The six *Tomato yellow leaf curl virus* genes expressed individually in tomato induce different levels of plant stress response attenuation

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Abstract *Tomato yellow leaf curl virus* (TYLCV) is a begomovirus infecting tomato plants worldwide. TYLCV needs a healthy host environment to ensure a successful infection cycle for long periods. Hence, TYLCV restrains its destructive effect and induces neither a hypersensitive response nor cell death in infected tomatoes. On the contrary, TYLCV counteracts cell death induced by other factors, such as inactivation of HSP90 functionality. Suppression of plant death is associated with the inhibition of the ubiquitin 26S proteasome degradation and with a deactivation of the heat shock transcription factor HSFA2 pathways (including decreased HSP17 levels). The goal of the current study was to find if the individual TYLCV genes were capable of suppressing HSP90-dependent death and HSFA2 deactivation. The expression of C2 (C3 and CP to a lesser extent) caused a decrease in the severity of death phenotypes, while the expression of V2 (C1 and C4 to a lesser extent) strengthened cell death. However, C2 or V2 markedly affected stress response under conditions of viral infection. The downregulation of HSFA2 signaling, initiated by the expression of C1 and V2,

was detected in the absence of virus infection, but was enhanced in infected plants, while CP and C4 mitigated HSFA2 levels only in the infected tomatoes. The dependence of analyzed plant stress response suppression on the interaction of the expressed genes with the environment created by the whole virus infection was more pronounced than on the expression of individual TYLCV genes.

Keywords Geminivirus · Viral proteins · Stress response · Cell death

Introduction

Plant innate immunity is the result of specific pre-programmed defense responses. In plant-pathogen interactions, the hypersensitive response (HR) is an active defense response, resulting from the activation of defense-related pathways, which lead to cell death (CD) (reviewed in Heath 2000). Plant infection with the bacterium *Pseudomonas syringae* or the fungus *Sclerotinia sclerotiorum* is followed by conspicuous HR and CD responses of the inoculated leaves, already after 1 or 2 days (Kabbage et al. 2013). Infection of *Nicotiana* spp. with the RNA virus *Tobacco mosaic virus* (TMV) also causes an HR similar to that induced by bacteria and fungi (Mandadi and Scholthof 2013). By comparison, monopartite begomoviruses such as *Tomato yellow leaf curl virus* (TYLCV) induce HR/CD only in some plants (e.g., *Nicotiana benthamiana*) (van Wezel et al. 2002), but not in others (e.g., tomato). As shown previously, HR/CD becomes visible in leaves of TYLCV-infected tomato plants at the very late stages of infection (50 to 60 days after the onset of infection, when plants become senescent (Gorovits et al. 2007; Gorovits and Czosnek 2007; Moshe et al. 2012). Similar to TYLCV, the bipartite begomovirus *Tomato leaf curl New*

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Delhi virus (ToLCNDV) was not able to induce HR/CD in infected *N. tabacum* and tomato plants (Hussain et al. 2007). However, the nuclear shuttle protein (NSP) encoded by the bipartite begomovirus ToLCNDV DNA B-induced CD when expressed under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter or when cloned in a *Potato virus X* (PVX) expression vector (Hussain et al. 2005). These results suggested that ToLCNDV-encoded protein(s) counteracted the NSP-dependent HR/CD responses. Analysis of the ToLCNDV-encoded genes pointed to the transcriptional activator protein (TrAP) (encoded by DNA A) as the factor mediating the anti-HR effect (Hussain et al. 2005). Later on, the V2 protein of two monopartite begomoviruses, *Papaya leaf curl virus* (PaLCuV) and *Cotton leaf curl Kokhran virus* (CLCuKoV), was shown to be a pathogenicity determinant and a target of host defense responses in *N. benthamiana* and *N. tabacum* plants (Mubin et al. 2010). C2/TrAP protein of PaLCuV or *Cotton leaf curl Multan virus* inhibited the HR induced by V2 in the virus-agroinfiltrated areas.

It is predictable that during the long-lasting begomovirus-plant relationship, the virus has developed means to ensure its survival and multiplication. In recent studies, we showed that TYLCV was able to maintain an environment suitable for its multiplication by suppressing HR/CD caused by other stresses (Moshe et al. 2016). CD, induced by the inhibition of HSP90 and its co-chaperone SGT1, was weakened in tomato plants infected by TYLCV. The inactivation of the HSP90-dependent ubiquitin-proteasome system (UPS) was alleviated in TYLCV-infected plants. In parallel, the activation of the transcription factor HSFB1, and of HSFA2 by itself and together with the HSFA2-regulated genes *Hsp17* (encoding the small HSP17) and *Apx1/2* (encoding the ascorbate peroxidases 1 and 2) (Nishizawa-Yokoi et al. 2010), was appropriately impaired in virus-infected cells. Because of the downregulation of the plant stress response, TYLCV can accumulate and cause disease in tomato plants.

