding plants were collected for each data point.

Development of *Oidium neolycopersici.* Leaf discs of 12 mm in diameter were collected or freshly prepared from inoculated plants 24 and 48 hpi, immersed in 100% acetic acid for 48 h, and later mounted in glycerol. Prior to the light microscopy (Olympus BX60 equipped with CCD camera Olympus DP70; Olympus C&S, Prague, Czech Republic), plant tissues were stained with 1% cotton blue (e.g. Piterková *et al*. 2011). For each treatment the ratio of germinated conidia and the number of germ tubes per conidia were evaluated on five leaf discs per each time interval. A minimum of 150 conidia, randomly selected on leaf discs, were evaluated per each treatment and time interval. Values are presented as mean and standard deviation.

RNA extraction and cDNA preparation. Total RNA from 100 mg of leaves was extracted using a TRIZOL Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). cDNA was synthesized from 1 µg of total RNA using oligo(dT)$_{15}$ primer and AMV transcriptase (2 units; Promega, Madison, USA). The reaction mixture was incubated at 42°C for 60 min followed by heat inactivation of reverse transcriptase by incubation at 70°C for 5 minutes.

Primer design, PCR product identity and sequencing. The gene sequence of Hsp70 of *S. lycopersicum* is available in the public GenBank database under the accession number EU195057.1 (http://www.ncbi.nlm.nih.gov/). Primer pairs for PCR and RT–PCR were designed using DNASTAR tool (DNASTAR Inc., Madison, USA). The following primers were used for *Hsp70* (fwd: 5'-GAGAAGCCATAGACGCGAAGAATCAA-3', rev: 5'-GTACCAGCACCAGGAGAAGGACCAG-3') and for reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (fwd: 5'-AACCGGTGTCTTCACTGACAAGGA-3', rev: 5'-CACCCACAACAAACATGGGAGCAT-3') to amplify 268 bp (Hsp70) and 110 bp (GAPDH) fragments.

Phire Plant Direct PCR Kit (Finnzymes, Espoo, Finland) was used to control the PCR product identity. The temperature profile was 94°C for 2 min; 35 times 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 7 minutes. PCR products were separated by 2% agarose gel electrophoresis. Bands with PCR products were cut out of the gel and the obtained DNA was extracted using a Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced (MacroGen Europe Laboratory, Amsterdam, the Netherlands).

Quantitative real-time polymerase chain reaction (qRT-PCR). Quantitative RT-PCR was performed in two steps: cDNA was prepared as described above and real-time PCR amplification was performed using an ABsolute SYBR Green ROX Kit (ABgene Ltd., Epsom, UK). The amplification of target genes and real-time detection of amplicon production were monitored on an Mx3000P QPCR System (Stratagene, La Jolla, USA). The SYBR Green fluorescent signal was standardized with an internal passive reference dye (1mM ROX) included in the SYBR Green PCR mix. The following program was applied: initial DNA polymerase activation at 95°C for 15 min, then 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 seconds. The specificity of the PCR amplification was checked with a melting curve program 55–95°C following the final cycle of the PCR. PCR conditions were optimised for high amplification efficiency > 95% for all primer pairs used. PCR reactions in the absence of template were also performed as negative controls for each primer pair. Pfaffl's method was applied for relative quantification of gene expression normalised to the housekeeping gene GAPDH (Pfaffl 2001). Results are presented as the means of three independent experiments at least.

Preparation of plant extracts and quantification of Hsp70 protein by Western blot. For Hsp analysis, leaves were homogenised at a 1:2 (w/v) ratio with 75mM Tris-HCl buffer, pH 7.0, containing 4.6% (w/v) SDS, 6.6% (v/v) glycerol and 0.007% (w/v) bromophenol blue. Extracts were centrifuged at 16 000 *g* at 4°C for 10 min and supernatants were stored at –80°C until used.

Proteins from leaf extracts were separated by SDS-PAGE using 4% stacking and 7% separating polyacrylamide gel. After the electrophoresis, proteins were transferred from gels onto nitrocellulose membranes by tank Western blotting. Membranes were blocked overnight with 3% gelatine in TBS, incubated for 2 h with monoclonal mouse anti-Hsp70 antibody (Sigma-Aldrich, St. Louis, USA) in 1% gelatine in TBS, washed extensively with Tween-20 solution in TBS and finally incubated for 2 h with anti-mouse IgG antibody conjugated with alkaline phosphatase (Sigma-Aldrich, St. Louis, USA) in 1% gelatine in TBS. This antibody has been confirmed to recognise plant Hsp70 proteins (Wimmer *et al*. 1997). Membranes were stained for alkaline phosphatase activity with nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (BCIP/NBT solution; Sigma-Aldrich, St. Louis, USA). The amount of Hsp was estimated from a densitometric analysis of stained membranes on a BioSpectrum imaging system (UVP, Cambridge, UK). Quantification of Hsp70 content in plant extracts was performed using a calibration curve obtained by the analysis of known amounts of the commercial standard of Hsp70 protein from the bovine brain (Sigma-Aldrich, St. Louis, USA). Commercial Hsp70 protein was also always applied in the same amount (1 µg per well) to each gel as an internal standard.

