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Chloroplast Proteome of *Nicotiana benthamiana* Infected by Tomato Blistering Mosaic Virus

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Abstract

Tymovirus is a genus of plant pathogenic viruses that infects several dicotyledonous plants worldwide, causing serious diseases in economically important crops. The known cytopathic effect on the host cell organelles involves chloroplast membrane deformation and the induction of vesicles in its periphery. These vesicles are known to be the location where tymoviral genomic RNA replication occurs. Tomato blistering mosaic virus (ToBMV) is a tymovirus recently identified in tomato plants in Brazil, which is able to infect several other plants, including tobacco. In this work, we investigated the chloroplast proteomic profile of ToBMV-infected *N. benthamiana* using bidimensional electrophoresis (2-DE) and mass spectrometry, aiming to study the virus-host interaction related to the virus replication and infection. A total of approximately 200 spots were resolved, out of which 36 were differentially abundant. Differential spots were identified by mass spectrometry including photosynthesis-related and defense proteins. We identified proteins that may be targets of a direct interaction with viral proteins, such as ATP synthase β subunit, RNA polymerase beta-subunit, 50S ribosomal protein L6 and Trigger factor-like protein. The identification of these candidate proteins gives support for future protein–protein interaction studies to confirm their roles in virus replication and disease development.

Keywords Plant proteomics · Plant-virus interaction · ToBMV · 2-DE · Mass spectrometry

1 Introduction

Tymovirus are single-stranded RNA viruses with positive sense that infect mainly dicotyledonous plants, including those from families Cucurbitaceae, Brassicaceae and Solanaceae, and cause serious diseases [1]. The genus Tymovirus comprises a total of 35 confirmed species, and belongs to the Tymoviridae family, which includes the Maculavirus and Marafivirus genera. In Brazil, five Tymovirus species have been reported: Eggplant *mosaic virus* [2], *Petunia*

vein-banding virus [3], *Passion fruit yellow mosaic virus* [4], cassia yellow mosaic-associated virus [5], and tomato blistering mosaic virus (ToBMV) [6].

ToBMV was isolated from Santa Catarina state, Brazil and tentatively named as tomato blistering mosaic virus (ToBMV) [7]. ToBMV was also able to infect several other plants, including *Nicotiana tabacum, N. benthamiana, Capsicum annuum* and *Solanum violaefolium* [8, 9]. This virus species was always found in virome studies in tomato and pepper plants using Next-generation sequencing (Tatsuya Nagata, unpublished data) and, therefore it is essential to investigate and understand the effects of this virus in the host plant since it represents a serious threat to tomato and pepper producing areas. A recent report revealed the detection of this virus affecting tomato in Argentina, which has a serious epidemiological impact [10]. A phylogenetic analysis was also performed and showed that ToBMV clustered with other common tymoviruses that infect solanaceous hosts [6, 7]. Recently, the complete genome of ToBMV was sequenced [6, 8, 9] revealing a size of about 6.3 kb and three ORFs: ORF 1 encodes the RNA replication polyprotein,

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(cleaved to metyltransferase/protease, helicase, and RNA-dependent RNA polymerase), ORF 2 the movement protein, and ORF 3 the coat protein. ToMBV causes the typical cytopathic effects usually seen on tymovirus infected tissue, including double-membrane vesicles on the periphery of the chloroplasts [7]. The vesicles, caused by tymoviruses, are the sites where viral genome replication occurs. In general, an RNA virus replicates on the surface of the organelle and (in many cases) viral replication-related proteins, such as helicase, methyltransferase or viral RNA-dependent RNA polymerase, interact directly with the host organelle membrane proteins. This replication site known as the virus replication complex (VRC) often forms vesicles, escaping from the RNA degradation pressure of plant cells by RNAi machinery [11].