In the current study, our goal was to find out if the individual TYLCV gene(s) could be responsible for the downregulation of the plant stress response. The TYLCV ssDNA genome strand encodes two genes, V1 (CP, coat protein) and V2 (essential for virus movement and TGS and PTGS). The complementary genome strand encodes four genes: C1 (Rep, initiates viral replication), C2 (TrAP, transcription activator), C3 (Ren, replication enhancer), and C4 (counteracts PTGS) (Díaz-Pendón et al. 2010). To identify the TYLCV genes involved in downregulating the plant stress response, we examined the ability of each of the six TYLCV genes to inhibit CD, induced by inhibition of *Hsp90* expression and by activating the HSFA2-dependent signaling. Our results suggest that the suppression of cellular stress response is dependent on the expression of individual TYLCV genes only to some extent, while the expression of the gene(s) with the environment

created by a whole virus infection was demonstrated to be more conspicuous.

Materials and methods

Sources of virus, insects, and plants

TYLCV was maintained in susceptible tomato plants (popular cultivar “Daniella,” also known as FA144 (Hazera, Israel). Whiteflies (*Bemisia tabaci* B biotype) used to inoculate tomato plants were reared on cotton plants grown in insect-proof cages at 26 °C. Plantlets (4 weeks after sowing) were caged with adult viruliferous whiteflies (approximately 50 whiteflies per plant at the onset of infection) for the duration of the experiments as previously described (Navot et al. 1991). All plants were grown in a temperature-controlled greenhouse under standard rearing conditions. Whiteflies were discarded before tissue sampling.

Gene silencing using a virus-induced gene silencing (VIGS) vector and developing of PVX constructs

The silencing *Tobacco rattle virus* (TRV) vector was licensed from PBL (UK). For *Hsp90-1* gene silencing, a fragment of 404 bp was amplified with the primers sense 5'-AATT TATGGAGGCCATTGCT-3' and anti-sense 5'-TTCA TCAACCTCCTCTACCTTT-3' using tomato cDNA as template. The fragment was T/A cloned into the TOPO vector (Invitrogen, USA), excised from the vector using *EcoRI* and *XhoI* and ligated to TRVII using the same enzymes. For cloning the TYLCV six genes into the PVX vector, each gene was amplified with a primer pair derived from the virus sequence (GenBank Accession number X15656; Navot et al. 1991). The sequences of the primers used to amplify C1, C3, C4, and CP are shown in Supplementary Table 1. C2 and V2 cloned in PVX vector were a gift from Prof. E. Bejarano (described in Luna et al. 2012). For the insertion of polymerase chain reaction (PCR) fragments into the PVX vector, restriction endonuclease recognition sites were included in virion-sense and anti-sense (complementary-sense) primers, respectively. PCR products were cloned into the PVX pGR106 vector (a gift from Prof. D. Baulcombe), at the *NotI* and *Sall* restriction sites.

The plasmids were introduced by electroporation into *Agrobacterium tumefaciens* C58. The cells were grown at 28 °C on LB medium containing Kanamycin and Rifampicin (50 µg/ml each) as previously described (Liu et al. 2002). All the leaves of tomato/*N. benthamiana* seedlings at the 4–6 true leaf stage (4 weeks after seeding) were infiltrated with the PVX constructs using a 1-ml syringe. Alternatively, a mixture of agrobacteria containing TRVI and TRVII-*Hsp90* was infiltrated into tomato seedlings. Twenty-

four hours after silencing, the treated tomatoes were agroinfiltrated with the PVX constructs. Each experiment was repeated at least five times.

Detection of H₂O₂

H₂O₂ accumulation was detected by endogenous peroxidase-dependent in situ histochemical staining with 3,3'-diaminobenzidine (DAB; Sigma, USA) as previously described (Gorovits et al. 2007). Samples were mounted on microscope glass slides in 60% glycerol. H₂O₂ was detectable as a reddish brown precipitate. Intensity and pattern of DAB staining were assessed under an Olympus SZET binocular coupled with a Nikon LCD camera DXM1200 and photographed.

Extraction of proteins from tomato leaves

For analyses by SDS polyacrylamide gel electrophoresis (SDS-PAGE), proteins were extracted from minced leaves (pooled from three plants), frozen in liquid nitrogen, and drill-homogenized in a standard PAGE loading buffer supplemented with 2% SDS. Samples, contained 50 µg proteins each, were subjected to 12% SDS PAGE.

Immunodetection of viral and plant proteins

Western blotting was performed as described (Gorovits et al. 2013a, b) using antibodies against the TYLCV CP (raised in our laboratory, Gorovits et al. 2013a), HSP70 (AS08371) and ubiquitin (AS08307) (Agrisera, Sweden), proteasome 20S alpha-beta (ab22673) (Abcam, UK), HSFA2 (a gift from Prof. K.D. Scharf; described in Scharf et al. 1998), and HSP17 (a gift of Prof. A. Grover; described in PhD thesis of Agarwal 2002). Incubation with the antibody was followed by ECL detection (Amersham, UK). Each immunodetection was repeated at least three times for each set of plants (pooled tissues from three plants).

