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Stress Responses to *Tomato Yellow Leaf Curl Virus* (TYLCV) Infection of Resistant and Susceptible Tomato Plants are Different

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Abstract

Two genetically close inbred tomato lines, one resistant to *Tomato yellow leaf curl virus* (TYLCV) infection (R), the other susceptible (S), showed completely different stress response upon TYLCV infection. S plants were stunted and do not yield, while R plants remained symptomless and yielded. Comparison of protein profiles and metabolites patterns in TYLCV infected R and S tomatoes revealed a completely different host stress response. S plants were characterized by higher levels of reactive oxygen species (ROS) and ROS compounds, the anti-oxidative, pathogenesis-related (PR) and wound-induced proteins were predominant. In contrast, infection of R tomatoes did not drastically activate the same host defense mechanisms as in S plants, while R homeostasis was much more effectively maintained by protein and chemical chaperones. Sources of carbon and nitrogen were less affected by TYLCV in R than in S plants, which could make R plants more balanced and more fit to sustain infection. Even though both tomato types contained comparable amounts of TYLCV at the specified stage of infection, the cellular immune responses were different. Presented results are preliminary and indicate not so much concrete data but the global tender in understanding of the cellular response to virus stress at the background of resistance and susceptibility to TYLCV.

Keywords: Virus; Plant stress response; Proteomic and metabolomic analysis

Introduction

Plant acclimation to stress is associated with profound changes in their proteome and metabolome. Since proteins are directly involved in plant response to biotic stresses, proteomics studies can significantly contribute to unravel the possible relationships between protein abundance and plant-pathogen interactions. Induction of specific metabolites acting as defense compounds is the most common feature by which plants control pathogen and environmental stresses.

The tomato (*Solanum lycopersicum*) provides an excellent model system to investigate plant-pathogen interactions. This crop suffers from the attack of a number of pathogens including viruses, bacteria, fungi and nematodes. Among the viruses infecting tomato, *Tomato yellow leaf curl virus* (TYLCV) has the most important economical impact, worldwide [1]. Unraveling the tomato plants reactions to pathogens is necessary in order to establish effective methods to control pests and to develop tolerant tomato cultivars. We discuss here the results of a comparative analysis of proteins and metabolites upon infection of TYLCV of two different tomato genotypes: one is resistant (R) and the other is susceptible (S) to the virus.

TYLCV is a whitefly-transmitted geminivirus, which threatens tomato production worldwide. While the domesticated tomato *S. lycopersicum* is susceptible to the virus, natural sources of resistance have been found in several wild tomato species such as *S. chilense*, *S peruvianum*, *S. pimpinellifolium* and *S. habrochaites*. Breeding resistant cultivars consisted of introgressing the resistance genes from the wild into the domesticated tomato [2]. As a result the resistant tomato contains chromosomal fragments from wild species bearing resistance loci identifiable with polymorphic DNA markers [3]. Five major loci (*Ty-1* through *Ty-5*) from wild tomato species associated with resistance to TYLCV and related begomoviruses have been identified so far and mapped on tomato chromosomes using such markers [4]. However, the genes conferring resistance to TYLCV are unknown.

In the course of a breeding program aimed at developing TYLCV-resistant tomato lines, we have used *S. habrochaites* as the resistant wild tomato genitor [5]. Two inbred lines have been developed; one is susceptible to the virus (S), while the other is resistant (R). Upon whitefly-mediated inoculation of TYLCV, S plants are stunted, rapidly accumulate large amounts of virus and do not yield, R plants remain symptomless, contain lesser amounts of virus and yield. To identify genes important for the resistant phenotype, we have performed a differential screen that has allowed identifying approximately 70 different cDNAs representing genes preferentially expressed in R plants at the early stage of TYLCV infection [6]. Among them a Permease I-like protein gene encoding a transmembranal transporter was upregulated in R plants following TYLCV inoculation. Similar results were obtained for the Hexose transporter gene *LeHT1* [7] and for a Lipocalin-like gene (unpublished). These three genes do not encode proteins known to be involved in stress response or in the regulation of the immune response. In parallel, to distinguish between S and R genotypes, we have compared the activities and expression patterns of canonic stress response proteins such as pathogenesis-related (PR) proteins, mitogen-activated protein kinases (MAPKs) heat shock proteins (HSPs) and proteases, before and after TYLCV inoculation [1,8,9]. The patterns of PRs accumulation in S and R tomatoes were

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comparable at the early stage of infection; differences were detected only 20-30 days (dpi) after the onset of inoculation. Similarly, at the early stages of infection, the patterns of regulatory proteins such as MAPK-like kinases and different HSPs/chaperones, including FtsH protease, were similar in both tomato genotypes. However, at later stages the decrease in the amounts of stress proteins was more pronounced in S than in R plants. The present report discusses the behavior of TYLCV resistance versus susceptible tomato genotypes at the proteome, transcriptome and metabolome levels in order to understand how the two tomato genotypes cope with the viral stress.

By investigating the extent of TYLCV coat protein (CP) aggregation in S and R plants, we observed that CP formed aggregates of increasing size as infection developed. The formation of large aggregates during the early stages of infection was a characteristic of S plants; in contrast, in R plants the formation of large aggregates was delayed [10]. In R plants, mid-sized CP aggregates persisted up to 21-35 days after the onset of infection, while in S plants massive CP-containing inclusion bodies appeared. At later infection stages CP was found to be associated exclusively with inclusion bodies in R as well as in S plants. We considered that the time span when the levels of CP aggregation are so different was adequate to launch a comparison of metabolites and proteins in infected R and S tomatoes. We postulate that this was the period when the plant immune response machinery succeeds or fails to withstand virus expansion.

Materials and Methods

Sources of virus, insects and plants and inoculation plants with TYLCV

TYLCV was maintained in tomato plants [11]. TYLCV resistant and susceptible inbred tomato lines were described [5]. All plants were grown in a greenhouse according to the regulations of the Israel Plant Protection Authorities. Tomato plants at their 3-5 true leaf stage were caged with viruliferous whiteflies for the duration of the experiments (about 50 insects per plant at the onset of infection). Whiteflies were discarded before tissue handling.

Extraction and immunodetection of plant proteins

Leaf tissues (100 mg of pooled 2-3 young leaves from three plants) were drill-homogenized in 30 mM Tris-HCl pH 7.5, 60 mM KCl, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.5% Nonidet P40, 1 mM dithiothreitol and Complete Protease Inhibitor Mixture (Roche, Mannheim, Germany). Homogenates were incubated on ice for 45 min with vortex and centrifuged at 1,200 g for 10 min at 4°C to discard debris. PAGE sample buffer with SDS was added to supernatants, and proteins were separated by SDS PAGE of 10-12%.

Western blotting was performed according to standard procedures. The source of antibodies (polyclonal) was as follows: anti-HSP70 cytosolic, chloroplastic, anti-BiP and goat peroxidase-coupled secondary antibodies were purchased from Agrisera (Sweden), anti-LapA was a gift from Prof. Walling (Frostburg State University, USA), Phospho-AMPKα Thr172 polyclonal antibody was supplied by Cell Signaling Technology (USA). Incubation with antibodies was followed by ECL detection (Amersham, UK). Each immunodetection was repeated at least three times (pooled tissues from three plants).

Sample preparation for mass spectrometry

Protein samples were run just 1 cm into the separating gel, then electrophoresis was stopped and the gels were stained with Coomassie blue. The total protein content of each fraction was cut out as a gel block and in-gel digested with trypsin (Roche, Germany) according to Shevchenko et al. [12]. After digestion the supernatant was removed from the gel pieces and transferred to a new tube. Gel pieces were extracted with 70% acetonitrile (ACN)/30% 0.1% formic acid (FA) (v/v) for 15 min and the supernatant was collected. The pooled supernatants were then lyophilized in a vacuum centrifuge and stored at -20°C. For Nano-LC-ESI-MS/MS experiments dried samples were resuspended in 0.1% formic acid.