During the last 10 years, more focus has been given to identify the host gene expression changes during viral infection using different functional genomic techniques, including proteomics based on bidimensional electrophoresis (2-DE) and liquid chromatography (LC) coupled to mass spectrometry (MS). Research in proteomics of plant-virus interactions has been extensively reviewed in Di Carli et al. [12]. In order to get a better view of plant responses to biotic and abiotic stresses, recent studies have focused on organelle subproteomes, such as those of chloroplast and mitochondria. 2-DE has proven to successfully reveal the protein profiles of both luminal and peripheral thylakoid proteins from *Pisum sativum* [13] and *Arabidopsis thaliana* [14]. However, much less is known about the effects that different biotic and abiotic-stress factors have on the chloroplast proteome. In virus-plant interaction, a study of the interaction between *N. benthamiana* and pepper mild mottle virus (PMMoV, genus *Tobamovirus*) pointed out changes in the abundance of several photosynthetic proteins as well as proteins of the Benson-Calvin cycle, nitrogen metabolism, and protein synthesis [15].

The most common symptoms of viral infection observed in plants are chlorosis and reduction in photosynthesis. In the case of ToBMV, due to the strong chloroplast interaction, photobleaching (whitening leaves) is also observed when the symptoms are very severe, probably as a result of compromised chlorophyll synthesis [7]. Several reports showed the down-regulation of photosynthetic proteins by viral proteins (reviewed in [16]), and the identification of these targets is one on the main goals in plant-virus interaction studies. ROS production and scavenging, for example, are important responses during plant-virus interaction and are crucial for disease resistance. Brizard et al. [17] co-purified different peroxidases with viruses from *Rice yellow mottle virus* resistant and susceptible rice and showed that viruses recruit many host proteins for their development. Chloroplasts are also the crucial site for hormone production, such as salicylic (SA) and jasmonic (JA) acids

[18, 19], involved in plant defense systems against viruses [20]. Proteins involved in photosynthetic electron-transport chain and the Benson-Calvin cycle were also reported in *N. benthamiana* in response to the PMMoV Spanish strain by analyzing the chloroplast proteome by 2-DE [15]. Although some chloroplast proteins interacting with virus proteins have been identified, the knowledge of this interaction is still limited. The understanding of chloroplast-virus interaction can certainly bring new insights for the elucidation of the tymovirus replication and infection mechanisms. Thus, considering the importance of plant chloroplast proteins in viral infection and development, the objective of the present work was to study the chloroplast subproteome of the host plant *N. benthamiana* (model plant for plant virology) upon ToBMV infection by 2-DE in order to obtain a new insight of the processes affected by this virus.

2 Materials and Methods

2.1 ToBMV Plant Inoculation and Chloroplast Enrichment

Nicotiana benthamiana plants cultivated in glasshouse at $\pm 25\,°C$ for 30 days were mechanically inoculated with ToBMV infected sap in phosphate buffer pH 7.2 with 0.2% of sodium sulfide and carborundum. The same mechanical damage with phosphate buffer and abrasive carborundum was applied to non-inoculated control plants. Three biological replicates were obtained for each sample and used for chloroplast enrichment.

Leaves were collected 10 days after inoculation, when plants started showing intense systemic symptoms, as determined by de Oliveira et al. [7]. Plants were maintained in a dark room for 24 h before leaves were harvested to reduce starch accumulation. Chloroplast enrichment was performed on collected leaves using Chloroplast Isolation Kit (Sigma-Aldrich, St. Louis, USA), with minor modifications. Fresh leaves were blended two to five times for 5 s in Chloroplast Isolation Buffer (CIB) 6:1 v:w (g) (0.3 M sorbitol, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM EDTA, 20 mM HEPES/KOH, pH 8.0, 10 mM $NaHCO_3$) [21]. The blended leaf sap was filtered in nylon cloth and centrifuged in 50 mL tubes at $1000\times g$ at 4 °C for 7 min to sediment the chloroplasts. The supernatant was discarded and the green pellet was suspended in CIB. A Percoll (GE HealthCare) gradient 40%/80% diluted in CIB was prepared by centrifugation at $3200\times g$ for 15 min at 4 °C. The intact chloroplasts at the interface between the 40 and 80% Percoll layers were collected, visualized by light microscope and stored at $-80\,°C$.

2.2 Chloroplast Protein Extraction and Quantification

Chloroplast protein extraction was performed according to Carmo et al. [22]. For each 100 μL of purified chloroplast 750 μL of extraction buffer (0.7 M sucrose, 0.5 M Tris–HCl, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, 40 mM DTT) were used. Samples were incubated for 15 min at room temperature and 750 μL of phenol were added. Samples were maintained under agitation in a vortex mixer for 15 min and centrifuged at 8050×g for 3 min. This step was repeated with 500 μL of phenol. Proteins were precipitated with 0.1 M ammonium acetate in methanol and washed with 80% acetone. Proteins were suspended in solubilization buffer (7 M urea; 1 M thiourea; 4% m/v CHAPS; 2% IPG buffer pH 3–10 NL; 40 mM DTT). Protein quantification was performed using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad), according to the manufacturer's instructions.