DNA, RNA extractions and qPCR/qRT-PCR

DNA was prepared from 100-mg leaf tissues as described (Gorovits et al. 2013a). For qPCR, 50 ng DNA was used in triplicate for each DNA sample. RNA was prepared from 100-mg leaf tissues using the Tri-Reagent method (Sigma-Aldrich, USA); cDNA was prepared using the EZ-first strand cDNA synthesis kit (Biological Industries, Israel) according to the manufacturer. cDNA was used for qRT-PCR in triplicate for each cDNA sample.

DNA or cDNA was subjected to qPCR or qRT-PCR, respectively, in the presence of SYBR Green I (Takara, Japan), using a Corbett Research Rotor-Gene 6000 cyclor. The reaction was as follows: 30 s at 94 °C followed by 40 cycles consisting of 10 s at 94 °C, 30 s at 59 °C, and 20 s at 72 °C.

The primers used to amplify a 182 bp fragment of TYLCV (X15656) were as follows: sense 5'-TCTGTTCAAGGATTTCGTTG-3' and complementary sense 5'-GCTGTCGAAGTTCAGCCTTC-3'. As an internal reference, a 183 bp fragment of the tomato "Expressed" housekeeping gene (SGN-U346908) was amplified using the sense 5'-CTAAGAACGCTGGACCTAATG-3' and complementary sense 5'-TGGGTGTGCCTTTCTGAATG-3' primers. For tomato *β-actin* gene (TC178617), a 180 bp fragment was amplified using the sense 5'-GGAAAAGCTTGCTATGTGG-3' and complementary sense 5'-CCTGCAGCTTCCATACCAAT-3' primers. For tomato *HsfA2* (CAA47870), a 66 bp fragment was amplified using the sense 5'-ACCTGTGTGGATCAGCTTGGTTTCC-3' and complementary sense 5'-AATAGTGGAGGAGGCCAGAGGAAC-3' primers. For tomato *Hsp17-C1* (AJ225046), a 77 bp fragment was amplified using the sense 5'-ACTTGGCATCGTGTGGAACG-3' and complementary sense 5'-TGATCCATCTTTGCGTTCTCTGG-3' primers. For tomato *Hsp90-1* transcript (SGN-U312354), a fragment of 77 bp was amplified using the sense (5'-TGCGTTCTTGTATGGAAGTCTGC-3') and antisense (5'-TGGACCACTTAGTACGACCAATC-3') primers.

Results

Induction of HR/CD by the expression of individually expressed TYLCV genes in tomato and *N. benthamiana*

PVX-derived vector has been used throughout this study to express TYLCV genes in all plant tissues. The PVX expression system was shown to be productive for the expression of various begomovirus individual genes in *N. benthamiana* and tomato plants (Hussain et al. 2007; Mubin et al. 2010; Sharma and Ikegami 2010; Luna et al. 2012). The recombinant PVX was infiltrated into *N. benthamiana* and tomato leaves, and the treated plants were maintained in a greenhouse for 30 days, during which symptoms were periodically recorded. In *N. benthamiana*, chlorotic lesions started to be seen in leaves 8 to 10 days post-infiltration, especially in plants expressing V2. At 12–16 days, the PVX-V2 plants declined, while those inoculated with the PVX vector alone exhibited the PVX typical mild yellowing symptoms (Supplementary Fig. 1a). The ability of the geminiviral V2 protein to be a HR-determinant in *N. benthamiana* has been documented already for PaLCuV and CLCuKoV (Mubin et al. 2010), *Tomato leaf curl Java virus-A* (ToLCJV-A) (Sharma and Ikegami 2010), and several others.

To follow the development of HR/CD in tomato plants, tomato leaflets were infiltrated either with the empty PVX vector or with PVX-based constructs expressing the six TYLCV genes. The production of H₂O₂, the marker of HR,

was visualized by the 3,3'-diaminobenzidine (DAB) method; in the presence of H_2O_2 , DAB polymerizes to produce a brown product. Figure 1a shows DAB staining of tomato leaflets 7 days after infiltration. At the sites of infiltration with each one of the PVX constructs, weak HR/CD symptoms were observed in tomato leaves. In the case of PVX-V2 (-C1 and C4), some increase of DAB stain was detected (Fig. 1a). The timing of the appearance of V2-induced HR symptoms did not completely coincide with a previous report indicating that the geminiviral V2 is a CD determinant (Mubin et al. 2010; Sharma and Ikegami 2010). To compare the strength of V2-dependent HR symptoms, DAB staining was performed on tomato leaflets inoculated with *Xanthomonas campestris* or *S. sclerotiorum*, both known to cause strong HR symptoms (Kabbage et al. 2013). DAB stain of tissues inoculated with the bacteria and the fungus was far more intense than that produced by PVX-V2 (Fig. 1a). Moreover, in contrast to *N. benthamiana*, where the infiltration of PVX-V2

caused plant growth decline, no significant effect on tomato growth and HR/CD symptoms was seen in tomato plants treated with the same PVX-V2 construct (Supplementary Fig. 1a).

