RESEARCH ARTICLE

Identification of host proteins modulated by the virulence factor AC2 of Tomato chlorotic mottle virus in *Nicotiana benthamiana*

Lilian Silveira Travassos Carmo[1,2], Renato Oliveira Resende[2], Luciano Paulino Silva[1], Simone Graça Ribeiro[1] and Angela Mehta[1]

[1] Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil
[2] Departamento de Biologia Celular, Instituto de Biologia, Universidade de Brasília, Brasília, DF, Brazil

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Tomato, one of the most important crops cultivated worldwide, has been severely affected by begomoviruses such as the *Tomato chlorotic mottle virus* (ToCMoV). Virulence factor AC2 is considered crucial for a successful virus–plant interaction and is known to act as a transcriptional activator and in some begomoviruses to function as an RNA silencing suppressor factor. However, the exact functions of the AC2 protein of the begomovirus ToCMoV are not yet established. The aim of the present study was to identify differentially expressed proteins of the model plant *Nicotiana benthamiana* in response to the expression of the AC2 gene, isolated from ToCMoV. *N. benthamiana* plants were inoculated with *Agrobacterium tumefaciens* containing the viral vector Potato virus X (PVX) and with the PVX-AC2 construction. 2DE was performed and proteins were identified by MS. The results showed that the expression of ToCMoV AC2 alters the levels of several host proteins, which are important for normal plant development, causing an imbalance in cellular homeostasis. This study highlights the effect of AC2 in the modulation of plant defense processes by increasing the expression of several oxidative stress-related and pathogenesis-related proteins, as well as its role in modulating the proteome of the photosynthesis and energy production systems.

Keywords:
Differential expression / Plant proteomics / Plant–virus interaction / ToCMoV

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1 Introduction

Tomato (*Solanum lycopersicon*) is one of the most important vegetable crops cultivated worldwide, and is severely affected by several pathogens, including nematodes, fungi, bacteria, and viruses. Whitefly-transmitted geminiviruses, which belong to the genus *Begomovirus* (family Geminiviridae), affect tomato productivity in several countries around the world [1], including Brazil, where losses can range from 40 to 100% [2].

In an attempt to minimize the damage caused by these viruses, characterization of the begomoviruses present in the tomato cultivating regions in Brazil has been carried out and revealed a highly diverse group, phylogenetically distant from the begomoviruses reported in other countries [3, 4]. *Tomato chlorotic mottle virus* (ToCMoV) is one of the five predominant begomovirus species and is prevalent in several tomato cultivating areas in Brazil. The control of begomoviruses is achieved by applying pesticides that target the whitefly vector and therefore are costly and not totally efficient. The

Correspondence: Dr. Angela Mehta, Embrapa Recursos Genéticos e Biotecnologia, Av. W5 Norte (final), 70770-917 Brasília, DF, Brazil
E-mail: angela.mehta@embrapa.br
Fax: +55-61-3340-3658

Abbreviations: ACMV, African cassava mosaic virus; **CP**, coat protein; **PRs**, pathogenesis-related proteins; **cyMDH**, cytosolic malate dehydrogenase; **PVX**, Potato virus X; **ToCMoV**, Tomato chlorotic mottle virus; **TMV**, Tobacco mosaic virus

Colour Online: See the article online to view Figs. 1–3 in colour.

development of resistant tomato plants seems to be the best alternative to be used together with integrated crop management of whitefly/begomoviruses. Therefore, the comprehension of the interaction between ToCMoV and the host plant is essential to establish more efficient disease management strategies.

The virulence factor AC2, isolated from different geminiviruses, is considered crucial for a successful interaction between the virus and the host plant. The AC2 gene encodes the multifunctional protein AC2 (also called C2, AL2, and TrAP), which is present in all members of the *Begomovirus* genus. The AC2 protein can be found in the nucleus and in the cytoplasm of the infected host cell [5, 6] and is known to activate the transcription of other virus genes, such as the coat protein and the nuclear shuttle protein genes [7, 8]. Moreover, the AC2 protein of some begomoviruses can act as a suppressor of RNA silencing by activating the transcription of host silencing suppressor genes [9] or by inactivating the host adenosine kinase [5], which is important for several processes such as the transmethylation cycle, expression of primary cytokinin-responsive genes [10], and inactivation of sucrose nonfermenting 1 kinase [11].

It has also been suggested that the AC2 protein may play a role in the inhibition of the methylation process, an important defense mechanism of the host plant against viral infection [12, 13]. Additionally, it has been shown that AC2 can interfere in the expression of important regulators of several cellular processes, such as SCFs (Skp1/Cullin1/F-box), enzymes associated to ubiquitination [12]. AC2 has also been reported to increase the host plant susceptibility to infections caused by viruses [14].

In spite of the large amount of information available regarding the multifunctionality of AC2, there is limited knowledge of the function of the AC2 protein isolated from the begomovirus ToCMoV or of its effect over the global protein expression of the host plant. Moreover, the function of ToCMoV AC2 as a silencing suppressor has not been established yet.