Spectrophotometric determination of chlorophyll content. The procedure was carried out at 4°C and dark. Leaf samples (0.1 g) were homogenised and extracted with 80% acetone (v/v). The extract was filtered through a filter paper and centrifuged at 5000 *g* at 4°C for 5 minutes. The supernatant was collected and the absorbance was determined at 663 and 647 nm. Concentrations of chlorophyll *a* and chlorophyll *b* were calculated in µg/ml extract solution according to the equations of Lichtenthaler and Buschmann (2001):

$$\text{Chlorophyll } a = 12.25\ A_{663} - 2.79\ A_{647}$$

$$\text{Chlorophyll } b = 21.50\ A_{647} - 5.10\ A_{663}$$

Statistical analysis. The statistical significance of the treatment differences was assessed using Student's *t*-test. The presented data are expressed as mean ± standard deviations of values obtained in three independent biological experiments of plant cultivation and stress treatment of plant leaf discs.

RESULTS

Our study reports differential changes in Hsp70 expression and protein accumulation induced by powdery mildew infection, caused by *Oidium neolycopersici,* combined with heat and cold stresses in three species of the genus *Solanum* (*Solanum lycopersicum* cv. Amateur, *Solanum chmielewskii*, and *Solanum habrochaites*). The three above-mentioned species were selected due to their different susceptibility and resistance to *O. neolycopersici* and their sensitivity and tolerance to extreme temperatures (Mieslerová *et al*. 2004). Traditionally, the development of tomato powdery mildew has been studied microscopically on leaf discs kept in temperature conditions optimal for mildew development (Mieslerová *et al*. 2004). Herein, we compare leaf discs with the whole potted plants to deepen our knowledge of plant responses to stresses in the environment.

Development of *Oidium neolycopersici* on leaf discs and whole plants of *Solanum* spp.

Dynamics of *O. neolycopersici* germination on detached leaf discs and potted plants was assessed during 48 hpi. Data for the resistant species, *S. habrochaites*, with only very limited growth of the pathogen, are not shown. In general, the powdery mildew development, the percentage of germinated conidia as well as the number of germ tubes per conidia, is usually more intensive on leaf discs compared to plants (Figures 1–3). The pathogen growth on leaf discs of *S. lycopersicum* is faster at an optimal temperature of 20°C whereas it is slowed down in the cold pre-treated tissues compared to potted plants. On the other hand, neither on leaf discs nor on whole plants the heat pre-treatment influenced significantly the dynamics of germ tube formation. The significant retardation of pathogen development on *S. chmielewskii* due to cold pre-treatment was recorded only at 24 hpi on leaf discs and at 48 hpi on potted plants. Pre-

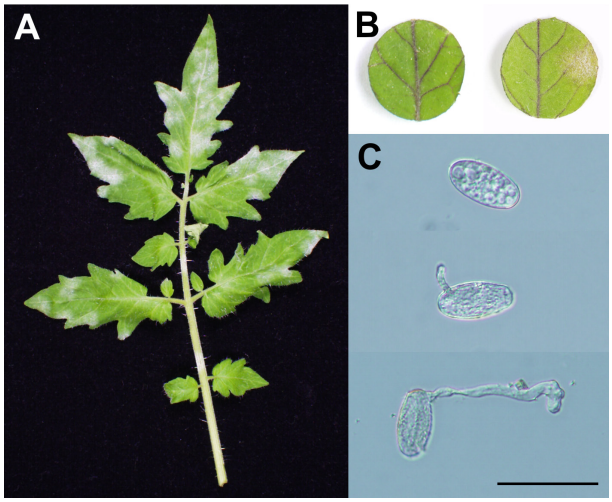

Figure 1. Symptoms of tomato powdery mildew on *S. lycopersicum* cv. Amateur: (**A**) leaf, (**B**) leaf discs 8 h (left), and 72 h (right) post inoculation with *O. neolycopersici*, and (**C**) microphotographs of conidium germination and formation of an appressorium at the apex of the germ tube. Bar corresponds to 50 µm

treatment with heat shock (40.5°C) had no influence on the formation of *O. neolycopersici* germ tubes compared to the control (20°C) at 24 hpi. On the other hand, its effect differed at 48 hpi, i.e. heat shock induced the formation of germ tubes on leaf discs while it reduced germ tubes on plants (Figure 3).