Mass spectrometry

Nano-LC-ESI-MS/MS experiments were performed on an ACQUITY nano-UPLC system (Waters, Milford, USA) directly coupled to a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher, Bremen, Germany). Tryptic digests of sucrose gradient fractions were concentrated and desalted on a precolumn (2 cm x 180 µm, Symmetry C18, 5 µm particle size, Waters, Milford, USA) and separated on a 20 cm x 75 µm BEH 130 C18 reversed phase column (1.7 µm particle size, Waters, Milford, USA) using a linear gradient of 1-50% ACN in 0.1% FA within 30 min. The LTQ-Orbitrap was operated under the control of XCalibur 2.0.7 software. Survey spectra (m/z = 250-1800) at a resolution of 60.000 at m/z = 400 were detected in the Orbitrap. Data-dependent tandem mass spectra were generated for the five most abundant peptide precursors in the linear ion trap. For all measurements using the Orbitrap detector internal calibration was performed using the polycyclodimethylsiloxane background ion m/z 445.1200 from ambient air as lock-mass [13].

MascotTM 2.2 software (Matrix Science, London, UK) was used for protein identification. MS/MS-Spectra were searched on an in-house Mascot server against the plant and virus subset of the NCBI protein sequence database downloaded as FASTA-formatted sequences from ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz. Search parameters specified trypsin as cleaving enzyme allowing two missed cleavages, a 3 ppm mass tolerance for peptide precursors and 0.6 Da tolerance for fragment ions. Carbamidomethylation of cysteine residues was set as fixed modification and S,T,Y phosphorylation and methionine oxidation were considered as variable modifications.

Scaffold (version Scaffold_3_00_03, Proteome Software Inc., Portland, OR) was used to validate peptide and protein identifications. Peptide and protein identifications were accepted if they show greater than 95.0% probability as specified by the Peptide Prophet algorithm [14]. Protein identifications were accepted if they show greater than 99.0% probability assigned by the Protein Prophet algorithm [15] and contained at least 2 identified peptides. Scaffold was also used for label free quantification based on spectral count values. Unweighted spectral count values were converted into normalized spectral count values to calculate fold changes between R and S samples.

Metabolite profiling

GC-MS analysis was performed as described previously [16]. Six replicates each consisting of three infected tomatoes independent were subjected to GC-MS analysis. Metabolite levels were determined in a targeted fashion using the Target Search software package [17]. Metabolites were selected by comparing their - retention indexes (+/– 2 s) and - spectra (similarity > 85%) against the- compounds stored in the Golm-Metabolome-Database [18], this resulted in metabolites kept in the data matrix. Each metabolite is represented by the observed ion intensity of a selected unique ion which allows for a relative quantification between groups.

Real time PCR analysis

RNA was extracted from tomato leaves using Tri-Reagent (Sigma-Aldrich, USA). cDNA was prepared using the EZ-first strand cDNA synthesis kit according to the manufacturer (Biological Industries, Israel). The primer pair used to amplify a fragment of 179 bp of *Pin 2* was F 5'- GCCAAATGCTTGCACC-3' and R 5'-GGGTTCAT-CACTCTCTCCTTC-3'. The primer pair used to amplify a 158 bp of *Proteinase A inhibitor* was F 5'-ATGGCTGTCCACAAGGAAGT-3' and R 5'-ATGCATATGGGATTTAGCGG-3'. The primer pair used to amplify a 130 bp of *bZIP34* was F 5'-TTGCATTTTTGGACCAT-CAA-3' and R 5'- CTCAATTTCTTTCTTCAATGCTTC-3'. The primer pair used to amplify a 180 nt of *β-actin* (used as calibrator) were F 5'-GGA AAAGCTTGCCTATGTGG-3 and R 5'-CCTGCAGCTTC-CATACCAAT-3'. The primer pair used for tomato gene SGN-4346908 expressed was F 5'-GCTAAGAACGCTGGACCTAATG-3 and R 5' -TGGGTGTGCCTTTCTGAATG-3', amplifying a fragment of 183-nt. Quantitative real-time PCR was carried using the Light- Cycler 480 Real-Time PCR System (Roche) with the following cycling conditions: 10 min at 95°C, 45 cycles consisting of 10 s at 95°C, 60 s at 59°C. The results were analyzed using the software provided by the manufacturer. All PCR experiments were repeated three times (three biological repeats and three technical repeats).

Results and Discussion

In our experiments, the tomato plants were continually caged with viruliferous whiteflies. Consequently, the plants were repeatedly inoculated with the virus and constantly submitted to this stress, as it occurs in the field. The amounts of TYLCV CP and DNA in S and R leaves increased steadily from the onset of inoculation; however CP and DNA accumulation was slower in R than in S plants (not shown). As a result of this continuous inoculation, 4 weeks after the onset of whitefly-mediated TYLCV inoculation, virus amounts in S plants were only about 300 times those in R plants. By comparison, at the early stages of virus infection or when the S and R tomato plants were inoculated for a mere 3-day-period (in the greenhouse) the amount of virus in S plants was higher than in R plants by three orders of magnitude [6]. This discrepancy can be explained by the continuous influx of virus in the present case, versus a short inoculation period in the later. In spite of the absence of dramatic difference in virus accumulation between the two tomato lines, R tomatoes remained symptomless and yielded.

Proteins and metabolites were extracted from tomato leaves four weeks after the onset of TYLCV inoculation (28 dpi). The selected time span was enough to allow the development of differential CP aggregation, as shown by ultracentrifugation through sucrose gradients (Figure 1; discussed in details in Gorovits et al. submitted). At this time, most of the CP was already highly aggregated in S plants while midsized aggregates were still conspicuous in R plants, a feature associated with R tomatoes. At later times, the levels of CP aggregation in R tomatoes were similar to those in S plants. We hypothesized that differential CP aggregation is a feature associated with resistance. Retarded CP aggregation indicates that the cellular mechanisms sustaining resistance are still active. Metabolite profiles were analyzed in the R and S tomato lines at 28 dpi. Additional analyses were also performed at earlier (at 10 and 15 dpi) and at later (at 42 dpi) times after the onset of infection.

ROS response in infected tomato plants

The oxidative burst, a rapid, transient, production of huge amounts of reactive oxygen species (ROS), is one of the earliest observable aspects of a plant's defense strategy. As a result of biotic and abiotic stresses the plant intracellular levels of ROS rapidly rise [19] (and references therein). The rapid ROS accumulation at the site of pathogen attack, a phenomenon called oxidative burst, is directly toxic to pathogens [20]. It can lead to a hypersensitive response (HR) that results in a zone of host cell death, which prevents further spread of pathogens [21] (and references therein). ROS/HR can be detected by measuring hydrogen peroxide (H_2O_2) in living tissues [22]. H_2O_2 is commonly localized in plant tissues by 3,3-diaminobenzidine (DAB), producing a brown stain [23]. TYLCV infected tomato leaves showed a brownish color; the intensity of the stain increased with the development of the infection and was much more pronounced in S than in R plants (Figure 2). Therefore, cells of S plants need potent mechanisms in order to detoxify this excess of ROS and keep the balance of ROS formation/removal.

Anti-oxidative proteins: ROS scavenging mechanisms in plants involve enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT). SODs act as the first line of defense against ROS, dismutating superoxide to H_2O_2. APX, GPX, and CAT subsequently detoxify H_2O_2. Most anti-oxidative enzymes were detected in TYLCV infected tomatoes. SODS, APX, thioredoxin peroxidase, ferredoxin-nitrite reductase were more abundant in S then in R plants (Table 1). Plant thioredoxins are the key factors in oxidative stress response. They are involved in detoxification of H_2O_2 via thioredoxin peroxidase [24]. The amount of thioredoxin peroxidase was higher in S plants, which

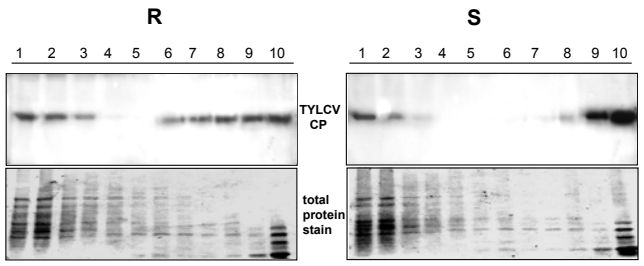

Figure 1: Distribution of TYLCV CP aggregates following sedimentation on linear 10-50% sucrose gradients. Leaf homogenates were prepared from R and S plants at 28 dpi. Gradients were divided into 10 fractions, 1 (top) to 10 (bottom). Aliquots were subjected to 12% SDS-PAGE. The gels were stained with Coomassie blue (total protein) and western blotted. CP was detected using anti-CP antibody.