2.3 2-DE and Image Analysis

A total of 400 μg of extracted proteins were used to rehydrate 13 cm Immobiline DryStrips, pH 3–10 NL for 16 h. Isoelectric focusing was performed using GE™ Healthcare Ettan™ IPGphor™ 3 Isoelectric Focusing System (GE Healthcare Life Sciences, Issaquah, USA), according to the manufacturer's instructions (step 1: 500 V, 60 min, 0.5 kVh; step 2: 1000 V, 60 min, 0.8 kVh; step 3: 8000 V, 150 min, 11.3 kVh; step 4: 8000 V, 55 min, 7.4 kVh). IPG strips were maintained in equilibration buffer [1.5 M Tris–HCl pH 8.8; 6 M urea; 30% (v/v) glycerol; 2% (w/v) SDS; 1% (v/v) bromophenol blue] with 1 M DTT for 15 min followed by 15 min in the same buffer containing 2.5% (w/v) iodoacetamide. The second dimension was performed on 12% polyacrylamide gel and the electrophoresis was run in a vertical system (Biometra V2) with glycine buffer [20 mM Tris HCl pH 8.3, 192 μM glycine and 0.1% (w/v) SDS]. Benchmark Protein Ladder (Thermo Fisher Scientific, Waltham, USA) was used as molecular mass marker. Gels were stained overnight with colloidal Coomassie Blue [0.1% (w/v) Coomassie G250; 2% (v/v) phosphoric acid; 10% (w/v) ammonium sulphate; and 20% (v/v) methanol] and destained with Milli-Q water.

One gel from each biological replicate was digitalized with the ImageScanner III (GE Healthcare Life Sciences) according to the manufacturer's instructions. Images were analyzed using the software ImageMaster™ 2D Platinum v7.0 (GE Healthcare Life Sciences). Spots were automatically detected and a manual adjustment was performed to minimize possible technical artifacts. Automated matching was performed and matches were manually checked in order to minimize possible errors. Only proteins present in, at least, two out of three replicates were considered for analysis. Spot quantification was performed using intensity, area, volume, and relative volume, according to ImageMaster™ 2D Platinum v7.0. Proteins were considered as differentially abundant only when differences were significant by Student's t test at a significance level of 95%.

2.4 Protein Identification by MS

The differentially abundant proteins were excised from the gel and hydrolyzed using Trypsin Profile IGD kit (Sigma-Aldrich), according to manufacturer's instructions. A total of 0.4 μg of trypsin was added to each spot and the digestion was incubated overnight at 37 °C. After the digestion, 1 μL of the solution was mixed with 1 μL of alpha-cyano-4-hydroxycinnamic acid (10 mg/mL in 50% acetonitrile and 0.1% trifluoroacetic acid) and applied manually onto an Anchorchip 800/384 MALDI target plate. Peptides were analyzed using Ultra-Flex III or Auto-Flex Speed MALDI TOF–TOF mass spectrometers (Bruker Daltonics, Billerica, USA) operating in positive reflector (MS) and LIFT TM (MS/MS) modes.

MS and MS/MS peak lists were generated using the FlexAnalysis 3.3 software (Bruker Daltonics), with a quality factor threshold of 30 and 3 as S/N, and were individually searched using the MASCOT server (Matrix Science, London, UK; http://www.matrixscience.com/search_form_select.html) in NCBIprot database against Viridiplantae (or Plant ESTs) and Virus. The protein identification parameters for peptide mass fingerprinting (PMF) searches were: 150 ppm mass tolerance, 1 missed cleavage and carbamidomethylation of cysteine residues as fixed modification and oxidation of methionine residues as variable modification. For MS/MS, the parameters used were the same described for PMF with an ion mass tolerance of 0.6 Da and charge state +1. When pI and molecular mass (Mw) were not available, these values were calculated using ExPASy Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html). The cutoff value for the Probability Based Mowse score calculated by MASCOT (at $p < 0.05$) was used to accept the identification.