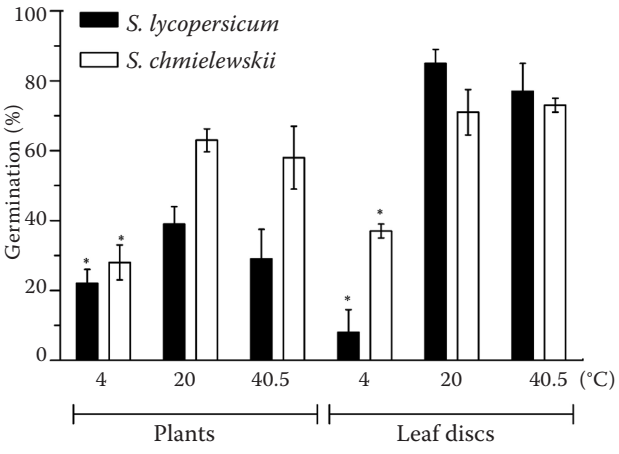

Figure 2. Relative rate of *O. neolycopersici* conidia germination on plants and leaf discs of *Solanum* spp. exposed to cold and heat treatment 24 hpi

Results are given as a mean (n = 9) ± SD, and the values statistically different from corresponding control values of plants or discs incubated at 20°C are marked as * (P = 0.05)

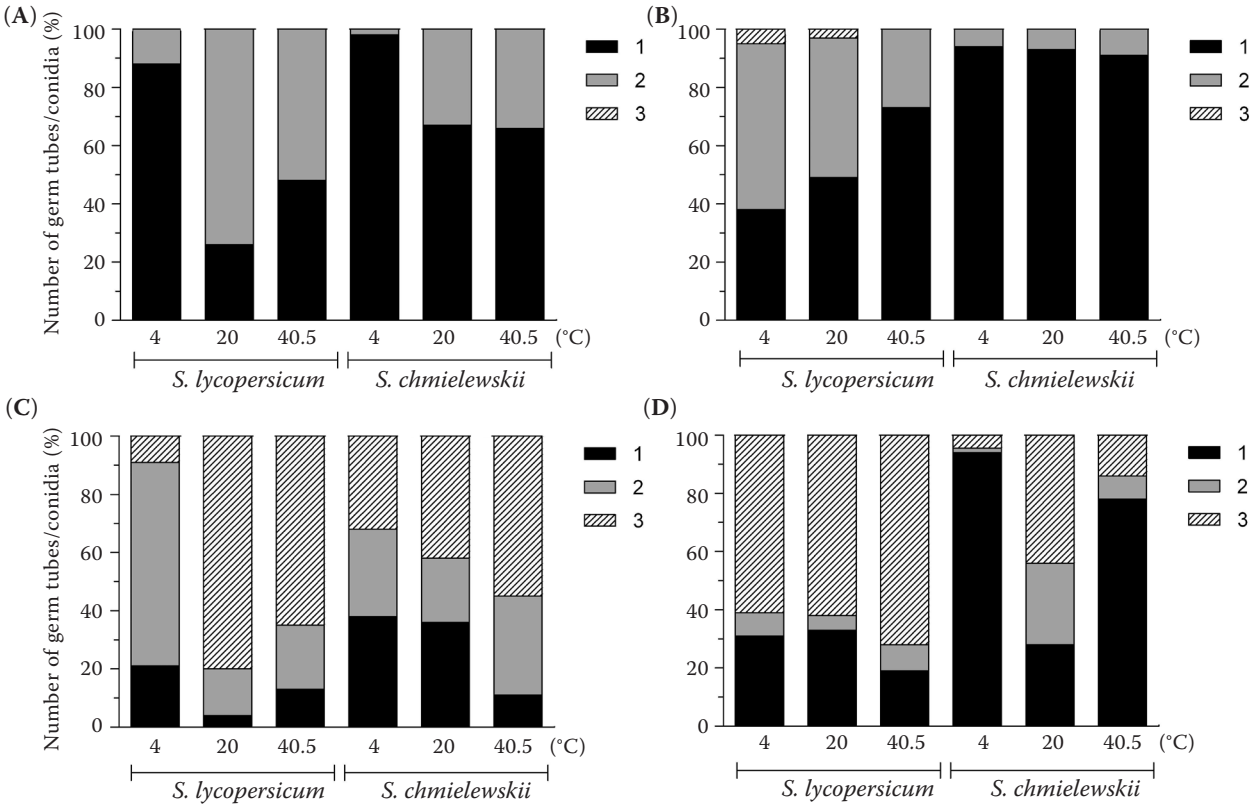

Figure 3. Proportion of *O. neolycopersici* conidia with developed 1, 2, or 3 germ tubes on leaf discs (**A**, **B**) and plants (**C**, **D**) of *Solanum* spp. exposed to cold and heat shock, 24 hpi (**A**, **C**) and 48 hpi (B, D)

The effect of temperature stress on tomato leaves and content of chlorophyll

The physiological state of whole leaves and tissues was evaluated macroscopically. Leaf curling was recorded in *S. lycopersicum* 24 h after both heat and cold stress. Changes recorded in *S. chmielewskii* and *S. habrochaites* were only limited. Moreover, the total amounts of chlorophyll *a* and chlorophyll *b* were determined for all three genotypes (Figure 4). The strongest gradual reduction of chlorophyll content was caused by the heat stress of 40.5°C whereas the cold stresses of 4 and 10°C and pathogen infection led to minor changes. An interesting trend was recorded in plants exposed to 10°C: this stress was manifested by a relatively rapid decrease in chlorophyll content during the first four hours, while a recovery of chlorophyll content was observed later at 24 h after the initiation of the stress (Table 1). Among the tested genotypes, the susceptible *S. lycopersicum* cv. Amateur showed the greatest changes in chlorophyll level under all stress factors. In moderately resistant *S. chmielewskii*, a genotype with the lowest content of chlorophyll, the *O. neolycopersici* pathogenesis led to only minimal changes in chlorophyll content, whereas an intensive reduction was recorded 24 h after the application of heat stress. In resistant *S. habrochaites* the trends of chlorophyll decrease were similar though not as intense as in *S. lycopersicum* (Figure 4C).