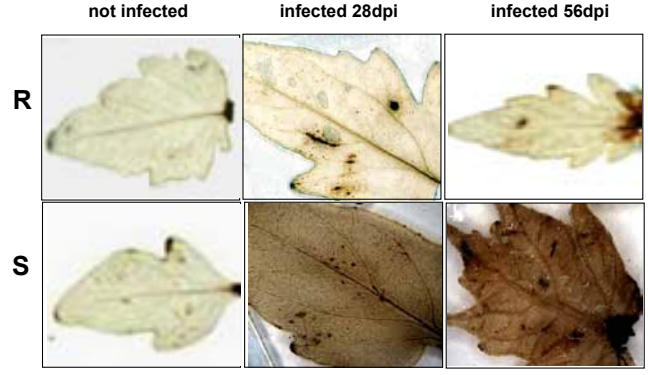

Figure 2: Oxidative burst-related damages detected with DAB. Leaves of S and R tomato genotypes were DAB-stained after 0, 28 and 56 dpi. Release of H2O2 was detectable as a reddish brown precipitate.

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S. No	Name of protein	accession N	R	S	R:S ratio
1.	ribulose-1,5-bisphosphate carboxylase large subunit (RuBisCO)	gi|349144	163	54	3
2.	malate dehydrogenase	gi|52139816	45	42	1
		gi|77999077	16	27	0.7
		gi|52139820	8	14	0.6
		gi|56562183	5	12	0.4
3.	mitochondrial ATP synthase subunit beta	gi|114421	57	34	1.7
4.	ATP synthase beta subunit (53 kDa)	gi|21633353	15	61	0.25
5.	phosphoglycerate kinase precursor	gi|3328122	47	28	1.7
6.	chaperonin-60 beta subunit	gi|1762130	49	11	4.5
7.	glucan endo-1,3-beta-D- glucosidase	gi|498926	36	20	1.8
8.	RuBisCO activase	gi|10720247	36	26	1.4
9.	dehydroascorbate reductase	gi|66475036	33	19	1.7
10.	inhibitor of yeast proteinase A	gi|8648959	0	47	∞
11.	glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic	gi|120661	27	15	2.8
12.	chlorophyll a-b binding protein	gi|115802	52	94	0.56
13.	glycine-rich RNA-binding protein	gi|45533923	23	14	1.6
14.	elongation factor 1-alpha	gi|119150	28	11	2.5
15.	wound-induced proteinase inhibitor II	gi|125053	0	36	∞
16.	mRNA binding protein precursor	gi|26453355	8	23	0.35
17.	RuBisCO small subunit 1	gi|132079	18	14	1.3
18.	photosystem II CP47 chlorophyll apoprotein	gi|114107158	8	21	0.38
19.	enolase	gi|119354	21	10	2.1
20.	arginase 2	gi|54648782	4	25	0.16
21.	photosystem I P700	gi|225198	9	16	0.56
22.	photosystem II protein D2	gi|164597815	4	20	0.2
23.	P14a	gi|157830592	0	24	∞
24.	basic chitinase	gi|544011	3	17	0.18
25.	leucine aminopeptidase	gi|1236654	6	17	0.35
26.	HSP68	gi|300265	14	6	2.3
27.	acidic endochitinase	gi|544010	5	15	0.3
28.	histone H4	gi|462243	13		1.9
29.	polyphenol oxidase precursor	gi|1172583	0	17	∞
30.	cytochrome b6	gi|114329999	4	13	0.3
31.	S-adenosyl-l-homocysteine hydrolase	gi|21362943	13	4	3.3
32.	glyceraldehyde 3-phosphate dehydrogenase	gi|2078298	12	4	3
		gi|15217555	15	0	∞
		gi|256862074	14	0	∞
33.	phosphoglycerate mutase	gi|4582924	12	4	3
34.	chlorophyll a-b binding protein 3C	gi|115825	0	75	∞
35.	subtilisin-like protease (P69B)	gi|2230959	2	13	0.15
36.	non-specific lipid transfer protein	gi|71360928	15	0	∞
37	plastid-lipid-Associated Protein	gi|2632090	2	0	∞
38.	putative ascorbate peroxidase	gi|11387206	5	10	0.2
39.	Chlorophyll a-b binding protein CP24	gi|115833	8	5	1.6
40.	chlorophyll a/b-binding protein CAB	gi|170388	2	11	0.2
41.	aspartate aminotransferase	gi|15236129	11	2	5.5
42.	photosystem II oxygen-evolving complex protein 3	gi|51457944	9	4	2.25
43.	Hsp90-1	gi|38154489	13	0	∞
44.	thaumatin-like protein (PR)	gi|146737976	2	10	0.2
45.	putative photosystem I subunit III precursor	gi|30013659	2	12	0.2
46.	adenine phosphoribosyltransferase	gi|82621166	8	4	2
47.	alpha-mannosidase	gi|301176645	4	8	0.5
48.	dihydrolipoamide dehydrogenase precursor	gi|23321340	9	2	4.5
49.	chain L Photosystem I Supercomplex	gi|149242538	2	8	0.25
50	endochitinase	gi|116349	0	14	∞
51.	actin	gi|110612122	6	2	3
52.	thioredoxin peroxidase	gi|21912927	2	6	0.3
53.	ferredoxin--nitrite reductase	gi|19893	0	8	∞
54.	enoyl-ACP reductase	gi|2204236	8	0	∞

55.	Os06 putative FtsH	gi|115469444	7	0	∞
56.	deoxyuridine triphosphatase	gi|416922	7	0	∞
57.	ascorbate free radical reductase	gi|50400860	0	7	∞
58.	F1-ATPase alpha subunit	gi|57115599	8	0	∞
59.	photosystem I reaction center II	gi|131166	26	41	0.6
60.	photosystem II cytochrome b559 alpha subunit protein	gi|11497544	5	0	∞
61.	aminomethyltransferase	gi|1707878	5	0	∞
62.	enoyl-ACP reductase	gi|2204236	8	0	∞
63.	deoxyuridine triphosphatase	gi|416922	7	0	∞
64.	fructokinase 3	gi|38604456	4	0	∞
65.	subtilisin-like protease	gi|2230959	2	13	0.15
66.	pectinesterase	gi|1944575	0	6	∞
67.	superoxide dismutase	gi|33413303	0	5	∞
68.	superoxide dismutase chloroplastic	gi|134682	0	4	∞

Table 1: The relative amounts of proteins, analyzed in infected R and S tomatoes at 28 dpi.

indicates a higher abundance of antioxidative proteins in this genotype. Several additional oxidases were also found to be more abundant in S plants, such as glycolate oxidases and polyphenol chloroplastic oxidase, which is known to be activated by virus infection and by wounding [25]. It has to be mentioned, that not all detected anti-oxidant enzymes are more expressed in infected S than R cells. For example, monodehydroascorbate reductase, one of the major anti-oxidants involved in the gluthatione-ascorbate cycle, was more abundant in R than in S plants. The same was true for dehydroascorbate reductase, which is induced by jasmonic acid (JA); together with the accumulation of ascorbate (amounts in R exceeding those in S, shown in section 3.4) they are important defense components against oxidative stress [26]. Since TYLCV suppresses JA production [27], this may explain the higher amount of dehydroascorbate reductase in R plants.