3 Results and Discussion

In the present study, the effect of ToBMV infection in protein accumulation in chloroplasts of *N. benthamiana* plants was analyzed by 2-DE and mass spectrometry. Inoculated plants showed typical symptoms, including veinal clearing, mottling, leaf distortion and stunting [7] (Fig. 1). Leaf samples from infected and control plants were collected and used for chloroplast isolation followed by protein extraction. The analysis of the obtained 2D maps from infected and non-infected plants showed approximately 210 spots per gel, varying in mass from 15 to 120 kDa. These results showed a number of proteins similar to those previously

Fig. 1 Symptoms caused by ToBMV in *Nicotiana benthamiana* plants. (**a**) Infected plant, showing necrotic spot in inoculated leaves, vein clearing and top distortion in upper leaves. (**b**) Infected leaf showing vein clearing and mottling. (**c**) Healty *N. benthamiana* plant
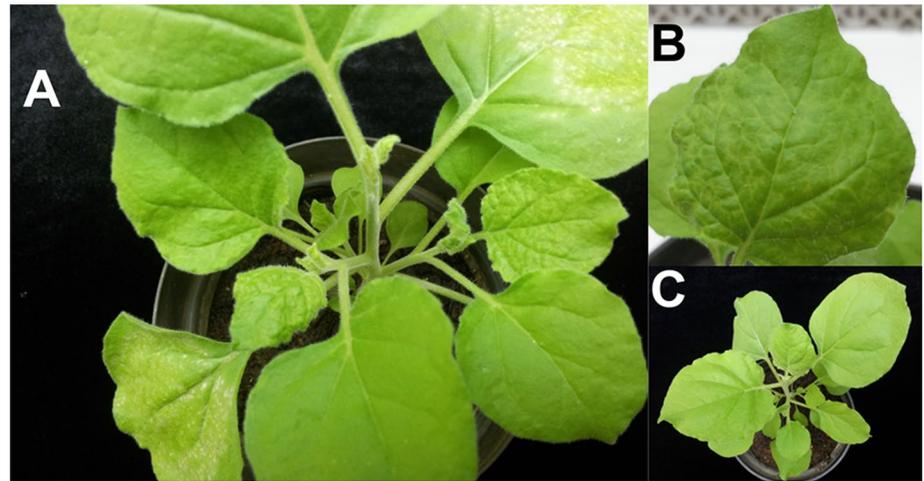
described for *N. bethamiana* chloroplasts [15]. The comparative analysis between both treatments (infected and control plants) showed a total of 36 differentially abundant proteins, including 12 increased, 17 decreased and 4 unique proteins in infected samples, as well as 3 unique proteins in control plants. All differential spots were excised for identification by mass spectrometry (MS), and a total of 16 differential proteins were identified (Table 1).

The cytopathic effect of the ToBMV on the host cell organelles involves chloroplast deformation and the induction of vesicles on its periphery. In general, it is well known that plant viral infection affects plant chloroplasts causing the reduction of several chloroplastic proteins [16, 23–27]. In this study, as expected, these proteins were, indeed, decreased in infected plants when compared to control plants, such as a Sedoheptulose-1,7-bisphosphatase (spot 130; Table 1; Fig. 2a, b) and Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (RubL) (Spot 211; Table 1; Fig. 2a), which was unique to control plants. The lower abundance of Sedoheptulose-1,7-bisphosphatase and RubL in infected plants was previously described in different plant-pathogen interactions [28, 29], and it has been shown that oxidative alterations affect the photosynthetic proteins causing their degradation during stress [30, 31]. This is consistent with the low levels of these proteins during PMMoV—*N. benthamiana* interaction [15] and also with the data obtained in our study. These results suggest that ToBMV also compromises the abundance of chloroplast-associated proteins and affects the photosynthetic apparatus.