Table 1. Relative decrease in chlorophyll content recorded 24 h after the application of stress factors. Values are expressed in percent of the value determined for control intact plants of the corresponding tomato genotype

Heat stress/inoculation	*S. lycopersicum*	*S. chmielewskii*	*S. habrochaites*
4°C	28.4	9.6	19.7
10°C	30.4	8.3	19.4
40.5°C	48.0	28.3	34.0
O. neolycopersici	17.6	3.4	14.0

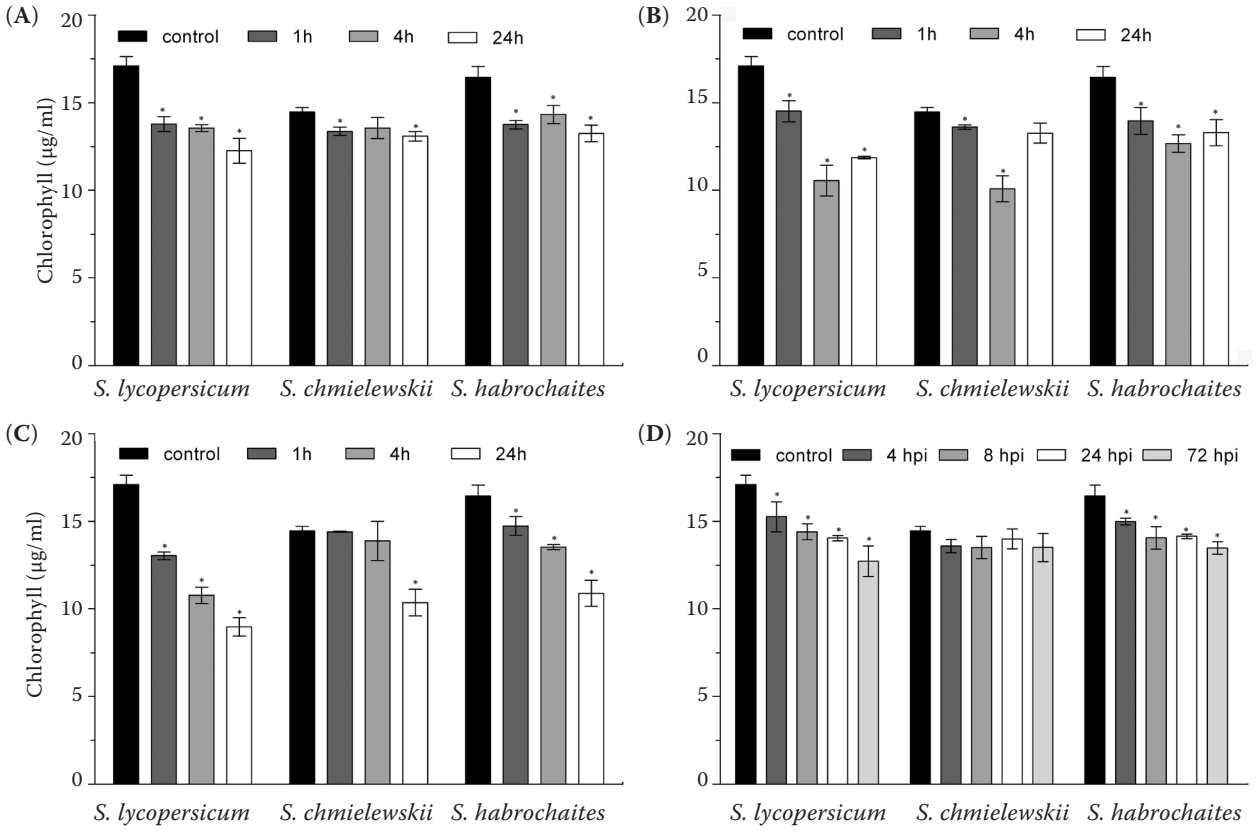

Figure 4. Changes of chlorophyll content in the leaves of three *Solanum* spp. genotypes induced by cold and heat stress and by *O. neolycopersici.* Plants exposed to cold stress at 4°C (**A**) and 10°C (**B**) or heat stress at 40.5°C (**C**) for 1, 4, and 24 h; plants inoculated with *O. neolycopersici* and cultivated at 20°C (**D**), the samples were collected at the 4th, 8th, 24th, and 72nd h post-inoculation (hpi). Results are given as a mean (n = 9) ± SD and the values statistically different from corresponding control values are marked as * (P = 0.05)

Influence of temperature on the expression and protein level of Hsp70 in *Solanum* spp.