In the absence of virus infection, the expression of each of the individual TYLCV genes does not affect the HSP90-dependent HR/CD

The inhibition of the HSP90 functions is known to cause HR/CD in *N. benthamiana*: silencing of *Hsp90* gene induces CD (Liu et al. 2004). The HSP90 co-chaperone SGT1 positively regulated CD (Wang et al. 2010). In tomato, the downregulation of the HSP90 machinery causes severe necrosis on the stems of inoculated plants (Moshe et al. 2016). To find out which TYLCV expressing gene influenced the HR/CD phenotype, caused by the inhibition of HSP90 machinery, a tomato *Hsp90-1* gene silencing TRV construct (TRV-*Hsp90*) was introduced into *N. benthamiana* and tomato plants before

Fig. 1 Changes in the intensity of HR/CD in tomatoes infiltrated with PVX constructs expressing individually the six TYLCV genes. **a** H_2O_2 in tomato leaves assayed with 3,3'-diaminobenzidine (DAB) staining, 7 days after infiltration with PVX-constructs and 2 days after infection with *X. campestris* and *S. sclerotiorum*. **b** HR/CD symptoms in the stems

of *Hsp90*-silenced plants with infiltrated PVX, PVX-C1, -C2, -C3, -C4, -CP, and -V2 from uninfected tomatoes 19 days after TRV-*Hsp90* infiltration (*top panel*) were compared with stems of tomatoes infected by TYLCV at 14 dpi, corresponding to 19 days after infiltration, plants (*bottom panel*). DAB staining was performed on stem cross-sections

infiltration of PVX-TYLCV gene constructs. Thus, at the first stage, plants (4–6 true leaves) were agroinfiltrated with TRV-*Hsp90*. At the second stage, 24 h later, PVX constructs containing each one of the six TYLCV genes were separately introduced in the *Hsp90*-silenced plants. In *Hsp90*-silenced *N. benthamiana*, the presence of the PVX vector alone did not change the growth pattern of the infiltrated plants, while the expression of PVX-V2, and to a lesser extent that of PVX-C1, resulted in severe leaf curling, followed by plant death (Supplementary Fig. 1b, shown for 15th day after introducing of the PVX-TYLCV gene constructs). TYLCV CP and especially C2 expression in *Hsp90*-silenced *N. benthamiana* improved plant growth. The expression of C3 had effects comparable with those of CP. The results pointed to differential effects of TYLCV genes in the suppression of CD in *N. benthamiana* plants.

Similarly to *N. benthamiana*, infiltration of tomatoes with TRV-*Hsp90* was followed by the inoculation of the PVX constructs containing the individual TYLCV genes. However, tomatoes, as the natural TYLCV host, were infected with TYLCV using viruliferous whiteflies. Thus, at the third stage of treatments, half of the tomatoes, infiltrated with TRV-*Hsp90* and PVX constructs, remained uninfected, while the second half was exposed to TYLCV 5 days after PVX infiltration. Neither TYLCV infection (Moshe et al. 2016) nor PVX expression (Supplementary Fig. 2) significantly changed the efficiency of *Hsp90* silencing.

Hsp90-silenced tomatoes ceased to grow approximately 2 weeks after the onset of silencing. The stems became necrotic and the plants collapsed several weeks thereafter. If the *Hsp90*-silenced tomato plants were infected with TYLCV, the CD symptoms weakened, and the plants looked much healthier for more than 1 month (Moshe et al. 2016). When separated PVX-expressed six viral genes replaced the whole TYLCV, no significant difference in *Hsp90*-silenced plant growth versus tomatoes with PVX vector was observed. The intensity of the HR/CD symptoms along the stem was comparable for all *Hsp90*-silenced plants (shown in Fig. 1b, at 19 days after agroinoculation with the TRV-*Hsp90*). The absence of CD suppression was detected in at least seven independent biological repeats during 30 days of growth. DAB staining revealed very small changes in the release of H₂O₂ from dying tissues (Fig. 1b). Sometimes, for a restricted period (10–14 days after infiltration), some decrease of DAB-browning was observed in tomatoes with PVX-C2 and PVX-CP, but as a whole, comparable levels of HR/CD efficiency were perceived in all tomatoes containing PVX without or with expression of TYLCV genes.

TYLCV-infected *Hsp90*-silenced plants with PVX expression vector carrying each of the six TYLCV genes started to show differences in growth and development of CD on the stem 12 to 16 days after the onset of infection (dpi) (Supplementary Fig. 1c). Tomatoes harboring PVX-C2 had

a healthier appearance than those with PVX alone, while plants with PVX-V2 started to collapse sooner than all the others. Visualization of HR/CD in stem and the corresponding results of DAB staining showed the strongest signals in the case of PVX-V2 and the weakest for PVX-C2, while the expression of PVX-CP, -C3, -C4, and -C1 presented an intermediate effect between that of C2 and V2 plants (Fig. 1b).