One of the strategies often used to analyze the effect of a given protein in a plant is heterologous expression using viral vectors, such as the Potato virus X (PVX) and Tobacco mosaic virus (TMV) [15]. Lacorte et al. [16] have constructed Gateway-compatible versions of these two vectors and showed that the targeted genes were efficiently cloned by recombination and successfully expressed. This approach has been used in several studies and changes in the transcription profile have been reported. Amin et al. [17] showed that the transient expression of genes from different begomoviruses, including the Trap/C2 gene, altered the phenotype of *Nicotiana benthamiana* plants and also modulated the miRNA levels. Similarly, Hong et al. [18] used the viral vector PVX to evaluate the effect of AC2 gene isolated from *African cassava mosaic virus* (ACMV) in transgenic model plant *N. benthamiana* and showed the potential of this system for AC2 functional investigation in planta.

Although crop plants such as *Phaseolus vulgaris* and tomato [19], and the model plants *Arabdopsis thaliana* and *Nicotiana spp.* have been used for virus gene functional studies [5, 9, 10, 12, 13, 19], *N. benthamiana* has been considered the best choice for plant–pathogen interaction investigations, since it presents homologous genomic regions to other agronomically important solanaceous plants such as tomato, potato, and pepper. Moreover, *N. benthamiana* is susceptible to several pathogens, including bacteria and viruses, among others [20]. It has been shown that *N. benthamiana* is an efficient host for studies involving virus-induced gene silencing and protein transient expression [21, 22].

In this study, we have used the model plant *N. benthamiana* and the PVX-based viral vector to analyze the effect of the AC2 protein encoded by the begomovirus ToCMoV in the global protein profile of the host plant in an attempt to better understand the changes modulated by this pathogenicity factor. We show that the AC2 protein interferes with several cellular processes, with major effects on the cellular homeostasis of the host plant, causing oxidative stress and the upregulation of defense-related proteins.

2 Materials and methods

2.1 Vector, plant material, and inoculations

The PVX-AC2 vector construction used in this study was previously obtained [23]. Briefly, the AC2 gene was PCR-amplified from a ToCMoV infectious clone [24] with specific primers containing attB-flanked sequences to allow Gateway® (Invitrogen) cloning. The PCR fragment was introduced by BP recombination, using BP-clonase II enzyme mix (Invitrogen) according to the manufacturer's instructions, into pDonr207. Subsequent LR recombination, using LR-clonase II enzyme mix (Invitrogen) according to the manufacturers' instructions, into the Gateway-compatible destination vector PVX-GW [16] originated PVX-AC2 (Supporting Information Fig. 1). *N. benthamiana* plants presenting 3–4 leaves were used for inoculation with *Agrobacterium tumefaciens* GV 3101 containing the PVX-AC2 and PVX empty vector as control. A total of 2 leaves were punctured around the central vein with a toothpick dipped in bacterium culture [25]. Leaves from the apical region were collected at 5, 10, 15, and 17 days after inoculation for protein and RNA extraction and the symptoms were monitored during this period (Fig. 1 and Supporting Information Fig. 2).

2.2 RNA extraction and RT-PCR analysis

Leaves from *N. benthamiana* inoculated with PVX-AC2 and PVX alone were collected at 5, 10, 15, and 17 dpi and pulverized in liquid nitrogen. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. For cDNA synthesis,

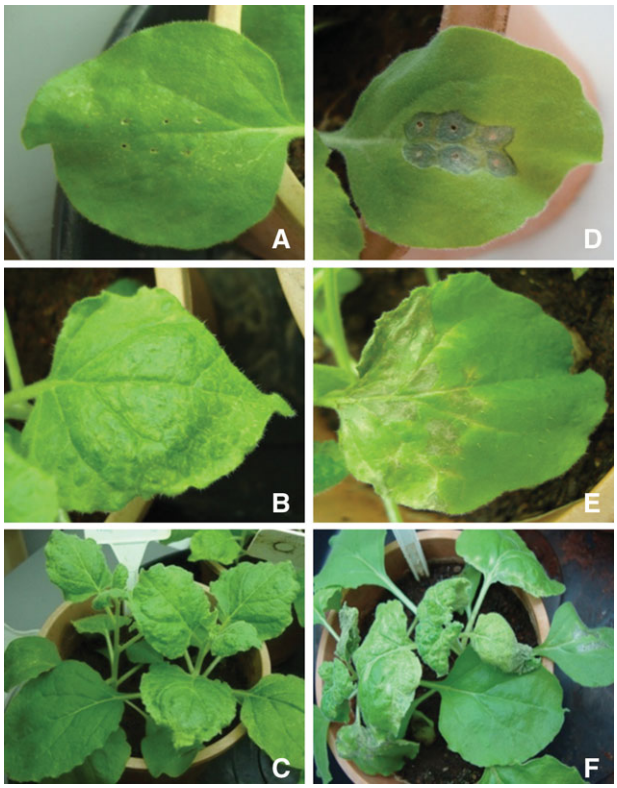

Figure 1. Symptoms of *N. benthamiana* plants infected with PVX (A, B, and C) and with PVX-AC2 (D, E, and F). (A, D) close-up view of leaves inoculated with *A. tumefaciens* containing PVX and PVX-AC2. (B, E) Systemic symptoms showing mosaic lesions and necrosis, respectively, at 17 days after inoculation. (C, F) Overview of *N. benthamiana* plants inoculated with PVX and PVX-AC2, respectively.