In the three *Solanum* spp. genotypes, Hsp70 mRNA levels were determined by qRT-PCR blot analysis (Figures 5A–C). The *Hsp70* gene expression was studied at 0, 1, 4, and 24 h after cold (4 and 10°C) and heat treatment (40.5°C). Plants kept at 20°C were used as controls. None of the studied tomato genotypes exposed to 10°C, with the exception of *S. habrochaites* in the 1st h, showed any significant change in the gene expression. However, the treatment at 4°C significantly influenced the gene expression mainly in the *S. lycopersicum* cv. Amateur, where gene expression increased 11-fold already after the 1st h under stress, and further 9- and 36-fold increases of gene expression were observed in the 4th and 24th h, respectively. At 40.5°C significant changes in gene expression were observed in all genotypes from the 1st h after the initiation of the treatment, compared to control plants incubated at 20°C, however the course of gene expression varied among the studied species. The heat stress resulted in an approximately 43 700-fold increase of Hsp70 expression in the *S. lycopersicum* cv. Amateur (Figure 5A), of the gene expression, 1800-fold increase in *S. chmielewskii* (Figure 5B), and 16 900-fold in *S. habrochaites* (Figure 5C). In the 4th h the expression of Hsp70 decreased 39 400-fold in *S. lycopersicum* and 160-fold in *S. chmielewskii* but it increased 48100-fold in *S. habrochaites*. A dramatic decrease of Hsp70 expression was detected in all genotypes in the 24th h compared to the 1st h of stress, though still higher than in control plants (6100-fold in *S. lycopersicum*, 13-fold in *S. chmielewskii*, and 460-fold in *S. habrochaites*).

We also determined Hsp70 protein levels in control and stressed plants by Western blot analyses (Figures 5D–F). Exposure to 10°C slightly decreased the Hsp70 protein level in *S. lycopersicum* and *S. habrochaites* but it did not influence the Hsp70 content in *S. chmielewskii*. Surprisingly, the cold

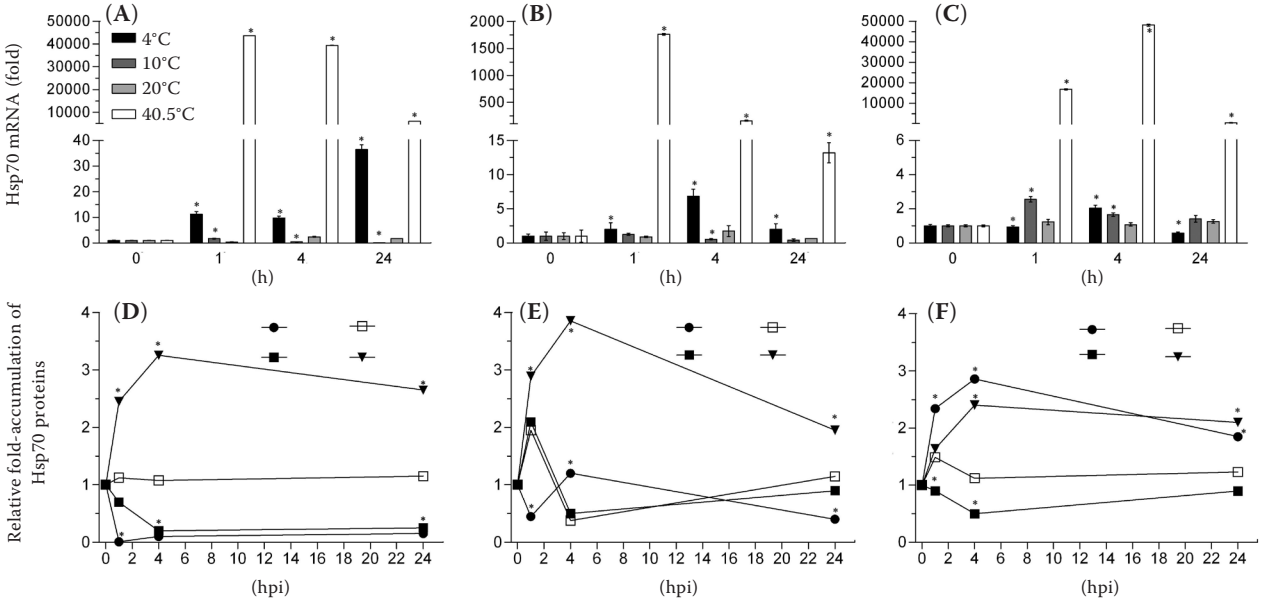

Figure 5. Effects of cold and heat stress on Hsp70 mRNA expression (**A**, **B**, **C**) and on Hsp70 accumulation detected by Western blot using a mouse anti-Hsp70 monoclonal antibody (**D**, **E**, **F**) in *S. lycopersicum* cv. Amateur (**A**, **D**), *S. chmielewskii* (**B**, **E**), and *S. habrochaites* (**C**, **F**). Plants were exposed to 4, 10, 20 and 40.5°C for 24 hours. The analyses of Hsp70 mRNA by qRT-PCR and protein accumulation from three experiments are presented. The data are expressed as fold induction compared to untreated plants at the start of the experiment (time point of 0 h). Moreover, the data of Hsp70 mRNA expression were normalised to GAPDH mRNA levels. Results are given as a mean (n = 9) ± SD and the values statistically different from corresponding control values of plants incubated at 20°C are marked as * (P = 0.05)