Interestingly, among the increased proteins in plants infected by ToBMV was a mixture of proteins, which included Trigger factor-like protein and ATP synthase β subunit (spot 18; Table 1; Fig. 2a, b). It has been reported that ATP synthase β subunit interacts with viral proteins [16]. Moreover, the Trigger factor-like protein may help

viral infection, since it is a folding chaperone [32, 33]. Host chaperones can contribute to viral infection by interacting with viral proteins [32, 33]. Another interesting protein is RNA polymerase beta-subunit (spot 165; Table 1; Fig. 2), which showed an increased fold change of 3.93 in infected plants when compared to the control. The involvement of this enzyme in viroid replication has been reported. Plant viroids, which have circular RNAs, encode no proteins and are able to redirect a host polymerase for its replication in the chloroplast (reviewed in Ahlquist [34]). Differently, the ToBMV genome presents an ORF that encodes the RNA replicase polyprotein. It is possible that ToBMV interacts with chloroplastic RNA polymerase to change the gene expression in chloroplast genes to benefit virus infection. Therefore, these proteins are interesting candidates to further investigation to establish their role in viral pathogenesis during ToBMV infection.

It is noteworthy that some proteins were unique in infected samples. Among them are 50S ribosomal protein L6 (spot 205; Table 1; Fig. 2a), ATP synthase F1 subunit 1—mitochondrion (spot 203; Table 1; Fig. 2a), and ToBMV coat protein (spot 157 and 206; Table 1; Fig. 2a). Studies showed that the protein synthesis by chloroplasts generally can be inhibited by the virus [35], compromising even the levels of chloroplast ribosomal RNA [36]. However, it is also known that viruses recruit host ribosomal subunits to translate viral mRNAs (reviewed in Walsh and Mohr [37]). Therefore, it is possible that the abundance of 50S ribosomal protein in infected plants could be caused by ToBMV to promote viral infection.

Table 1 Differentially abundant proteins identified by mass spectrometry in *N. benthamiana* control and ToBMV-infected plants

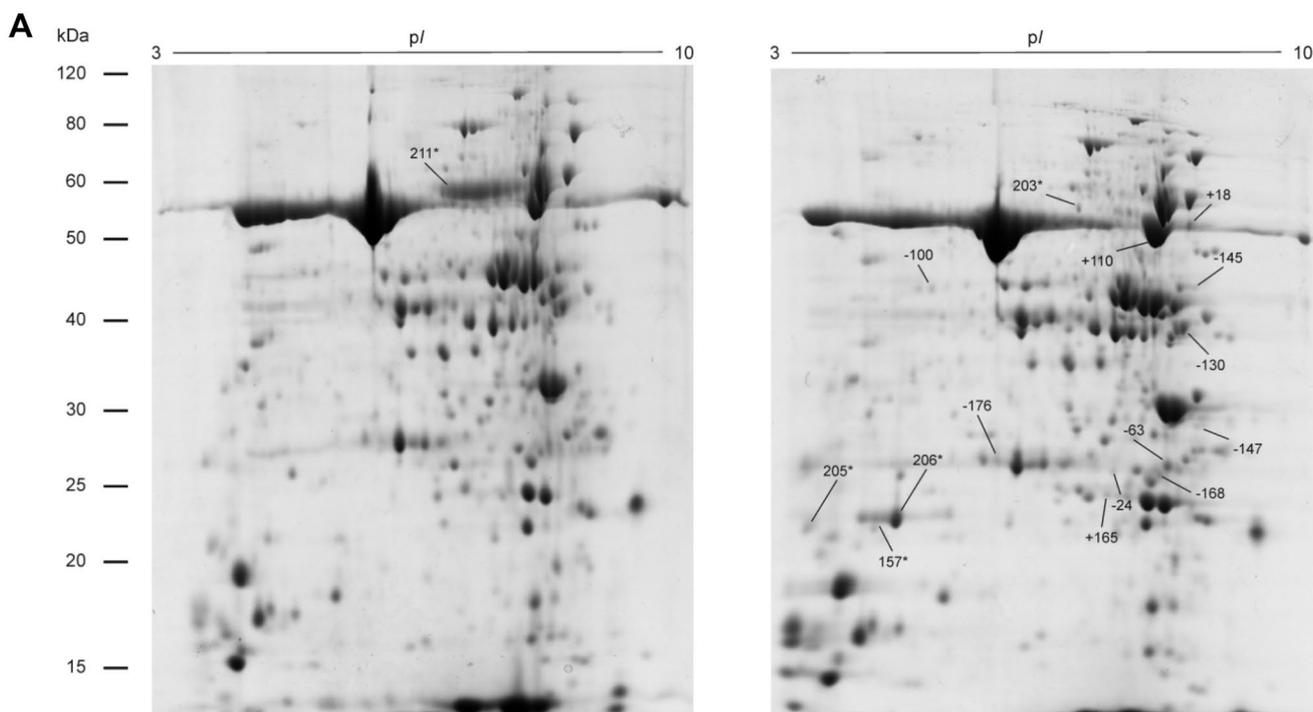| Protein spot	Protein description	Peptide sequence[a]	Organism	Acession[b]	Mass (kDa) theor	Mass (kDa) exp	pI theor	pI exp	Mascot score	Coverage (%)/No. of matched peptides (MS)	Ratio[c] Inoculated/control
18	Mixture: Predicted: trigger factor-like protein TIG (chloroplastic); ATP synthase beta subunit, partial (chloroplast)	–	*Nicotiana tabacum* / *Sarcandra glabra*	XP_016435236 / AAD37040	61 / 51	57	5.03 / 5.13	4.73	129 / 117	36/18 / 38/17	2.76
24	Triosephosphate isomerase, chloroplastic-like	–	*Nicotiana tomentosiformis*	XP_009590743	35	28	6.14	5.70	94	22/7	0.70
63	Chlorophyll *a, b* binding protein 40, chloroplastic like	K.AKPVSSGSPWYGPDR.V	*Nicotiana tomentosiformis*	XP_009593856	28	28	5.47	5.00	92	5/1	0.70
100	Aminomethyltransferase, mitochondrial	– / K.GGDVSWHIHDER.S	*Nicotiana sylvestris* / *Nicotiana tomentosiformis*	XP_009782825 / XP_009591091	45 / 45	46	8.78 / 8.78	7.89	95 / 93	18/9 / 2/1	0.60
110	ATP synthase beta subunit, partial (chloroplast)	–	*Neillia thibetica*	AEI27430	45	55	5.16	5.18	175	46/19	1.46
130	Sedoheptulose-1,7-bisphosphatase, (chloroplastic)	–	*Nicotiana sylvestris*	XP_009780073	43	41	5.74	4.83	102	34/13	0.74