approximately 2 μg of total RNA were mixed with 500 ng oligo d(T) primer and 0.5 mM dNTP and the reaction was incubated for 5 min at 65°C. A total of 1 μL Superscript Reverse Transcriptase II (200 U/μL), 1 X First strand buffer (Invitrogen), and 10 mM DTT were added and the reaction was incubated for 60 min at 42°C, followed by 15 min at 70°C. PCR reactions were performed using the primers specific for the PVX coat protein (CP) (forward: ATGTCAGCACCAGCTAGCAC and reverse: GTTATGGTGGTGGTAGAGTG) and ToCMoV AC2 gene (forward: 5′-GGTACCGCGCGGCCGCATGCGCAATTCATCTT-3′ and reverse: 5′-GTCGACGCGGCCGCCTATTTAAATATGTCATCCC-3′). A total of 0.5 μL of the synthesized cDNA, 0.15 μM of each primer, 150 μM dNTP, 1 U Taq DNA Polymerase, and 1X of the corresponding buffer (Invitrogen) were used for PCR reactions in the following conditions: 4 min at 95°C, 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C, and a final extension of 4 min at 72°C. The amplified fragments were separated in 1.0% agarose gels and visualized after ethidium bromide staining under UV light.

2.3 Protein extraction

Total proteins were extracted from approximately 2 mg of ground tissue pooled from the different sampling points. Only plants successfully infected were used for protein extraction. Two biological replicates were performed and at least five gels from each sample were produced. Proteins were extracted according to Mot and Vanderleyden [26] by adding 750 μL of extraction buffer (0.7 M sucrose, 0.5 M Tris-HCl, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, and 40 mM DTT) to the plant material and the same volume of phenol. Samples were agitated for 15 min and centrifuged for 3 min at 12 000 rpm. The upper phase was transferred to a new tube and reextracted twice with extraction buffer. Proteins were precipitated in 0.1 M ammonium acetate in methanol and washed with 80% acetone. Proteins were resuspended in rehydration buffer (7 M urea; 1 M thiourea; 4% m/v CHAPS; 2% IPG buffer pH 3–10 NL; 40 mM DTT) and the amount was estimated using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) by following the manufacturer's instructions.

2.4 Western blot

For Western blot analysis, proteins were extracted from approximately 1 mg of ground tissue from the different sampling points (5, 10, 15, and 17 dpi) separately. Approximately 40 μg of total proteins were submitted to SDS-PAGE using a 12% polyacrylamide gel. Samples were transferred to a PVDF membrane (Hybond-P/GE Healthcare Life Sciences), which was blocked with 5% milk in PBS-T (68 mM NaCl, 58 mM Na_2HPO_4, 17 mM NaH_2PO_4 pH 7.4, and 0.1% v/v Tween-20) overnight at 4°C and washed twice with 0.5% milk in PBS-T for 10 min. The membranes were incubated for 2 h at room temperature with the primary anti-CP antibody for PVX coat protein (diluted 1:2500 in 0.5% milk in PBS-T) and anti-Tomato golden mosaic virus (TGMV) AC2 for AC2 (diluted 1:2000 in 0.5% milk in PBS-T). After two washes with 0.5% milk in PBS-T, secondary antibody Anti-Rabbit IgG (Sigma) alcaline phosphatase conjugate, diluted 1:10 000 in 0.5% milk in PBS-T, was added and membranes were incubated for 1 h at room temperature. Membranes were washed with 0.5% milk in PBS-T and immunodetection was performed using SIGMA*FAST*™ BCIP® (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets (Sigma) dissolved in 10 mL of water.

2.5 2DE and image analysis

Immobiline DryStrips of 13 cm, pH 3–10 NL were rehydrated with 280 μL of rehydration buffer and traces of bromophenol blue containing approximately 600 μg of proteins for 16 h. The first dimension was performed using a Multiphor II Electrophoresis system (GE Healthcare), according to the manufacturer's instructions (phase 1: 300 V, 2 mA, 5 W,

0:01 h; phase 2: 3500 V, 2 mA, 5 W, 1:30 h; phase 3: 3500 V, 2 mA, 5 W, 4:00 h).

The strips were maintained in equilibration buffer (1.5 M Tris-HCl pH 8.8; 6 M Urea; 30% Glycerol; 2% SDS; 1% bromophenol blue) with 1 M DTT for 15 min followed by 15 min in the same buffer containing 2.5% iodoacetamide. The second dimension was performed by SDS-PAGE using 13% polyacrylamide gels and the molecular mass marker "Benchmark Protein Ladder" (Invitrogen). Proteins were stained with Coomassie Blue G-250 and the gel replicates were digitalized with the ImageScanner III (GE Healthcare).

Three gels from each condition showing highest spot resolution and homogeneity in the electrophoretic pattern were selected and used for image and data analysis with the software Image Master 2D Platinum version 7.05 (GE Healthcare). Spots were automatically detected, followed by manual editing to improve detection and eliminate technical artifacts. In order to compensate for slight variations in sample loading, gel staining, and destaining, data were normalized by expressing protein abundance as relative volume (% vol). Automated matching was performed and spot alignment was improved by manual spot detection and matching. The proteins were accepted as having been differentially expressed when they displayed a fold change of 1.5 and differences were significant in Student's *t*-test at a significance level of 95%. Image analysis was performed on the set of reproducible spots from each stage and only spots present in two of three replicates were considered for the differential expression analysis.