stress of 4°C induced high Hsp70 accumulation in *S. habrochaites* (Figure 5F). As expected, the exposure to 40.5°C led to an increase of the Hsp70 protein level in all studied genotypes, starting from the 1st h and reaching its maximum in the 4th hour. The protein level in the 24th h showed a more dramatic decrease in *S. chmielewskii* than in the other two genotypes (Figure 5E).

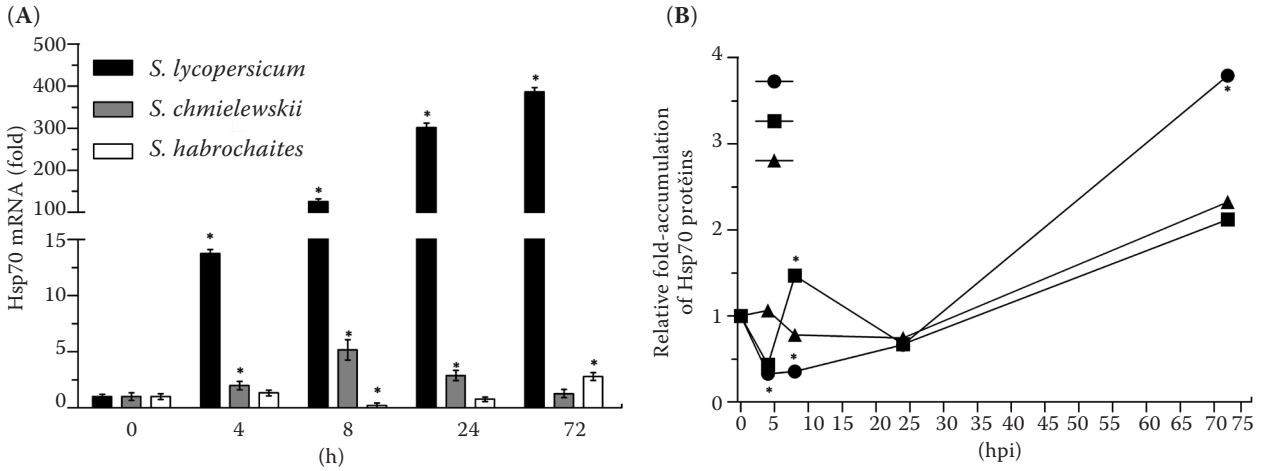

Figure 6. Changes in *Hsp70* gene expression (**A**) and protein accumulation (**B**) in three *Solanum* spp. genotypes induced by *O. neolycopersici* infection. Expression of the tested gene mRNA was determined using qRT-PCR in leaf samples from control and *O. neolycopersici* inoculated plants, collected 0, 4, 8, 24, and 72 hpi. The data are expressed as fold induction over control samples without inoculation. The *Hsp70* gene expression was normalised per GAPDH mRNA levels. The Hsp70 content was detected by Western blot using a mouse anti-Hsp70 monoclonal antibody and expressed as fold induction compared to untreated plants at the start of the experiment (0 h). Results are given as a mean (n = 9) ± SD and the values statistically different from corresponding values of control plants are marked as * (P = 0.05)

The influence of pathogen infection on Hsp70 levels in *Solanum* spp.

Methods of qRT-PCR and Western blot were also applied to quantify the levels of Hsp70 mRNA and protein content in the studied *Solanum* spp. infected by powdery mildew (Figure 6). An increase in gene expression was found in susceptible *S. lycopersicum* leaves already 4 hpi when most conidia of *O. neolycopersici* had already germinated and the mycelium started to grow intensively. The gradual increase of mRNA level continued up to 24 hpi. On the other hand, Figure 6A illustrates that the *Hsp70* gene expression did not change dramatically in moderately resistant *S. chmielewskii* or resistant *S. habrochaites*. The highest mRNA level was observed 8 hpi in the case of *S. chmielewskii*.

The accumulation of Hsp70 protein shown in Figure 6B was slower compared to changes in mRNA. No increase of Hsp70 was detected in all species until 72 hpi. At this time interval, the highest increase in Hsp70 protein content was observed in the susceptible *S. lycopersicum* cv. Amateur.