Compounds involved in ROS metabolism: Glucosamine (GlcN) is a well known metabolic compound involved in ROS metabolism. This amino sugar is produced by amidation of fructose-6-phosphate. The decrease of GlcN amounts following the onset of TYLCV inoculation of R and S tomatoes was followed by a sharp increase at 25-28 dpi; the increase was stronger in S than in R tomatoes. Such a pattern is in accordance with the burst of ROS during this period, higher in R than in S plants (Figure 3a). Genetic manipulation of endogenous GlcN levels can effectively lead to the generation of stress-tolerant transgenic crop plants. For example, the reduction of GlcN-induced ROS in *Arabidopsis* enhanced drought tolerance. Scavenging of endogenous GlcN by ectopic expression of *E. coli* glucosamine-6-phosphate deaminase conferred enhanced tolerance to oxidative, drought, and cold stresses. On the contrary, overproduction of GlcN by the ectopic expression of *E. coli* glucosamine-6-phosphate synthase induced cell death at an early stage [28]. Consistent with these data, the lower GlcN levels in R vs. S plants may point to the potential tolerance of R tomatoes to several abiotic stresses [9].

Pathogenesis-related (PR) proteins and wound response proteins

The localized cell death associated with the HR may help to prevent the pathogen from spreading to uninfected tissues. Just before or concomitant with the appearance of a HR is the increased synthesis of several families of pathogenesis-related (PR) proteins in the inoculated leaves. Many of these proteins have been shown to possess anti-pathogenic activity either *in vitro* or *in vivo*. PR proteins are concurrent with the development of a long lasting, broad-based resistance known as systemic acquired resistance (SAR), interestingly, although plants lack a circulatory system and do not produce antibodies [29] (and references therein).

PR proteins: PR proteins are a family of host-encoded plant proteins induced and accumulated in the plant tissues under a number of biotic or abiotic stresses. A role for these proteins as defensive tools against bacteria and fungi has been proposed on the ground that some PR proteins display hydrolytic activities such as chitinase, 1,3-glucanase and protease [30]. Chitinases and endoglucanases are PR proteins capable of hydrolyzing chitin and b-1,3-glucans of fungal cell walls. The induction of these two enzymes has been well documented in several plant tissues as a response to a range of biotic and abiotic stress [31]. TYLCV prolonged infection (after 28 dpi) was accompanied by a low increase of total chitinase activity and to a strong increase in β-1,3-glucanase activity [32]. S and R tomatoes presented similar patterns upon virus stress. The current proteomic analysis revealed different patterns of several other PR proteins. After 28-30 dpi several specific chitinases (basic endochitinases with MW 30 kDa, 27 kDa and 35 kDa, acidic endochitinases with MW 27 and 35 kDa) were more abundant in S than in R leaves (Table 1). Glucanases were not detected. The expressions of both chitinases (PR-3) and glucanases (PR-2) were shown to be slightly induced by another plant virus, *Tobacco mosaic virus* TMV [33].

The PR-1 protein P14a was detected in S but not in R leaves one month after the onset of TYLCV inoculation (Table 1). P14a is the most abundant acid-extractable tomato leaf protein upon infection with pathogens [34]. P14a is regulated by salicylate, an inducer of SAR. Another PR protein, P69, was discovered in *S. lycopersicum* by screening a genomic library with a cDNA probe encoding a subtilisin-like protease; as a result, four different subtilisin-like protease genes with high homology were isolated [35]. In our experiments, we found one subtilisin-like protease with MW 79 kDa. Its amount was 6.5 times higher in S than in R tomatoes. Tomato P69 was shown to be induced at the transcriptional level following pathogen attack [36]. Plant genome analyses have not revealed clear orthologues of caspase genes, indicating that enzyme(s) structurally unrelated yet possessing caspase specificity have functions in plant PCD. Recent data showed that some caspase-like activities are attributable to plant subtilisin-like proteases [37], regulating PCD during both abiotic (oxidative and osmotic stresses) and biotic (virus infection) stresses. If the S predominant 79 kDa subtilisin-like protease possesses a caspase activity, it might be possible to link it to the increased levels of cell death observed in infected leaves of S tomatoes. An additional regulated PR protein was a thaumatin-like protein (MW 23 kDa). Its amount in TYLCV infected S plants was five times higher than in infected R plants (Table 1). Thaumatine

was reported to accumulate in tomato leaves upon Citrus exocortis viroid (CEVd) infection [38]. Pectinesterase was found only in infected leaves of S plants. This enzyme is known to be involved in cell wall modification. Pectinesterase is one of the components of insects saliva, together with alkaline phosphatase, polygalacturonase, peroxidase, and sucrose, that can act individually or in concert stimulate host plant defenses, directly or indirectly [39-41].

Wound-induced proteins: The wound response of tomato plants has been extensively studied. The principal markers that have been used in these studies were genes encoding proteinase inhibitor (pin) proteins. Activation of pin genes occurs in the wounded leaf and in distant unwounded leaves of the plant [42]. The expression levels of wound-induced proteinase inhibitor II (Pin2) were shown to be essential for coping with damages caused by biotic stresses to plant cells [43]. In R tomatoes, virus replication and spread is restrained, and the leaves remain asymptomatic. Since there is much less damage to plant cells in R than in S plants, we expected that R plants will express *Pin2* at lesser levels than in S tissues, where cellular damage is accompanied by the induction of protease inhibitors. However, the proteomic analysis has not detected this important marker of wound response in R 28 dpi, while in S tomatoes the level was very high (Table 1). We used real-time PCR to determine the kinetics of *Pin2* expression in TYLCV infected tomato leaves of R and S plants (Figure 4). The basal levels of *Pin2* transcripts in R plants were lower by two orders of magnitude compared to S plants. With the onset and the development of infection (7-14 dpi), *Pin2* transcript levels increased more rapidly in S than in R plants; at the later stages (28-42 dpi), when TYLCV amounts in both lines were comparable [10], the levels of *Pin2* transcript in the two genotypes were comparable. Very similar patterns were detected for the other known wound-induced protein, the inhibitor of yeast proteinase A (Figure 4).

Furthermore, the present proteomic analysis did reveal that another plant wound-response protein, leucine aminopeptidase (LapA), was present in amounts three times higher in S than in R tomatoes (Table 1). These results have been confirmed by immunodetection with anti-LapA antibodies in protein extracts of infected tomato leaves (not shown). LapA was found to play an essential defense role against herbivores by promoting late wound responses, and by acting downstream of JA biosynthesis and perception [44]. It was also shown to be upregulated by TMV in tomato fruits [33]. Pin2 is regulated by LapA; Pin2 RNA disappeared more rapidly in wounded leaves of silenced LapA-SI than in wild-type plants. In tomatoes where LapA-OX was over-expressed, Pin2 RNA persisted for longer periods of time [44]. Both Pin2 and LapA are well characterized wound-response proteins, known to be JA-responsive and dependent on the jasmonate receptor JAI1 [45].

In addition to Pin2 and LapA, late wound-response proteins such as polyphenol oxidases (PPOs) and arginase were identified in TYLCV-infected tomato leaves. The amounts of chloroplast PPO in R and S tissues were similar. However, PPO precursor was found in S but not in R tomatoes. The amounts of arginase were approximately six times higher in S than in R leaf tissues (Table 1). Stress-susceptible plants are characterized by massive induction of arginase and also by the accumulation of proline [46]. There were approximately equal amounts of proline in R and S plants at 28 dpi, while prolonged TYLCV infection led to decrease of proline in R and increase in S.