2.6 Protein identification by MS

Gel spots were washed for 15 min in 50% acetonitrile and 25 mM ammonium bicarbonate and then dehydrated with 100% acetonitrile for 10 min [27]. The gel spots were rehydrated in a 15 μL solution of trypsin Sequencing Grade (Promega) 0.1 μg/μL prepared in 50 mM NH_4HCO_3 and incubated at 37°C for 22 h. After 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 the MALDI plate. Peptides were analyzed by Ultra-Flex III or Auto-Flex Speed MALDI TOF-TOF (Bruker Daltonics) mass spectrometers operating in positive reflector (MS) and LIFT™ (MS/MS) modes.

Peak lists were generated using the FlexAnalysis 3.3 software (Bruker Daltonics). The sophisticated numerical annotation procedure algorithm was used to detect the monoisotopic peak values, with a quality factor threshold of 30 and 3 as S/N threshold. All MS and MS/MS peak lists were individually searched using the MASCOT search engine (Matrix Science, UK) with the NCBInr protein database and Viridiplantae taxonomy. The mass tolerance used in the searches was 150 ppm and one missed cleavage was allowed. Carbamidomethylation of cysteine residues and oxidation of methionine residues were considered as fixed and variable modifications, respectively. The cutoff value for the Probability Based Mowse score calculated by MASCOT (at $p < 0.05$) was used to accept the identification. For MS/MS data, the peptide mass tolerance was 150 ppm, MS/MS ion mass tolerance at 0.6 Da, allowance of 1 missed cleavage, and charge state +1. When the pI and MW of matched proteins were not available, these values were calculated using ExPASy Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html).

3 Results and discussion

AC2 proteins have been studied in several begomoviruses and some have been described as gene silencing suppressors [9, 19, 28–30]. Although the silencing suppression property of ToCMoV-AC2 has not been established yet, it has been shown that PVX-ToCMoV-AC2 causes enhanced symptoms in tomato, as well as *N. benthamiana* and *Datura stramonium* plants [23]. These results indicate that ToCMoV-AC2 is a pathogenicity determinant, which is a characteristic shared by several virus silencing suppression proteins [31]. Similar enhanced symptoms have been reported when the Tomato leaf curl virus (TLCV) C2 gene was expressed in *N. benthamiana* plants [29], showing that this gene has an important role in pathogenicity. In this study, we have analyzed the proteomic profile of *N. benthamiana* plants inoculated with PVX viral vector expressing the AC2 protein of the begomovirus ToCMoV to better understand the effect of this pathogenicity factor on the global protein expression of the host plant. Symptoms of *N. benthamiana* inoculated with *A. tumefaciens* containing PVX-AC2 and PVX control vectors were compared at 5, 10, 15, and 17 days after inoculation. Plants inoculated with PVX alone showed typical symptoms of mild mottling, whereas PVX-AC2 inoculated plants presented chlorotic and necrotic lesions, curled leaves, and systemic necrosis (Fig. 1), as previously observed [23]. The successful infection and expression of AC2 was also confirmed by RT-PCR using PVX CP and ToCMoV AC2-specific primers (Fig. 2). The RT-PCR analysis showed that the CP and AC2 mRNAs could be observed at 5 dpi with increasing intensities at 10, 15, and 17 dpi. Although slightly lower AC2 mRNA levels could be observed at 17 dpi when compared to 15 dpi, the results show that AC2 is expressed at an early stage of infection and its levels increase during disease development.

The expression of the AC2 protein was also detected by Western blotting analysis (Fig. 2). The results show that the AC2 protein could be detected at 15 dpi, which is consistent with the RT-PCR results that show an earlier AC2 mRNA detection at 5 dpi. Although the detection of AC2 protein was only observed at 15 dpi, it is probable that this protein is present at an earlier stage in low amounts, but was beyond the detection capacity of the Western blot analysis and therefore could not be visualized. Moreover, since the antibody used was developed against an AC2 protein from another virus (TGMV) showing 70% protein sequence identity to ToCMoV AC2 (Fig. 2), it is possible that the lower levels of