DISCUSSION

Defence mechanisms that contribute to plant survival in suboptimal conditions involve various short- and long-term changes of their primary and secondary metabolism. Successful plant survival under stress conditions requires the maintenance of protein functionality (Vierling 1991). The 70 kDa heat shock proteins (Hsp70) are constitutively expressed members of the Hsp families which are involved in the cell protection to a variety of stresses (Wang *et al*. 2004). In the present study we compared the influence of both abiotic and biotic stress stimuli on *Hsp70* gene expression and Hsp70 protein accumulation. Three genotypes of *Solanum* spp. were used, which differ both in their susceptibility and resistance to tomato powdery mildew (*O. neolycopersici*) and in sensitivity and tolerance to extreme temperatures (Mieslerová *et al*. 2000). We have recently contributed to the knowledge of the role of hormones, reactive oxygen and nitrogen species in signalling and defence mechanisms in *Solanum* spp. in interactions with *O. neolycopersici* (Piterková *et al.* 2009, 2011, 2013).

The effect of extreme temperatures on leaves and pathogen

Short-time exposure of tomato plants or detached leaf discs to temperatures of 4, 10, or 40.5°C modified the tissue turgor and altered their appearance. The *S. lycopersicum* cv. Amateur, which originates from lower elevations, proved to be the most sensitive to low as well as to high temperatures. On the contrary, wild *S. chmielewskii*, native to high-altitude tropic-alpine habitats in restricted areas of the southern Peruvian Andes, and *S. habrochaites*, originating from habitats slightly warmer, drier and less exposed to the sun (Nakazato *et al*. 2010), were more tolerant to cold and heat exposure. Reactions of individual genotypes are also conditioned by the morphology and anatomy of plant leaves.

It was demonstrated in many pathosystems that the host resistance or susceptibility can be affected by temperature. Heat pre-treatment can lead to the induction of resistance, but also to an increased susceptibility. Schweizer *et al.* (1995) and Vallélian-Bindschedler *et al.* (1998) reported that a short exposure of susceptible barley cultivar to a higher temperature (50°C for 30–60 s) induced resistance and reduced subsequent infection by powdery mildew (*Blumeria graminis* f.sp. *hordei*). However, an adverse effect, i.e. increased susceptibility following a heat shock, was reported in other plant-pathogen interactions (Chamberlain & Gerdemann 1966; Chen *et al.* 2003). In our experiments, a significant deceleration of pathogen development was recorded due to cold pre-treatment. Experiments carried out on the leaf discs represent an ideal humidity conditions for the development of *O. neolycopersici*. A comparative study performed on leaf discs and intact plants showed higher Hsp70 production in the case of leaf discs, which may be caused by mechanical damage to discs (Piterková *et al*. 2013). Cheong *et al*. (2002) identified the genes corresponding to mechanical injury in *Arabidopsis*, and found that a large part of them plays a role in resistance to the pathogen. The leaf disc assay is a common and fast method of screening of host susceptibility/resistance to the pathogen. It is widely used in many pathosystems (e.g. *Lactuca* spp.–*Golovinomyces cichoracearum*; *Solanum* spp.–*O. neolycopersici*; cucurbitaceous plants–*Golovinomyces orontii*, *Podosphaera fusca*; sweet cherry–*Podosphaera clandestina*; Cohen 1993; Mieslerová *et al.* 2000;

Olmstead *et al.* 2000; Lebeda *et al.* 2011, 2013). In most of the studied pathosystems, results obtained from the leaf disc assay correlated well with results obtained with whole plants, indicating that the disc assay may accurately predict responses of whole plants. However, for an interaction of *Lactuca–G. cichoracearum*, Schnathorst and Bardin (1958) reported that results obtained in field experiments and in laboratory detached-leaf experiments partly differed. The reasons for this fact are not clear, but environmental conditions (e.g. microclimate) might play an important role. Results of our study correspond with Piterková *et al.* (2013), when heat stress minimally influenced the pathogen development on *S. chmielewskii* and significantly suppressed the pathogen development on the susceptible *S. lycopersicum* cv. Amateur.

Our previous experiments showed the pre-treatment of leaf discs with high (45°C) or low temperature (3°C), and mainly their combination influenced the development of *O. neolycopersici* on both susceptible *S. lycopersicum* cv. Amateur and moderately resistant *S. chmielewskii,* expressing a hypersensitive reaction (Mieslerová & Lebeda 2010). Experiments of Nožková-Hlaváčková *et al.* (2013) focused on a longer time interval up to 9 days post inoculation brought new insights into the variation of *O. neolycopersici* development in susceptible *S. lycopersicum* and moderately resistant *S. chmielewskii* plants exposed to 40.5°C. Moreover, a higher incidence of necrosis and chlorosis was observed together with the accumulation of salicylic and abscisic acid, and increased peroxidase activity. In the work of Prokopová *et al.* (2010) the rapid formation of chlorosis and necrosis in heat-stressed inoculated leaves was linked to a decrease of chlorophyll *a* and *b* contents, stomatal closure, and inhibition of the CO_2 assimilation rate. The chlorosis and necrosis are usually detectable in this pathosystem a few days after infection (Lebeda & Mieslerová 2010). However, pre-treatment with heat stress followed by an inoculation and infection process probably amplifies the stress to such a great extent that the plants lose their turgor and start to die.