Table 1 (continued)

Protein spot	Protein description	Peptide sequence[a]	Organism	Acession[b]	Mass (kDa) theor	Mass (kDa) exp	pI theor	pI exp	Mascot score	Coverage (%)/No. of matched peptides (MS)	Ratio[c] Inoculated/control
145	Fructose-1,6-bisphosphatase chloroplastic-like	–	*Nicotiana atttenuata*	XP_019242936	45	46	5.46	4.67	80	19/6	0.40
		K.YIDDLKDPGPSGKPYSAR.Y	*Nicotiana tabacum*	XP_016461409	45		5.26		55	4/1	
147	Unknown protein DS12 from 2D-PAGE of leaf, chloroplastic-like	–	*Nicotiana sylvestris*	XP_009762796	31	31	5.20	4.61	82	27/5	0.59
157	Putative coat protein	K.TPTATLQIR.G K.LLAYSPAPTTPSK.T	Tomato blistering mosaic virus	AIG05829	20	24	8.60	8.63	69 71	4/1 6/1	Unique to inoculated
165	RNA polymerase beta subunit (chloroplast)	–	*Sartidia dewinteri*	YP_009141152	17	26	7.16	5.78	86	5/8	3.93
168	Chlorophyll *a, b* binding protein CP26, chloroplastic	K.YQAYELIHAR.W	*Nicotiana tabacum*	XP_016458800	30	28	5.73	5.13	66	3/1	0.60
176	Proteinase inhibitor I-B-like	–	*Solanum tuberosum*	XP_006354531	13	28	5.23	7.08	80	59/5	0.74
203	ATP synthase F1 subunit 1 (mitochondrion)	–	*Nicotiana tabacum*	BAD83524	56	60	5.84	6.14	80	14/7	Unique to inoculated
		R.EAFPGDVFYLHSR.L	*Arabidopsis thaliana*		55		6.01		64	2/1	

Table 1 (continued)