As whole, these results demonstrated higher ROS (and consequently anti-oxidative) activities in S compared to R TYLCV infected tomatoes, as well as the increased amounts of other markers of host defense, PRs and wound-induced proteins. This situation could reflect the elevated defense level of host cellular machinery against virus invasion and virus-caused damages occurring in S tomatoes at 25-30 dpi. As mentioned above, during that time, in S cells the virus CP or whole virions concentrate in large aggregates/inclusion bodies where they could be protected from degradation and where they could develop virus factories promoting replication of the virus genome, similar to those shown for various mammalian viruses [47]. Moreover, at this time the typical TYLCV symptoms become obvious in S tomatoes. Our current data suggest that at this time of infection, TYLCV finally overcome the defenses of S plants, while R plants continue their regular

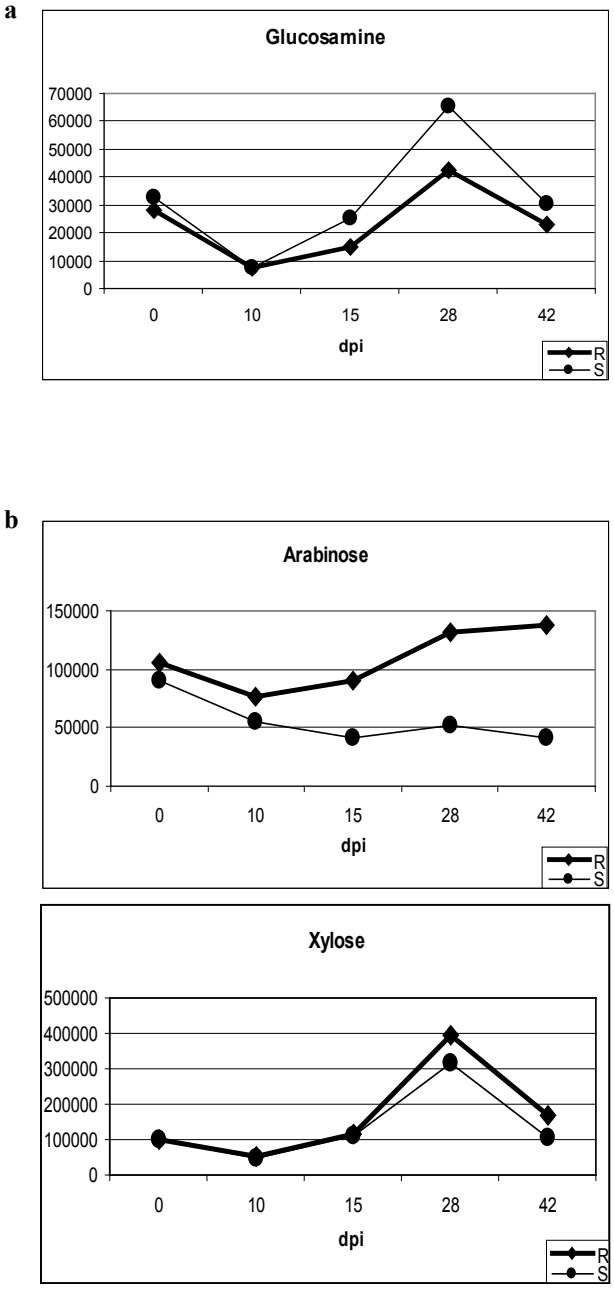

Figure 3: Changes of several metabolites in tomatoes, infected by TYLCV for 0, 10, 15, 28, 42 dpi. a. Glucosamine patterns. b. Arabinise, Xylose patterns. Metabolite contents were expressed as micromoles per gram units.

development without the massive activation of immune defense mechanisms characteristic of infected S tomatoes. To strengthen these findings, there is a need to investigate the patterns of the main signaling compounds, salicylic acid (SA), JA, abscisic acid (ABA), and ethylene, as well as the *de novo* production of defense-related secondary metabolites such as phytoalexins and various phenols.

Maintenance of homeostasis in TYLCV infected tomatoes

Chaperones/heat shock proteins (HSPs): The HSPs are a diverse subset of molecular chaperones that generally promote the proper folding of proteins after translation and also prevent their aggregation during cellular stress. The general function of the intracellular molecular chaperones has been suggested mainly to involve housekeeping and cytoprotection against various environmental stresses. The expression of many chaperones is induced during viral infection, either to facilitate viral pathogenesis or to participate in cellular response mechanisms to alleviate the stress caused by infection [48,49]. While increased expression of HSPs was suggested to be a biomarker for some viral infections [50], we have shown previously that TYLCV did not induce the expression of tomato HSP60, 70, 90, 100 and FtsH [8]. Prolonged infection led to a decline in the amounts of HSPs, which was more pronounced in S than in R plants.

Several key chaperones have been detected by MS analysis in infected leaves. Chaperonine60, HSP68/DNA K, Chaperone90 and putative FtsH were more abundant in R than in S samples (Table 1). At the same time, the amounts of 71 kDa HSP70-3 and of chloroplast HSP70-2 were similar in both tomato lines. F1-ATPase α subunit, which share homology with HSP60 [51] and HSP70 [52], was detected in R but not in S plants.

The levels of expression of cytosolic, chloroplastic and endoplasmic reticulum HSP70s (known as BiP) were appraised by immunodetection. During the first 2-3 weeks after the onset of TYLCV infection, the patterns of cytosolic and chloroplastic HSP70 were stable, while BiP was slightly induced BiP. Prolonged infection caused the decline of the HSP70s (Figure 5a). The decline in the abundance of HSP70s became evident during the fourth week of infection, emphasizing the relevance of the time selected for comparative MS analysis. At this time, the decline in the abundance of HSP70s was more pronounced in S than in R plants. Interestingly, the expression of HSP70s was not induced during early infection, which could be explained by the fact that TYLCV causes only gentle damages to plant tissues; HSP70s were not upregulated even at the later periods, when TYLCV CP developed large aggregates, especially in S cells. In contrast, the induction of HSP70 was shown to be correlated with the amount of aggregated viral CP during TMV infection [53]. Using sucrose gradient ultracentrifugation, we have detected an increase in amounts of HSP70s associated with large insoluble aggregates (not shown), which points to a possible co-localization and interaction of virus CP and various host HSP70s. Indeed, the abundance of aggregated HSP70s in S was higher than in R plants, while soluble HSP70s were predominant in R plants. Hence we suggest that in R plants HSPs play mostly a housekeeping role in the maintenance of homeostasis on the background of low biotic stress. In contrast, in S cells the virus utilizes the host HSPs to develop virus factories, shown to be involved in the insoluble inclusion structures [47,54]. Our future investigations will be towards this hypothesis.

Among the HSPs analyzed, only the expression of BiP was slightly induced by TYLCV. The transcription factor bZIP34 that activates BIP was analyzed by qPCR. *bZIP34* is a transcription factor that was shown to induce BiP1 expression in *Arabidopsis* [55]; the tomato homologue of the *Arabidopsis* gene atbZIP34 has not been characterized yet. A BLAST analysis has allowed us to discover a tomato BiP2 gene. The amounts of BiP2 increased upon TYLCV infection (7 to 21 dpi) in both