These results demonstrated that the expressions of separated TYLCV genes significantly affected the HSP90-dependent CD in tomatoes only under conditions of whitefly-mediated TYLCV infection. It must be mentioned that the obtained differences were observed during a restricted period, before the appearance of pronounced TYLCV symptoms (Supplementary Fig. 1c). High levels of accumulated TYLCV diminished the influence in individually expressed viral genes.

Ubiquitin-proteasome system (UPS) patterns in tomatoes containing TYLCV expressing genes

Inactivation of the HSP90 machinery reduces the 26S proteasome activity and, consequently, the UPS function in plant cells (Nishizawa-Yokoi et al. 2010). We have shown previously that in *Hsp90*-silenced tomatoes, ubiquitinated proteins and the core 20S proteasome accumulated (Moshe et al. 2016), pointing to UPS inactivation (Yamano et al. 2008). Upon TYLCV infection, 20S proteasome accumulation decreased (Moshe et al. 2016). In the current study, the patterns of polyubiquitinated proteins and 20S proteasome were analyzed in leaf extracts from *Hsp90*-silenced tomatoes containing each of the six PVX-TYLCV gene constructs, before and after TYLCV infection. Figure 2 shows that the presence of PVX and PVX-TYLCV genes did not significantly change the levels of UPS markers, whether virus-infected or not (shown for PVX, PVX-C2, and PVX-V2). There was some more intensive UPS activation in TYLCV-infected tomatoes agroinoculated with PVX-V2 (see the abundance of polyubiquitinated proteins and 20S subunit at 14 dpi in Fig. 2). There were no significant changes in UPS patterns in the case of the other TYLCV-expressed genes, C1, C3, C4, and CP (not shown). Therefore, the expression of individual TYLCV genes in tomatoes does not affect the UPS function. On the background of a notable downregulation caused by the presence of the whole virus, the possible input of separated genes (whether as transcripts or as proteins) is minor or undetectable by Western blot analysis (Fig. 2). As a control of proteins, extracted from the leaves of uninfected (19, 26, 33 days post-infiltration) and TYLCV infected (14, 21, 28 dpi post-infection, which corresponded to 19, 26, 33 days post-infiltration) tomatoes, the patterns of HSP70 were used. The decline of HSP70 abundances in extracts from silenced plants at the late stages of treatments pointed on the total plant decease. Previously, the high stability of HSP70 patterns was

Fig. 2 Patterns of ubiquitin–proteasome system (UPS) in *Hsp90*-silenced tomatoes infiltrated with PVX constructs. Western blot immuno-detection of ubiquitinated proteins (UbqP), the 20S core subunit of the 26S proteasome (20S), and HSP70 as unrelated control in proteins

extracted from leaves of uninfected (19, 26, 33 days post-infiltration) and TYLCV infected (14, 21, 28 dpi post-infection, which corresponded to 19, 26, 33 days post-infiltration) tomatoes

shown for uninfected tomatoes, while TYLCV stress caused its decrease (Gorovits et al. 2007).

Effect of individual TYLCV genes on the accumulation of HSFA2 and HSP17 in heat-treated tomato leaves

The inactivation of HSP90 increased the expression of HSFA2 and consequently, the expression of HSFA2-regulated genes, as shown in *Arabidopsis* cell cultures (Nishizawa-Yokoi et al. 2010) and in tomato leaves (Moshe et al. 2016). To increase the basal HSFA2 levels, the plant tissues were subjected to heat shock (HS). Detached leaves were incubated successively at 39 °C for 45 min, at room temperature for 2 h and finally either at 43 °C for 40 min (for RNA extraction) or at 43 °C for 2 h (for protein analysis). Such conditions were claimed to be optimal to obtain maximal amounts of HSFA2 and HSFB1 proteins and optimal *HsfA2* gene expressions (Mishra et al. 2002; Mittal et al. 2012).

HS induced a strong increase of *HsfA2* expression in all the plants with PVX constructs (shown in Fig. 3a for PVX minus HS compared with PVX plus HS). Western blot analyses detected changes in HSFA2 patterns caused by the expression of TYLCV genes, 7–10 days after infiltrating the PVX constructs (Fig. 3a). HSFA2 induction was more pronounced in tomatoes containing the PVX vector alone, PVX-C2, -C3, -CP, -C4, but not PVX-C1 and -V2. Similar differences in HSFA2 levels were detected in leaf extract 19 days after introducing the PVX constructs. It is important to mention that sometimes HSFA2 levels changed very fast during short time periods. For example, in PVX-C2 tomatoes, compared to PVX alone,

a sharp decrease was seen after 7 days, followed by an increase 3 days later. Despite the sudden, fast, and irregular changes of HSFA2 levels in tomatoes containing the PVX constructs, it can be concluded that HSFA2 downregulation depends on V2 and C1 expression, to a lesser extent on CP and C4, but not on C2 and C3 (Fig. 3a). Similar results were obtained in parallel for HSP17, a HSFA2-regulated heat stress protein. TYLCV infection strengthened these effects (Fig. 3a). The results of immunodetection were confirmed by qRT-PCR analyses, which estimated the changes in *HsfA2* transcription levels in the same plant tissues (Fig. 3b). The effects were best seen about 2 weeks after infiltration; at the late stage (after 3 weeks, for example), these differences vanished.