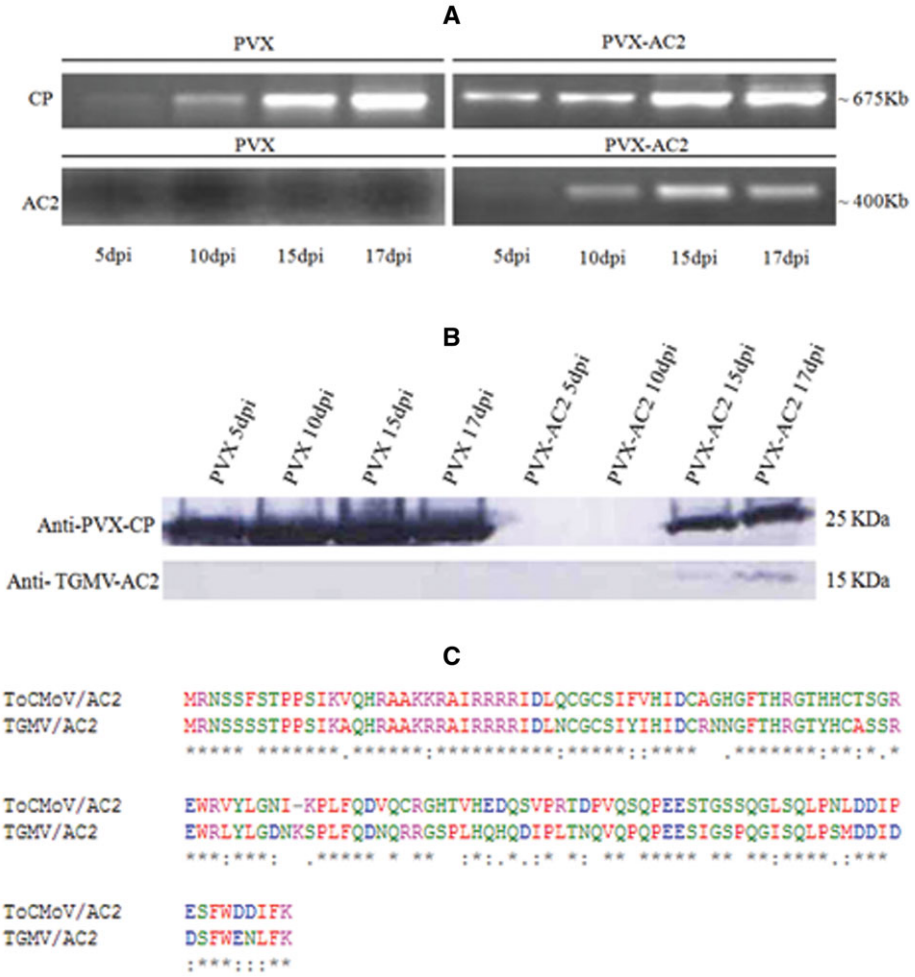

Figure 2. Expression of CP (PVX coat protein) and AC2 genes/proteins in *N. benthamiana* plants infected with PVX-AC2 and PVX vectors. (A) RT-PCR analysis of the expression levels of CP and AC2 genes at 5, 10, 15, and 17 days after inoculation. (B) Western blot analysis of the expression levels of CP and AC2 proteins at 5, 10, 15, and 17 days after inoculation. (C) Alignment of TGMV-AC2 and TocMoV-AC2 protein sequences, showing an amino acid identity of 70%.

detection observed may also be due to the lower specificity of the antibody.

Regarding the plant response, it is probable that even low amounts of AC2 protein trigger host response and cause changes in protein expression. This raises the question about the ideal sampling point for host plant expression analysis. The decision on the stage of plant infection to be analyzed is challenging and debatable, since the exact moment when the plant begins to respond to the pathogen is not known. Moreover, one has to bear in mind the limitations in detecting these changes. At early stages, e.g. the amount of tissue infected is still limited and probably few cells are affected. Moreover, it takes time for the plant to respond and produce visible symptoms. One way to overcome this dilemma is by pooling samples from different points. This strategy has been reported in other differential expression studies [32, 33], and it is certain that, although a broader overview of the biological process can be observed, some loss of information is inevitable. In this study, we have used this strategy and pooled samples from different dpi in an attempt to observe changes from the earlier to the later stages of infection. Total proteins were extracted from these pools and analyzed by 2DE.

The 2D gels analyses revealed approximately 500 protein spots per gel and, as expected, most proteins were observed in the pH range from 4 to 7 and in mass from 15 to 100 kDa (Fig. 3). The gel replicates from the control condition (empty PVX inoculated plants) revealed a correlation coefficient (r^2) ≥ 0.96, indicating a high reproducibility among the replicates. Similarly, the analysis of the 2D map replicates from the PVX-AC2 inoculated plants showed an $r^2 \geq 0.98$ (Fig. 3). When the 2D maps of both samples (PVX and PVX-AC2) were compared, a mean r^2 of 0.84 was obtained, which suggests that the AC2 protein interfered in the proteomic profile of the host plant.

When the protein profile of the PVX-AC2 inoculated plants was compared to the control condition, a total of 42 protein spots showed statistically significant variation above a minimum ratio of 1.5, including 24 up- and 13 downregulated, as well as three proteins exclusive to PVX-AC2 inoculated plants and two only observed in PVX inoculated plants (Figs. 3 and 4). A total of 36 differential protein spots were successfully identified by MS and are shown in Table 1 and Supporting Information Table 1. Interestingly, most of these proteins were upregulated in the presence of the AC2 gene (Fig. 4)