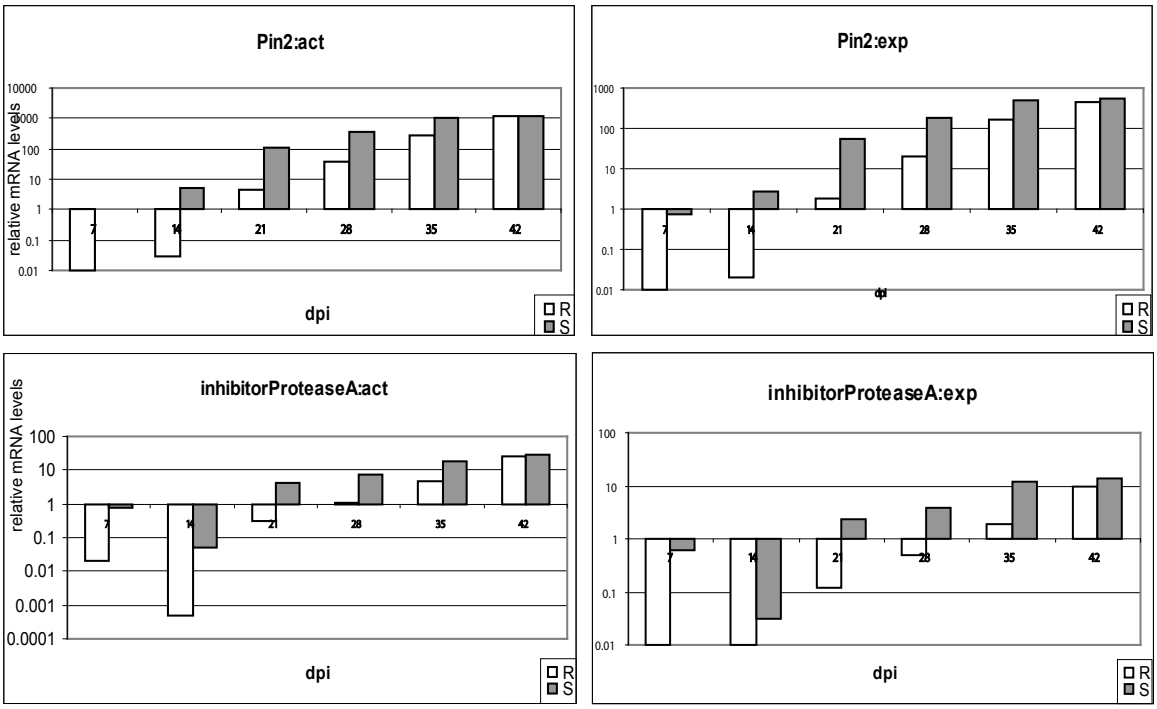

Figure 4: Relative transcript levels of the genes *pin2* and *proteinase A* in TYLCV infected tomatoes. Values were obtained by real-time quantitative RT-PCR after 7, 14, 21, 28, 35, 42 dpi and normalized to the host *β-actin* gene and tomato gene SGN-4346908 Expressed.

S and R tomato genotypes, then decreased, more abruptly in S than in R plants (Figure 5a). The induction of BiP by TYLCV at the early stage of infection coincided with transcriptional upregulation of its transcription factor bZIP34 (Figure 5b). Hence the increase followed by the decrease in the amounts of BiP chaperone in R and S plants under prolonged infection may occur by means of transcriptional regulation. Chaperons like BiP play an important role in protein synthesis and in the protection of cellular structures during stress-related processes. Therefore, the increased stability of protein and chemical chaperons in R plants may result in the development of a balanced state, allowing cells to protect themselves from virus-induced damages.

Sugar balance in infected tomatoes: Such chemical chaperons as sugars were detected by GCMS in infected R and S leaves. Glucose and mannose showed similar stable patterns in R and S tomatoes during prolonged virus infection. TYLCV caused down-regulation of arabinose and xylose, followed by a later increase (Figure 3b). At later stages of virus infection, the amounts of these sugars were higher in R than in S tomatoes. Moreover, the abundances of derivatives of these sugar, such as glucose 6-phosphate, fructose 6-phosphate and fructose-1,6-phosphate were more elevated in R than in S plants, all along the infection.

Hence, in TYLCV stressed plants, homeostasis was provided not only by proteins, but also by chemical chaperons. The stability of the patterns of both types of chaperons was characteristic of the resistant plants.

Photosynthesis components: Chlorophyll content is an important indicator of photosynthesis activity and of reversible and irreversible changes of photosystem II reaction centers in plants. In a previous study, we showed higher chlorophyll content in R compared to S plants under stress application [9]. Here MS analysis revealed three times higher amounts of ribulose-1,5-bisphosphate carboxylase large subunit (RuBisCO) in R than in S leaves (Table 1). RuBisCO activase was about 1.4 times higher in R than in S plants. Protein analysis by non-denaturing gel electrophoresis showed that *Arabidopsis* chaperonin-60 was associated with RuBisCO activase in a high molecular mass complex. This association was dynamic, increasing with the duration and intensity of the stress, suggesting that chaperonin-60 plays a role in acclimating photosynthesis to stress, possibly by protecting RuBisCO activase from denaturation [56]. Increased amounts of RuBisCO, RuBisCO activase and chaperonin-60 in R plants may point to a similar acclimation of photosynthesis to TYLCV, which is more effective in infected R then S tomatoes.

Many photosynthesis proteins such as oxygen-evolving enhancer protein 2, photosystem I subunit VII, photosystem II D1 and HSF136 binding proteins had similar amounts in the S and R lines (Table 1). Other photosystem II proteins, such as oxygen-evolving complex protein 3 and cytochrome b559, have been detected in amounts higher in R than in S plants. On the other hand, several proteins were much more abundant in S than in R tomatoes at 28 dpi; these included

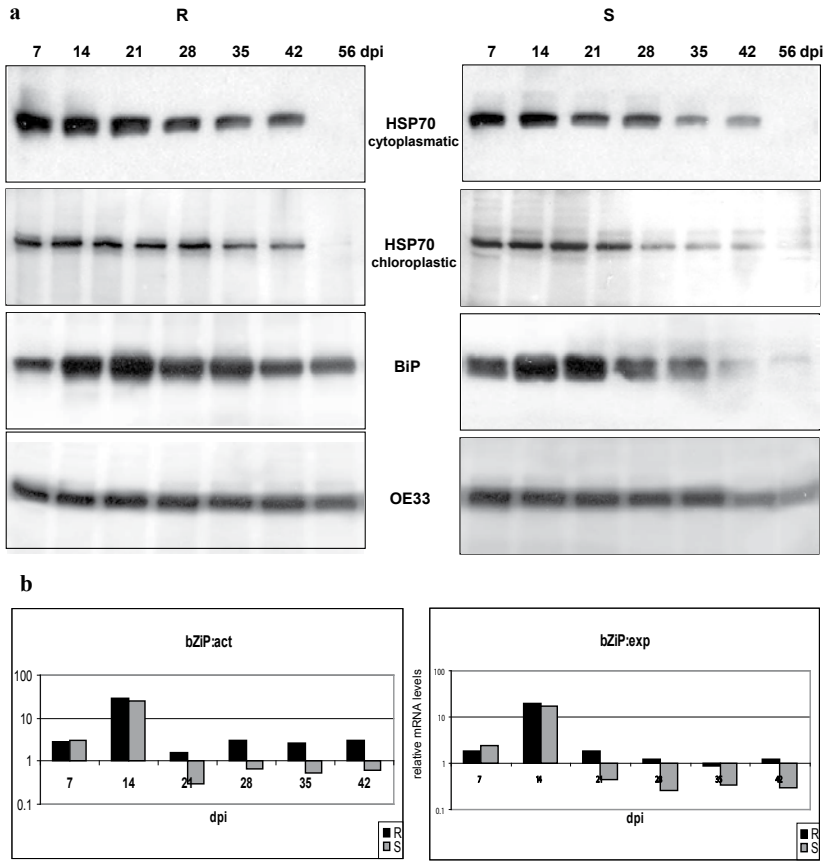

Figure 5: Kinetics of accumulation of plant HSPs in TYLCV R and S infected tomatoes. a. Immunodetection of cytoplasmic, chloroplastic HSP70 and BiP in leaf tissue after 7, 14, 21, 28, 35, 42, 56 dpi. HSPs were detected using anti-HSP70 cytosolic, chloroplastic and ER antibodies. Patterns of plant protein OE33 were used as internal control. b. Relative transcript levels of BiP's transcription factor bZIP34, normalized to the host genes. Values were obtained by real-time quantitative RT-PCR in leaves of tomatoes, infected during prolonged time.

photosystem II CP74, D2, photosystem I P700, III precursor, 20 kDa Subunit, and chlorophyllic a-b binding proteins.

Amino acids balance: TYLCV infection induced a decrease in the amount of many amino acids in tomato leaves (not shown). The ratio of amino acid amounts in R vs. S plants showed that this decrease was more pronounced in S than and in R plants. This ratio increased with time: at 28 dpi (at the time disease symptoms start to be visible), it was 1.2-1.4 for Phenylalanine, 1.2-4 for Alanine and Alanine-β, 2 for Asparagine, 2.7 for Aspartic acid, and 2.2 for Glycine (Figure 6a). One exception was Lysine: its amount was higher in S than in R leaves.