Hence, the TYLCV V2 and C1 genes are the most likely candidates for the role of suppressor of heat-induced HSFA2 activation. This conclusion was first suggested from the experiments performed in the absence of the whole TYLCV. At the next step, the changes in HSFA2 levels were analyzed in TYLCV-infected tomatoes agroinfiltrated with the PVX constructs containing each of the six viral genes 19 days after agroinfection and 14 dpi after virus infection. The patterns of HSFA2 mRNAs and proteins in TYLCV-infected plants indicated the following: (1) there was still no suppression of HSFA2 activation in plants with PVX-C2 and -C3; (2) there was still a suppression of HSFA2 by C1 and V2 in addition to that caused by the whole virus; (3) TYLCV infection changed the behavior of CP and C4 from neutral to prominent in down-regulating the activation of HSFA2 (Fig. 3). Western blot and qRT-PCR analyses of HSP17 detected similar changes as those observed for HSFA2.

Fig. 3 Influence of TYLCV genes on the expression of the transcription factor HSFA2 and HSP17 in heat stressed (HS) tomatoes. **a** Detached leaves from *Hsp90*-silenced tomatoes infiltrated with PVX constructs 10 and 19 days after infiltration and 14 days (dpi) after TYLCV infection (corresponding to 19 days post-infiltration were heat shock treated). Specific antibodies were applied to detect HSFA2 and HSP17. **b** qRT-PCR gene expression analyses in uninfected (19 days post-

infiltration) and TYLCV-infected (at 14 dpi, corresponding to 19 days) tomatoes with PVX-expressed TYLCV genes. After heat shock, the relative transcriptional levels of *HsfA2* and *Hsp17* were calculated in relation to the uninfected plant samples infiltrated with the PVX vector alone. The results were normalized using the β -actin gene. Bars represent the average and standard deviation of the relative expression from three independent biological repeats

Accumulation of TYLCV in tomatoes during changing stress responses

During infection of TYLCV-susceptible tomatoes, TYLCV DNA and CP accumulate steadily until they reach stable levels up to 60 dpi (Gorovits et al. 2013a). Virus CP and DNA were monitored in tomatoes with silenced *Hsp90*, agroinfiltrated with various PVX constructs (Fig. 4a shows the results in tomatoes agroinfiltrated with PVX, PVX-C2, and PVX-V2). The pattern of CP accumulation in silenced *Hsp90*-PVX plants was different from that of non-silenced (control) tomatoes: CP amounts just before the appearance of symptom (15 to 30 dpi) were significantly higher under conditions of HSP90 downregulation. The expression of viral C2 resulted in a stable TYLCV accumulation during a prolonged infection (until 35 dpi). In plants with PVX-V2, a sharp CP accumulation at the early stage of infection (14 dpi) was followed by a dramatic decrease of protein abundance later on (starting from 28 dpi) (Fig. 4a). CP patterns in plants with PVX-CP and PVX-C3 resembled those of C2 but to a lesser extent, while patterns related to PVX-C1 and PVX-C4 were comparable with PVX-V2 (not shown).

TYLCV DNA levels showed similar patterns to those of CP accumulation (Fig. 4b). In TYLCV susceptible tomatoes, the virus amount constantly increased until it reached a plateau maintained for up to 60 dpi (Gorovits et al. 2013a; Anfoka et al. 2016). In infected *Hsp90*-silenced tomatoes, despite the plant decline being partially suppressed by TYLCV infection, virus CP and DNA accumulated at least until 35 dpi (Moshe et al. 2016; Fig. 4b). As in the PVX-untreated plants, a sharp increase in viral CP and DNA was conspicuous at 14, 21, and 28 dpi in plants infiltrated with PVX alone and with PVX-TYLCV genes; however, a severe decrease was observed thereafter (Fig. 4b). Such a decrease was correlated with tomato plant decay (Fig. 1b, Supplementary Fig. 1c). Tomato plants infiltrated with PVX-C2 constituted an exception; they presented an improved physiological state compared to other *Hsp90*-silenced plants. In these plants, the expressed viral C2 was accompanied by a lesser decline of CP and DNA levels past 28 dpi (Fig. 4b). Conversely, the PVX-directed expression of V2 was accompanied by a dramatic decline of CP and DNA after 28 dpi. The results supported the conclusion that TYLCV multiplication was correlated with the successful suppression of CD.