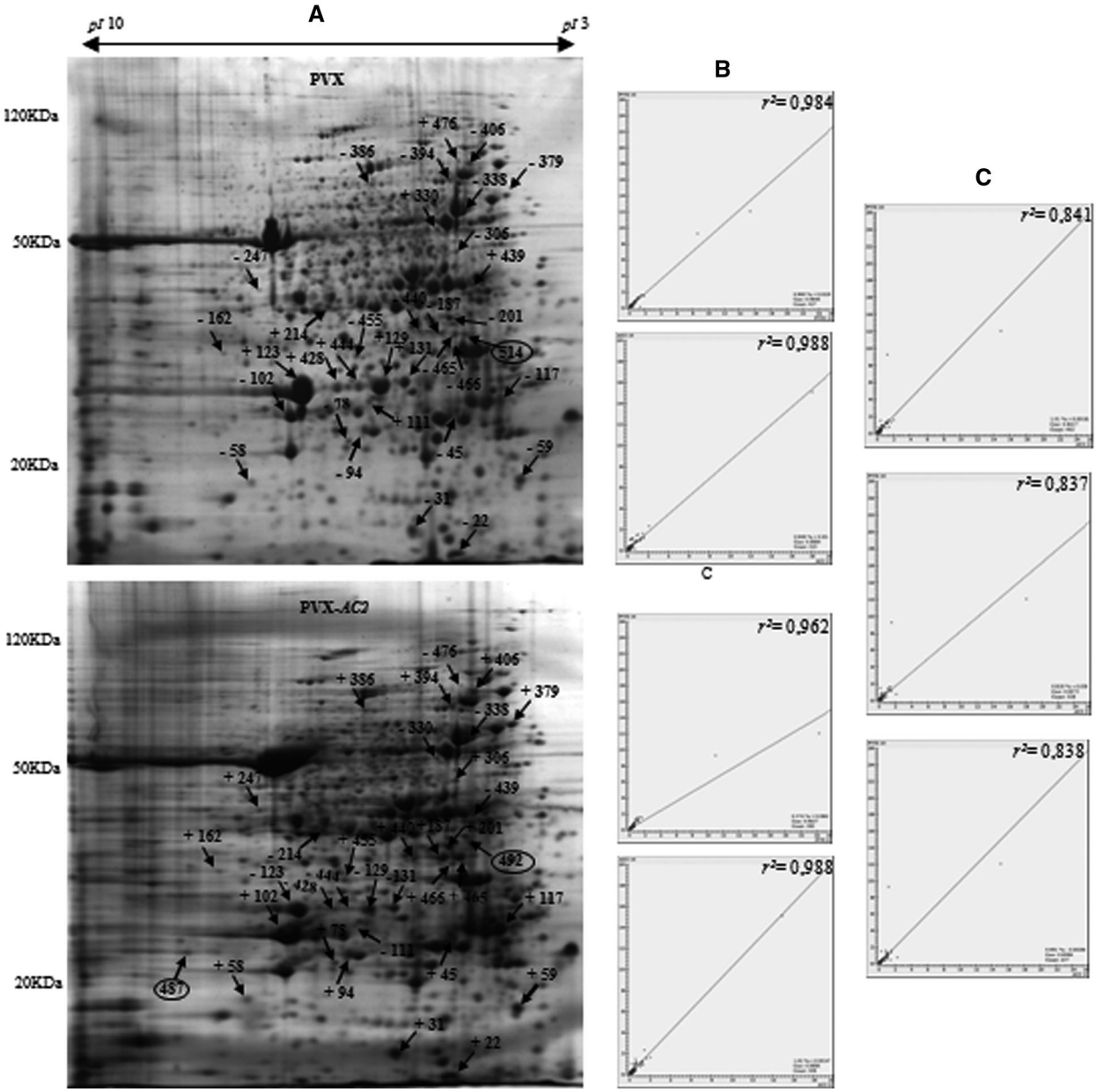

Figure 3. 2D maps of *N. benthamiana* plants inoculated with PVX and PVX-AC2 (A), as indicated, and scatter plot analysis of protein spots generated by the ImageMaster 2D Platinum 7.0 program (GE Healthcare) (B–D). Arrows indicate the differentially expressed proteins: (+) upregulated, (–) downregulated. Circled spots indicate putative exclusive proteins. (B) Comparison between the PVX gel replicates ($r^2 \geq 0.96$). (C) Comparison between the PVX-AC2 gel replicates ($r^2 \geq 0.98$). (D) Comparison between PVX and PVX-AC2 gel replicates.

and their identities indicate that different biological processes could be affected. An attempt to categorize the proteins identified was performed based on the literature and their biological roles are discussed below. Additionally, a metabolic pathway diagram depicting the influence of AC2 on host proteins was generated using SRI International Pathway Tools (version 15.5) based on the protein EC number (Supporting Information Fig. 3).

3.1 Viral proteins expressed in PVX-AC2 and PVX infected plants

Several spots corresponding to PVX viral proteins, such as TGB1 (25 kDa viral movement) (spot 102) and the CP (spots 131, 129, 123, 428, and 444) were identified in the 2D gel maps of PVX and PVX-AC2 inoculated plants (Figs. 3 and 4). The 25 kDa protein (TGB1) presented higher expression