The assimilation of nitrogen onto carbon skeletons has significant effects on plant development and yield. Studies have shown that nitrogen deficiency encourages disease development, presumably because nutritionally stressed plants are weaker and therefore are less able to defend themselves against pathogens [57]. In this view, the increase in the amino acids content in R vs. S leaves under prolonged infection acquires a specific importance. A novel cycle that generates asparagines for more energy-economical nitrogen remobilization was shown to be important in plants subjected to darkness, stress and starvation [58,59]. Aminomethyltransferase and glycine decarboxylase are involved in the breakdown of glycine, which when present in the form of glycine betaine is involved in osmotic adjustment during drought stress [60]. Aspartate aminotransferase was 5.5 times higher in R than in S plants. It has to be noted that Aspartate aminotransferase is upregulated by the transcription factor bZIP34 [55], which according to our qPCR results (Figure 5b) expressed higher in R than in S plants. The gene encoding aspartate aminotransferase, *Asp2*, was found to be induced upon infection with the necrotrophic pathogen *Botrytis cinerea* in *Arabidopsis* [61].

From these results, we postulate that the sources of carbon and nitrogen are less affected by TYLCV in R than in S plants. Moreover, enzymes connected to these sources seem to be more abundant and possibly more stable in R than in S plants, which make R plants more balanced and fit against the viral biotic stress.

Energy production

Glycolysis converts glucose to pyruvate resulting in a gain of ATP. GC-MS analysis showed that the amounts of the main glycolysis components, glucose 6-phosphate, fructose 6-phosphate, fructose-1,6-phosphate were higher in R than in S plants. One of the key glycolysis enzymes, glyceraldehydes 3-phosphate dehydrogenase (GAPDH), was found to be much more abundant in R than in S plants (Table 1). Respiration rates have been known to increase during resistance responses, suggesting that the pressure on cellular metabolism increases to provide energy to sustain stress [62]. Increase of GAPDH upon TYLCV infection may not only enhance the capacity of the plant respiration, but also provides the additional energy needed to cope with the biotic stress. Indeed, the increased amount of pyruvate (approximately 2 times more in R vs. S all along infection, Figure 6b) could point to a more effective pyruvate flux into the energy-generating tricarboxylic acid (TCA) cycle. The TCA cycle is a central

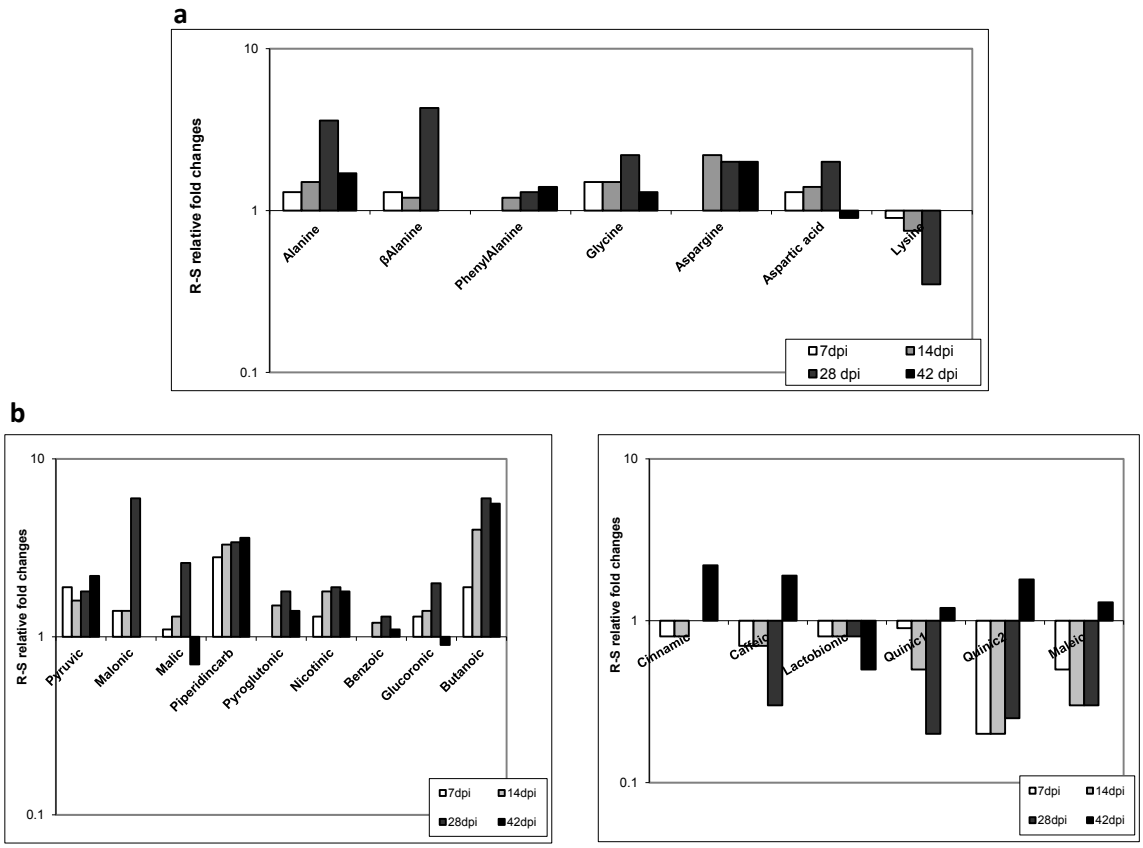

Figure 6: Effect of TYLCV infection on amino- and organic acids contents in leaves of R and S tomatoes. Detections were done after 7, 14, 28 and 42 dpi. a. Relative levels of aminoacids contents: R: S relative folds of changes. b. Relative levels of organic acids contents: R: S relative folds of changes.

metabolic pathway for aerobic processes and is responsible for a major portion of carbohydrate, fatty acid, and amino acid oxidation that produces energy [63]. Another pathway involved with the TCA cycle is the 4-aminobutyrate (γ-aminobutyrate) (GABA) shunt, a pathway that produces succinate using either glutamate or α-ketoglutarate as substrates [64]. Interestingly, there was more butanoic acid in R than in S plants: 4 times more at 15 dpi, and about 6 times more at 28 dpi (Figure 6b). At the same time the amounts of its derivative, 3-amino-n-butanoic acid, was not detected, and the amounts of 4-amino butanoic acid were similar in R and S plants.

Several organic acids have been detected in fruits of TYLCV-infected tomato plants [65]. However, changes in amounts of organic acids during the development of the TYLCV disease have not been monitored. In the current study, GC-MS metabolite profiling showed a reduction in the amounts of primary amino and organic acids in TYLCV infected tomato leaves. Pyruvic, malonic, malic, pyroglutonic, nicotinic, benzoic, glucoronic acids were more abundant in R than in S tomatoes, while cinnamic, caffeic, maleic, quinic, and lactobionic acids were more abundant in S plants (Figure 6b). In addition to butanoic acid, 2-iperidinecarboxylic acid was found in higher amounts in R than in S plant leaves; its amount increased three times upon virus inoculation. The role played by this acid is not known. It is involvement in a specific drug, Picaridin (2-(2-hydroxyethyl)-1-piperidinecarboxylic acid 1-methylpropyl ester), used as a repellant against biting flies, mosquitoes, chiggers, and ticks (Placer Mosquito and Vector Control District, US).

GABA shunt may be involved in supporting the resistance response to pathogens [66]. Under particular energy demanding conditions, pyruvate from glycolysis can be converted to acetyl CoA, allowing R plants to utilize the excess of pyruvate for energy production. Enolase, also known as phosphopyruvate dehydratase, is a metalloenzyme responsible for the catalysis of the conversion of 2-phosphoglycerate to phosphoenolpyruvate and reversably in glycolysis. Enolase is present in all tissues and organisms capable of glycolysis or fermentation. In plastids the energy-rich metabolite is the glycolytic intermediate phosphoenolpyruvate, provided by the plastidic enolase; it is vital for plant growth and development. The current MS analysis detected only one 48 kDa enolase (N56); its amount was two times higher in R than in S plants (Table 1). Enoyl-ACP reductase, shown to be a negative regulator of cell death and to be induced by abiotic stresses (salt for example, [67]) was predominant in R plants. The same was true for the cell death inhibitor deoxyuridine triphosphatase. R proteins showed a 3-fold increase of the other carbohydrate metabolism enzyme, phosphoglycerate mutase, 4,5-folds of dihydrolipoamide dehydrogenase precursor, known to be a a component of the pyruvate dehydrogenase and the α-ketoglutarate dehydrogenase complexes. The amount of fructokinase, which specifically catalyzes the transfer of a phosphate group from ATP to fructose as the initial step in its utilization, was higher in R than in S plants. The main role of fructokinase is in sucrose and fructose metabolism, according to the following equation: ATP + D-fructose = ADP + D-fructose 1-phosphate. Increased amount of fructose 1-phosphate in R plants has been mentioned above. Phosphoglycerate kinase is a transferase enzyme used in the seventh step of glycolysis, its precusor was found to be in 1.7 times higher in R vs. S (Table 1).