Fig. 4 The influence of separated TYLCV proteins on viral accumulation in infected tomatoes. **a** Immunodetection of TYLCV CP at 14, 21, 28, and 35 dpi in control and in *Hsp90*-silenced tomatoes infiltrated with PVX, PVX-C2, and PVX-V2. HSP70 as unrelated control was used to demonstrate the decline of total protein content in

deceased plants. **b** Quantitative polymerase chain reaction (qPCR) estimation of TYLCV DNA amounts. The results were normalized using the tomato *expressed* gene as an internal DNA marker. Bars represent the average and standard deviation of the relative expression from three independent biological repeats

Discussion

For an efficient infection, a virus needs to create a healthy environment for its survival and multiplication. To ensure a prolonged infection cycle, begomoviruses seem to restrain their destructive effect on the host cells and to prevent drastic plant responses. For example, a begomovirus representative TYLCV does not induce an HR/CD upon whitefly-mediated infection of virus-susceptible tomato plants until the infected tomatoes become senescent. Furthermore, TYLCV is able to

mitigate CD caused by other factors such as the inactivation of the HSP90 machinery (Moshe et al. 2016). TYLCV-dependent suppression of the plant heat stress response was demonstrated to occur through the downregulation of HSF2 expression (Anfoka et al. 2016), which controls key aspects of the stress response (especially heat stress) by regulating the transcription activity of a wide range of genes involved in numerous signaling and metabolic pathways (von Koskull-Döring et al. 2007).

TYLCV, with its six multi-function proteins, is able to modulate host (plant and insect) proteins and alter a large array

of developmental and defense processes (Díaz-Pendón et al. 2010; Hanley-Bowdoin et al. 2013; Ghanim and Czosnek 2016). Little is known about the induction and inhibition of plant HR/CD by the different geminivirus gene products. For the bipartite begomoviruses, ToLCNDV and *Bean dwarf mosaic virus*, the elicitor of HR was shown to be NSP (Garrido-Ramirez et al. 2000; Hussain et al. 2005). ToLCNDV C1/Rep protein induced plant CD (Hussain et al. 2005). C1/Rep of two distinct begomoviruses, the bipartite *African cassava mosaic virus* (ACMV) and the monopartite *Tomato yellow leaf curl China virus* (TYLCCNV), elicited a reaction resembling HR, when expressed from a PVX vector in *N. benthamiana* (van Wezel et al. 2002). The co-expression of C1 with either ACMV-C4 or TYLCV-C4, expressed from overlapping open reading frames, triggered systemic necrosis in infected tissues, but C4 alone was neither an inducer nor an enhancer of the HR-like phenotype. Complexes between viral C1/Rep and cellular RBR triggered plant CD (Jordan et al. 2007). In contrast, ToLCNDV TrAP was capable of countering NSP-induced hypersensitive CD (Hussain et al. 2007). The monopartite begomovirus V2 could elicit an HR in *N. benthamiana*, while the expression of C2 inhibited V2-induced CD when expressed at the site of inoculation under the control of the CaMV 35S promoter (Mubin et al. 2010; Amin et al. 2011).

In the current study, we confirmed that TYLCV V2 functions as an HR-determinant in *N. benthamiana* (Supplementary Fig. 1a). The data correlated with the ability of V2 from other begomoviruses to trigger HR/CD (Hussain et al. 2007; Mubin et al. 2010; Sharma and Ikegami 2010). However, in tomatoes, TYLCV V2 expression did not affect plant growth (Supplementary Fig. 1a), and V2-dependent elicitation of HR associated with H₂O₂ production was much less than that caused by tomato bacterial/fungal pathogens (Fig. 1a). According to the literature, C2, which is essential for the expression of full geminiviral pathogenicity, has been shown not only to suppress V2-induced HR/CD, but also the jasmonate (JA) triggered defense and secondary metabolism. Plants expressing C2 are more susceptible to pathogen attack (Rosas-Díaz et al. 2016). The disease symptoms of *Hsp90*-silenced *N. benthamiana* plants were weaker in the presence of PVX-expressed TYLCV C2 and CP, while the expression of V2 and C1 increased plant decline (Supplementary Fig. 1b).

The estimation of TYLCV expressing gene capacity to affect the HR/CD phenotype was examined on tomatoes with inactivated HSP90 machinery. Marked CD symptoms were detected on stems of tomato plants with silenced *Hsp90* (Fig. 1b). PVX-directed expression of TYLCV proteins in *Hsp90*-silenced tomatoes did not significantly change plant growth, severity of stem necrosis, and of HR/CD symptoms (Fig. 1b). Hence, we concluded that the expression of individual TYLCV genes is not essential for the CD phenotype