Protein spot	Protein description	Peptide sequence[a]	Acession[b]	Organism	Mass (kDa) theor	Mass (kDa) exp	pI theor	pI exp	Mascot score	Coverage (%)/No. of matched peptides (MS)	Ratio[c] Inoculated/control
205	50S ribosomal protein L6, chloroplastic	–	XP_009785468	Nicotiana sylvestris	25	23	9.99	9.01	155	30/12	Unique to inoculated
		K.KLQLVGVGYR.A	5MLC_H	Spinacia oleracea	25		10.09		65	4/1	
		K.SEIGQFAASIR.R	OIW08024	Lupinus angustifolius	27		10.08		63	4/1	
206	Putative coat protein	K.TPTATLQIR.G	AIG05829	Tomato blistering mosaic virus	20	24	8.60	8.29	72	4/1	Unique to inoculated
211	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (plastid)	–	AFJ96235	Quararibea spatulata	49	61	6.12	6.10	115	25/12	Unique to control

[a]The symbol "–" in the peptide sequence column indicates that peptide mass fingerprinting (PMF) was performed and therefore no peptide sequence was generated

[b]More than one accession cited indicates different hits for peptides identified from the same protein

[c]Ratio inoculated/control values over 1 determine increased proteins, while values below 1 correspond to decreased proteins. Unique proteins to each treatment are indicated. The protein spot abundance is expressed as a spot ratio, comparing inoculated and control spot volumes
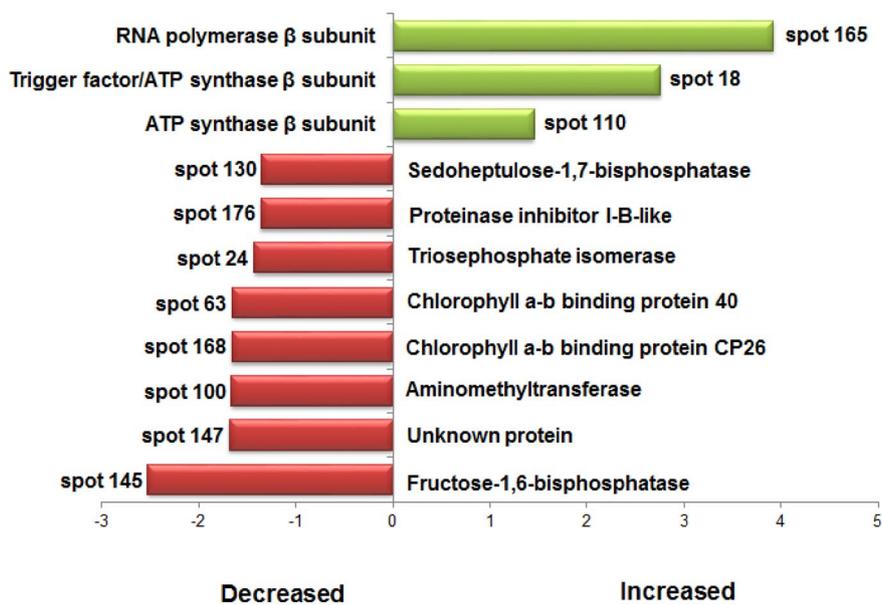
Fig. 2 (**a**) 2D maps of chloroplast preparations from *Nicotiana benthamiana* plants in the control (left) condition and inoculated with ToBMV (right). The symbols + and − indicate increased and decreased proteins, respectively, when compared to the control. The

* symbol indicates unique proteins to one of the conditions. (**b**) The graph represents the fold change of differential proteins in plants inoculated with ToBMV when compared to the control. The unique proteins of each condition are not shown

4 Conclusions

This is the first comparative report showing chloroplast proteins involved in the plant response to ToBMV infection. The results indicate that this virus has an effect similar to other viruses, affecting severely the protein metabolism in chloroplasts. Proteins potentially involved in the infection process were also identified, including an RNA polymerase beta-subunit and a 50S ribosomal protein L6, which were highly abundant or unique in infected tissues analyzed. Furthermore, this study identified some proteins that may be targets of a direct interaction with viral proteins, such as ATP synthase β subunit and Trigger factor-like protein. The identification of these targets is highly important in understanding the pathogenicity of ToBMV since they may be recruited by the virus to favor the infection. Therefore, it would be interesting to further investigate these chloroplast-related proteins to elucidate their roles in ToBMV pathogenesis.

Compliance with Ethical Standards

Conflict of interest Authors have no conflict of interest to declare.

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