Glycine rich-RNA binding proteins (GR-RBPs) have been implicated in cell functions linked to the metabolism of mRNA molecules. These include processing, transport, localization, translation and stability of mRNAs [68]. Strikingly, transcripts of GR-RBPs are up-regulated in response to number of biotic and abiotic stresses we have seen an increase in glycine rich-RNA binding protein in TYLCV infected R plants (Table 1).

ATP synthase creates energy for the cell to use through the synthesis of ATP. In plants, ATP synthase is present in mitochodria and chloroplasts (CF_1F_O-ATP synthase). The overall structure and the catalytic mechanism of the chloroplast ATP synthase are almost the same as those of the mitochondrial enzyme. However, in chloroplasts, the proton motive force is generated not by the respiratory electron transport chain but by primary photosynthetic proteins. ATP synthases were detected by proteomic analysis of tomato leaves. Amounts of ATP synthase CF1 α and β chains, chloroplastic ATP synthase γ chain were similar in R and S lines, whereas the mitochondrial enzyme was 1.7 times higher in R than in S plants, and the 53 kDa ATP synthase β subunit was 5 times higher in S than in R plants (Table 1). These results indicate that the importance of ATP supply is involved in keeping the resistance response of R plants or to activate specific defense mechanisms in S tomatoes.

As a whole, most of the detected enzymes and metabolites involved in energy production are more abundant in infected R than S tomatoes. Under conditions of stress, energy resources are shunted into metabolic reactions that increase stress tolerance independently of the resistant cellular background. However, the achievement of R acclimation is expressed mostly in maintaining homeostasis, while S management of energy resources is directed on the needs of acute defense activation and repairing of damaged cellular components. We suggest that maintenance of homeostasis in R tomatoes is a less energy-requiring process than in the energy-costly survival of S plants, which resulted in the induction of large amounts of energy-producing components.

SnRK1/AMPK/SNF1 as an example of key signaling proteins

Along this study, we aimed the understanding how elevated levels of nitrogen and carbon sources in connection with certain protein regulators could support resistance to TYLCV in infected tomatoes. Adenine, the other origination of nitrogen, adenine, was found to be higher in R than in S plants. Moreover, the amount of adenine phosphoribosyltransferase, which functions as a catalyst in the reaction between adenine and phosphoribosyl pyrophosphate to form AMP, was two times higher in R than in S plants. These results point to a possible increase in AMP synthesis by adenine phosphoribosyltransferase.

AMP-activated kinase (SnRK1/AMPK/SNF1) is a protein kinase that phosphorylates a variety of proteins including enzymes, transcription factors, and ion channels. It has a central role in plant glucose-mediated stress signaling [69,70]. SnRK1 has been considered as a molecular "fuel gauge" or "metabolic sensor." SnRK1 might be thought of as the conductor of an orchestra composed of diverse players that mediate stress responses in the cell. Geminivirus infection was shown to up-regulate the expression of two *Arabidopsis* protein kinases related to AMPK/SNF1 [71,72]. Our current proteomic analysis has not detected SnRK1 in TYLCV infected leaves. Hence SnRK1 patterns in infected S and R plants were followed by western blot analysis using a human Phospho-AMPKα Thr172 polyclonal antibody, which recognizes AMPKα only when phosphorylated at threonine 172 (Cell Signaling Technology). Thr172 phosphorylation occurs under activation of AMPK by AMP and an upstream kinase. A specific signal was obtained in mock and TYLCV infected tomato protein extracts (Figure 7). The virus may not up-regulate the kinase, but it is less degraded in R than in S leaves.

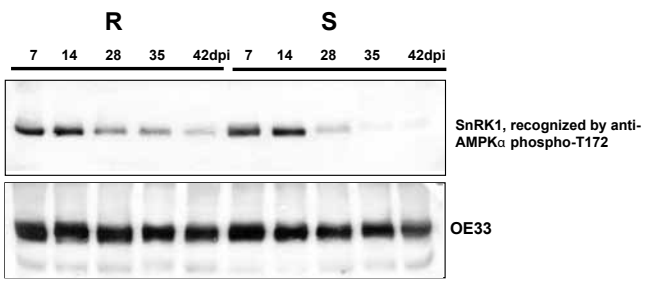

Figure 7: Patterns of SnRK1-like kinase in TYLCV infected tomatoes. Western blot analysis of polypeptides, extracted from infected R and S plants during 7, 14, 28, 35 dpi, recognized by anti-AMPKα Ph-T172 antiserum. Patterns of plant protein OE33 were used as internal control.

Several MAPKs in infected tomatoes behaved similarly to SnRK1 [73]. We propose that upon sensing the energy deficit associated with TYLCV stress, increased stability of SnRK1, MAPKs and HSPs triggers extensive transcriptional changes that contribute to restoring homeostasis, promoting cell survival and elaborating longer-term responses for adaptation, growth and development in R plants. Remarkably, regulation of gene expression by SnRK1 is not restricted to few genes; it triggers the reprogramming of transcription of numerous genes in mesophyll cells [70]. At the same time, only few SnRK1 target genes have been identified; examples of such genes are a sucrose synthase activated by sucrose in potato, and a wheat α-amylase gene repressed by glucose [73,74]. Because SnRK1 affects a variety of networks, we cannot make a direct connection between the SnRK1-like protein and its potential targets in infected tomato leaves in the frame of present study. More important, the higher stability of activated phosphorylated SnRK1 in R than in S plants, may point to its contribution in restoring homeostasis, promoting survival and elaborating longer-term responses for adaptation to TYLCV infection.

Conclusions

Current comparison of infected R and S tomatoes at the level of proteomic and metabolomic analyses demonstrates the activation of different host responses to the virus stress. Higher levels ROS compounds, anti-oxidative, PR- and wound-induced proteins were predominant in S plants. The activation of host plant responses could reflect the elevated levels of cellular defense against virus invasion. In R infected tomatoes, the host defense mechanisms were much less activated, and the protein and chemical chaperones were triggered to maintain tissue homeostasis. Sources of carbon and nitrogen were less affected by TYLCV in R than in S plants, which make R plants more balanced and fit against the virus biotic stress. Moreover, more stable patterns of key cellular regulators, such as SnRK1 and MAPKs, were characteristic in R plants.

Proteomic analyses did not reveal the sharp increases in expression of well known stress proteins in R plants. The contrary was found for several PR- and wound-induced proteins in S plants, as discussed in paragraph 2.1. These observations supported our findings showing that such PR proteins as chitinase, β-1,3-glucanase, peroxidase were not induced in infected R plants [8]. Recently, Garcia-Neria and Rivera-Bustamante [75] failed to observe a virus-dependent induction of PRs in leaves of resistant plants infected with the begomovirus *Pepper golden mosaic virus* PepGMV. Moreover, proteins that were found to be essential in establishing the resistance phenotype of R tomato plants (Permease, Hexose transporter, Lipocalin-like protein) do not belong to PRs, but rather to primary metabolism proteins that likely play role in providing energy for the resistance response.

The innovation of our research is in a unique comparison of genetically closed plant lines contained comparable amounts of virus but showing completely different stress response upon TYLCV infection. The proteomic/metabolomic relationships were studied at the specific time period when the R plant immune machinery succeeds and S fails to withstand virus invasion. Future analyses will cover additional time hallmarks along with TYLCV infection in R vs. S tomatoes. They will also involve the hormonal balance (SA, JA, Ethylene) and basic regulators of stress response.

Acknowledgments

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