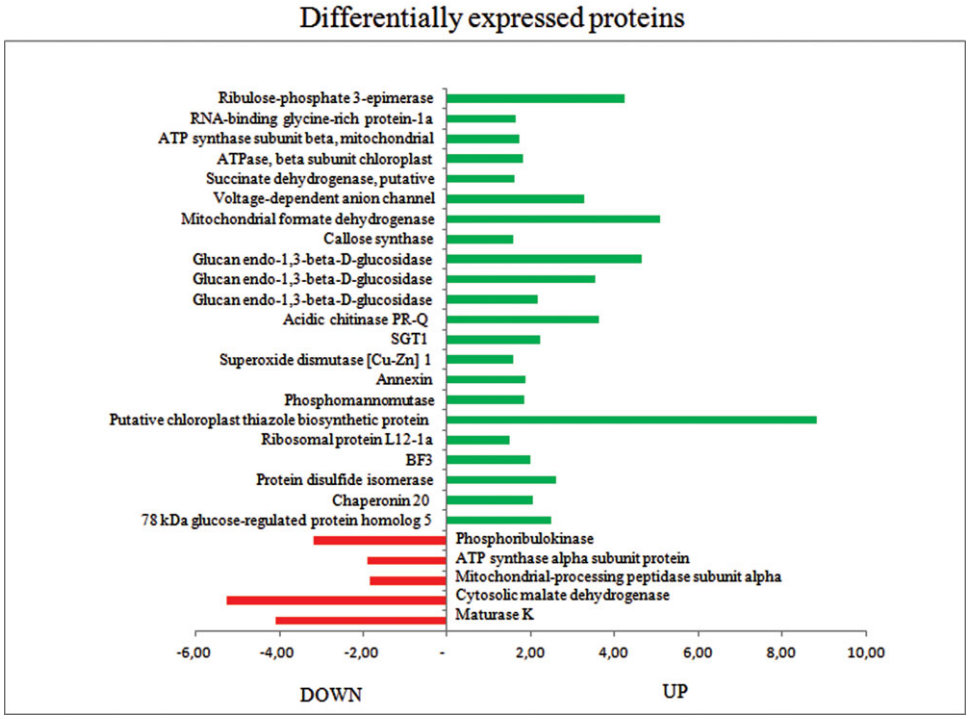

Figure 4. Diagram showing proteins that were up- (green bars) and downregulated (red bars) in *N. benthamiana* plants inoculated with PVX-AC2. The X axis represents the expression ratio detected in PVX-AC2 when compared to PVX (PVX-AC2/PVX).

(2.73-fold increase) in the plants inoculated with PVX-AC2. This protein was shown to be important for viral movement from cell to cell, via plasmodesma, in different host plants [34, 35] and can prevent the signaling of gene silencing [36]. In addition to the TGB1 protein, the coat protein is also required to facilitate viral movement in the host cells [37]. However, in our study, the expression of the coat protein was lower in plants inoculated with the PVX-AC2 construction (Table 1). Similar findings have been reported earlier by Chapman et al. [15], and according to these authors, although there was a lower accumulation of coat protein mRNA in PVX-GUS infected plants, PVX-GUS did infect the plant systemically, as was observed in our study for PVX-AC2 (Fig. 1).

Several spots of approximately 28 kDa with distinct isoelectric point were identified as coat protein (Table 1). It is well known that this protein undergoes several posttranslational modifications such as glycosylation and phosphorylation, affecting the physical and chemical structure of the protein surface [38, 39], which may account for the presence of several spots from the same protein with different pIs. Additionally, an anomalous behavior of the PVX CP electrophoresis mobility in SDS-PAGE was previously observed [40].

The fact that AC2 was not observed in the 2D gels can be explained by the molecular mass of this protein, which is 14.6 KDa (theoretical). Our gels had a concentration of 13% acrylamide, and therefore the limit for spot detection was around 15 KDa.

3.2 AC2 interferes in the photosynthetic processes and energy production

A successful viral infection is highly dependent on creating a suitable environment for viral multiplication and movement, which involves using the cellular machinery of the host and at the same time counteracting host defense responses. Viruses regulate several processes in the host cell such as transcription, translation, and posttranslational modifications in order to fulfil this task. Therefore, the study of host proteins modulated during successful viral infection represents an important approach in identifying potential targets for engineering viral resistance.

In the present study, it was observed that the presence of the AC2 protein produced a cellular imbalance, mainly of proteins involved in the metabolic and photosynthetic processes, which are also commonly affected during viral infections. Two proteins involved in photosynthesis (Ribulose-phosphate 3-epimerase and RNA-binding glycine-rich protein-1a) were upregulated, although usually biotic stresses negatively regulate genes related to photosynthesis [41]. Two other proteins were downregulated including phosphoribulokinase, which showed a fold change of –4.08 (spot 439) and the oxygen-evolving enhancer protein 1 (PSBO), a subunit of the chloroplast photosynthetic complex II that was exclusive to PVX infected plants. Phosphoribulokinase is involved in the regulation of the sugar flow in the Calvin cycle [42], and is regulated under stress conditions [43]. Similar results were also observed by Soitamo

Table 1. Protein identifications of differential spots from 2DE analysis by MALDI-TOF MS and MS/MS