caused by the inhibition of the HSP90 machinery. It seems that the strength of HSP90-dependent HR/CD phenotype in tomatoes exceeds the strength of the effect caused by V2 alone and suppressed by C2, as shown also in another study (Mubin et al. 2010). The same recombinant constructs demonstrated an extra effect on CD/HR phenotypes in TYLCV-infected tomatoes (Fig. 1b), demonstrating that the PVX-based vectors are useful for the expression of TYLCV genes in tomatoes. TYLCV infection by itself improved the survival of *Hsp90*-silenced tomatoes, while the additional expression of C2 unambiguously led to improved endurance of the infected plants. Some CD suppression was observed in tomatoes harboring PVX-C3 and PVX-CP, but to a lesser extent compared to PVX-C2. It is possible that the highest capacity of C2 to downregulate HSP90-dependent CD can be connected with the C2 functioning as a suppressor of gene silencing, both post-transcriptional and transcriptional (Dong et al. 2003; Luna et al. 2012). However, the minor changes observed in uninfected *Hsp90*-silenced tomatoes expressing C2 suggested additional mechanism(s) to be involved in CD phenotype mitigation. The influence of C4, C1, and especially V2, on CD mitigation and tomato survival, was just the opposite (Fig. 1b; Supplementary Fig. 1c). Therefore, we propose that individual TYLCV genes alone are not able to abolish the HSP90-dependent CD phenotype; they do so only by interacting with the whole viral machinery working as an HR/CD suppressor. The mechanisms of such interactions are under investigation.

We have indicated that UPS is involved in the degradation of TYLCV proteins in the infected tomato plant and in the whitefly vector (Gorovits et al. 2014; Gorovits et al. 2016). The geminivirus C2 protein was shown to be an important regulator of the plant UPS machinery by interacting with the CSN5 catalytic subunit of the constitutive photomorphogenesis 9 (COP9) signalosome (CSN) (Lozano-Durán et al. 2011). However, separated TYLCV proteins had no significant influence on the UPS pattern (Fig. 2). When the levels of polyubiquitinated proteins and 20S proteasome subunit were estimated in TYLCV-infected tomatoes, it was not possible to discriminate between the effects caused by the whole virus from the effect of individual proteins. Probably, the other kind of proteolysis, autophagy, and/or apoptosis-induced caspase-like proteases might be affected much more by separated individual TYLCV proteins.

In contrast, individually expressed TYLCV proteins were able to regulate the other tomato stress response associated with the induction of HSFA2. In the presence of the whole virus, V2, C1, C4, and CP countered the HS activation triggered by the transcription factor. V2 and C1 were able to downplay this activation, even in uninfected plants (Fig. 3). Similar changes of protein and RNA patterns were detected not only for HSFA2, but also for HSFA2-regulated HSP17. An in vitro protein-protein complex between tomato HSFA2 and viral proteins has been already described (Anfoka et al.

2016). All the six TYLCV proteins were able to capture one of the main cellular regulators of the stress response. By comparison, HSP70 interacted with only three viral proteins (Gorovits et al. 2013b). Such virus potential to capture cellular HSF A2 may point to the importance of virus-regulated suppression of the cellular stress response.

Indeed, the decreased abundances of the key HSPs, such as HSP60, HSP70, and HSP90, were repeatedly described in TYLCV-infected plants (Gorovits et al. 2007; Gorovits and Czosnek 2007; Moshe et al. 2012). The loss of host's protein-based stress response in mammalian cells infected by various viruses has been known for a long time (Collins and Hightower 1982). Such viruses as influenza A, West Nile virus, herpes simplex virus, and hepatitis C induce chronic fatigue and malaise act to limit the host stress response, including reduced HSP synthesis—which set hosts up for adverse symptoms. However, while raising HSPs may be protective in the case of some viral infections, in other cases, increasing HSPs could augment viral replication. (Hooper et al. 2012).

Following suppression of plant HR/CD, TYLCV is able to replicate and accumulate in a permissive environment. Indeed, tomato plants infiltrated with PVX-C2 showed the highest capacity to alleviate CD, survived better than the plants infiltrated with the other PVX-expressing genes, and contained the highest amounts of virus (Fig. 4). However, these differences were observed during restricted periods, as a function of the strength of TYLCV infection. On the other hand, the extent of HSF A2 downregulation was minimal in C2 tomatoes (Fig. 3).

From our studies on the downregulation of CD/HR and HSF A2 signaling, it is clear that the whole virus is more essential than the expression of isolated viral genes. The multifunctionality of each TYLCV protein in numerous interplays with the host protective mechanisms could be the basis of such a result. Hence, the development of transgenic tomatoes expressing an individual TYLCV gene for the improvement of heat-resistant tomatoes (e.g., to high summer temperatures in the Middle East) is inadequate. Instead, we suggest growing commercially available TYLCV-resistant tomatoes (see for example, <http://www.ctahr.hawaii.edu/sustainag/news/articles/v8-sugano-tylcv.pdf>) and pre-inoculating them with TYLCV. In such tomatoes, the virus minimally affects the physiology, growth, and mostly important the yield and protects plants from some other death-causing stresses. We are presently investigating the molecular mechanisms underlying these phenomena. Unraveling the mechanisms of virus-dependent regulation of cellular responses to various environmental stresses will provide new insights into the development of crops tolerant to changing conditions in the field, and in developing plant lines tolerant to multiple stresses.

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