Chlorophyll content is one of the markers used to gauge the physiological state of plants (Gorovits & Czosnek 2008). Many stresses affect chlorophyll levels and degradation of inherently stable chloroplast proteins, accompanied by the degradation of several photosystem-II (PSII) proteins. The chlorophyll biosynthesis and chloroplast development are mainly inhibited (Adam & Clarke 2002). The impaired chlorophyll biosynthesis can be part of a protective mechanism against stress during limited time periods. A high temperature provokes severe damage to the photosynthetic apparatus (Camejo *et al.* 2005). A time-dependent decrease of chlorophyll content induced by inoculation, cold and especially heat stress in our study seems to be connected with changes in photosynthetic parameters of *Solanum* spp. as shown by Prokopová *et al.* (2010). Both in susceptible *S. lycopersicum* cv. Amateur and moderately resistant *S. chmielewskii* only minimal impairment of photosynthesis was reported during 9 days post inoculation with *O. neolycopersici*. Changes in *S. chmielewskii* were ascribed to HR, wilting and necrosis of the infected leaves. Following a heat-shock pre-treatment identical to our study (40.5°C, 2 h), the response of *S. chmielewskii* plants was not affected, whereas chlorosis/necrosis developed and CO_2 assimilation rate and PSII efficiency decreased in the infected leaves of *S. lycopersicum* (Prokopová *et al.* 2010).

Heat shock protein 70 gene expression and protein level

Hsp70 proteins are expressed constitutively at low levels in most plants (Al-Niemi & Stout 2002). Wild tomato species are known to grow in the Andes Mountains at a high altitude (up to 2800 m above sea level) and at lower temperatures as surveyed e.g. by Venema *et al.* (1999) and Nakazato *et al.* (2010). Due to this fact we expected higher resilience to temperature changes in *S. chmielewskii* and *S. habrochaites*, compared to the *S. lycopersicum* cv. Amateur. Our results are consistent with this hypothesis. Different sensitivity to high temperatures has also been proposed by Prokopová *et al.* (2010), who found *S. lycopersicum* more sensitive to heat shock (40.5°C) in combination with *O. neolycopersici* infection than *S. chmielewskii*. In our experiment, significant changes in *Hsp70* gene expression and protein accumulation were detected after incubation of plants at 40.5°C for 1 h in comparison with the cold-stressed plants, as previously addressed by Piterková *et al.* (2013) using leaf discs. In research carried out by Sabehat *et al.* (1996) an increased synthesis of Hsp70 proteins in the *Solanum lycopersicum* cv. Daniella was observed after exposure to heat stress (38°C),

whereas tomatoes exposed to 2°C accumulated only low levels of Hsp70. A similar effect was reported previously for spinach (*Spinacia oleracea*), pepper (*Capsicum annuum*), and orange (*Citrus sinensis*) (Li *et al.* 1999; Garavaglia *et al.* 2009).

Several previous reports described Hsp/Hsc induction upon plant challenge with bacterial and fungal pathogens. Hsp70/Hsc70 were induced in tomato cell culture by avirulent strains of *Ralstonia solanacearum* as part of the defence response together with the induction of PAL activity and maintenance of cell viability 24–48 h after the infection (Byth *et al*. 2001). Small Hsp17 was induced in tobacco plants by both pathogenic and non-pathogenic strain of *R. solanacearum* and its expression was induced also by treatment with heat, aminocyclopropane carboxylic acid, hydrogen peroxide, methyl jasmonate, and salicylic acid (Maimbo *et al*. 2007). It was suggested that small Hsps might have a role in HR-independent defences in tobacco. A recent report described Hsp70 as the major target of HopI1, a virulence effector of pathogenic *Pseudomonas syringae* (Jelenska *et al*. 2010). The binding of HopI1 actively affects Hsp70 activity and subverts its defence-promoting activity. Interestingly, heat shock or high temperature treatment resulted in similar effects suggesting that the Hsp70 pool could be diverted to stress functions at the expense of defence responses.

In the present study, we tested whether differences in resistance to powdery mildew are associated with differences in the transcription and translation of Hsp 70 during *O. neolycopersici* pathogenesis and under abiotic stress factors (heat, cold). Increased *Hsp70* gene expression found in *Solanum* spp. infected by powdery mildew seems to link to time intervals of intensive development of *O. neolycopersici*. In susceptible *S. lycopersicum* the mycelium developed rapidly the haustoria at 4 hpi to feed from the host cells. Oppositely, in the case of resistant genotypes of *S. chmielewskii* and *S. habrochaites*, no significant changes were observed. In resistant *S. habrochaites*, where the pathogen growth is strongly restricted, only minor changes in Hsp70 mRNA were recorded at 72 hpi. However, all three species showed a high expression of the *Hsp70* gene during heat stress (40.5°C). Moreover, in the *S. lycopersicum* cv. Amateur, which is the most sensitive to cold stress among the studied *Solanum* species, an increased expression of Hsp70 was recorded also during cold stress (4°C).

Additional research focused on the study of combined effects of abiotic and biotic stress can bring about a better understanding of defence mechanisms in plants and biochemical changes that occur in plants.

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Received for publication June 6, 2013
Accepted after corrections October 4, 2013

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