Spot number	Protein identification	Organism	Acession # (NCBInr)[a)]	Fold change[b)] (PVX-AC2/PVX)
Viral movement and assembly				
102	25 kDa movement protein	Potato virus X	gi|138400	2.73
131	Coat protein	Potato virus X	gi|61430	–1.79
129	Coat protein	Potato virus X	gi|61430	–5.02
123	Coat protein	Potato virus X	gi|61430	–6.95
428	Coat protein	Potato virus X	gi|61430	–5.20
444	Coat protein	Potato virus X	gi|61430	–12.35
Photosynthesis and energetic metabolism				
94	Ribulose-phosphate 3-epimerase	Solanum tuberosum	gi|2499728	4.24
22	RNA-binding glycine-rich protein-1a	Nicotiana sylvestris	gi|469070	1.63
439	Phosphoribulokinase	Mesembryanthemum crystallinum	gi|125578 gi|15222551	–4.08
514	Oxygen-evolving enhancer protein 1, chloroplastic (OEE1)	Nicotiana tabacum	gi|11134054	Exclusive (PVX)
476	ATP synthase, alpha subunit chloroplast	Nicotiana sylvestris	gi|78102516	–5.26
394	ATPase, beta subunit chloroplast	Nicotiana rustica	gi|114557	1.82
338	ATP synthase, beta subunit mitochondrial	Nicotiana plumbaginifolia	gi|114421	1.73
330	Mitochondrial-processing peptidase subunit alpha	Solanum tuberosum	gi|266567 gi|587562	–1.83
386	Succinate dehydrogenase, putative	Ricinus communis	gi/255579273	1.62
162	Voltage-dependent anion channel	Nicotiana tabacum	gi|161788874	3.28
247	Mitochondrial formate dehydrogenase	Nicatiana attenuata Solanum lycopersicum	gi|116739352 gi|350538487	5.10
78	Callose synthase	Arabidopsis thaliana	gi|334184624	1.59
Defense and oxidative stress				
465	Glucan endo-1,3-beta-*D*-glucosidase	Nicotiana tabacum	gi|100324	4.66
187	Glucan endo-1,3-beta-*D*-glucosidase	Nicotiana tabacum Solanum tuberosum	gi|100324 gi|3395595	3.55
440	Glucan endo-1,3-beta-*D*-glucosidase	Nicotiana tabacum	gi|100324	2.15
117	Acidic chitinase PR-Q	Nicotiana tabacum	gi|116342 gi|19773	3.63
487	27 kDa pathogenesis-related protein (NtPRp27)	Nicotiana tabacum	gi|5360263	Exclusive (PVX-AC2)
306	Suppressor of G2 allele of Skp1 (SGT1)	Nicotiana benthamiana	gi|58760268 gi|29468339	2.22
492	Epoxide hydrolase	Nicotiana benthamiana	gi|189306755	Exclusive (PVX-AC2)
31	Superoxide dismutase (Cu-Zn)	Nicotiana Plumbaginifolia	gi|134616 gi|134612	1.58
201	Annexin	Nicotiana tabacum	gi|2467253	1.88
455	Phosphomannomutase	Nicotiana tabacum	gi|122194125	1.85
466	Putative chloroplast thiazole biosynthetic protein	Nicotiana tabacum	gi|30013665	8.83
Transcription and translation				
214	Cytosolic malate dehydrogenase	Nicotiana tabacum	gi|10798652	–1.90
59	Ribosomal protein L12–1a	Nicotiana tabacum	gi|20020	1.49
58	Basic transcription factor (BTF3)	Nicotiana benthamiana	gi|90823167	2.00
111	Maturase K, chloroplast	Chamerion angustifolium	gi|379039935	–3.17
Chaperones				
379	Protein disulfide isomerase	Nicotiana benthamiana	gi|257222620	2.59
451	Chaperonin 20	Arabidopsis thaliana	gi|15242045	2.05
406	78 kDa glucose-regulated protein homolog 5	Nicotiana tabacum	gi|729623	2.48

a) More than one accession cited indicates different hits for peptides identified from the same protein.
b) The "–" signal indicates downregulation.

et al. [44] who showed that transgenic tobacco plants expressing AC2 from ACMV, had several photosynthesis genes downregulated, including PSBO. They suggest that these alterations in sugar metabolism-related gene expression imply a carbon metabolism imbalance. Soitamo et al. [45] analyzed transgenic tobacco plants, which constitutively expressed the helper component-proteinase (HC-Pro), an RNA-silencing suppressors derived from potyviral genome. These authors also showed the downregulation of photosynthetic proteins such as chlororespiration and cyclic electron transport proteins. It seems that, similarly to HC-Pro, the presence of the AC2 protein also resulted in a carbon metabolism imbalance in *N. benthamiana* plants.

Other proteins involved in electron transport in chloroplast and mitochondria were also identified in the present study. Interestingly, the α subunit of the chloroplast ATPase was downregulated in PVX-AC2 plants (spot 476; –5.26-fold), while the β subunit was upregulated (spot 394; 1.82-fold). The chloroplast ATP synthase is formed by nine subunits, three of which are encoded in the nucleus (atpC, atpD, and atpG) while the other six by two separate operons in the chloroplast: the atpB–atpE (atpB/E) operon and the atpI–atpH–atpF–atpA cluster [46]. Differences in expression of the α and β ATPase subunits have been reported during the interaction of tomato plants resistant and susceptible to Tomato yellow leaf curl virus (TYLCV) [47]. It was shown that the α subunit of ATP-synthase was only observed in the resistant interaction, while the β subunit was upregulated in the susceptible interaction [47]. An additional role for the ATPase β subunit has been recently demonstrated by [48]. These authors isolated a mitochondrial ATP synthase β subunit responsive to fumonisin B1 (FB1), a mycotoxin secreted by fungi from the genus *Fusarium* that initiated programed cell death in *Arabidopsis* [49], and propose that this subunit is a novel plant cell death regulator. Although these studies were performed with mitochondrial ATPAse β subunit, choloplast ATPase β subunit was also identified as responsive to FB1. It is possible that the differences found in the expression patterns of α and β chloroplast ATPase subunits in this study may be due to a possible multifunctionality of these subunits.

Regarding the mitochondria ATP synthase, the β subunit was upregulated (spot 338; 1.73-fold, respectively). Our results showed that three mitochondrial proteins identified in PVX-AC2 plants are involved in oxidative phosphorylation: mitochondrial processing peptidase (α-MPP) (spot 330; –1.83-fold), β subunit of ATP synthase, and succinate dehydrogenase (spot 386; 1.62-fold). Two other proteins showed upregulation in PVX-AC2 infected plants: voltage-dependent anion channel (spot 162; 3.28-fold) and mitochondrial formate dehydrogenase (spot 247; 5.1-fold).

It has been reported in planta that βC1 silencing suppressor protein from the begomovirus-associated DNA-β satellite was able to regulate host genes involved in photosynthesis and respiratory processes [50]. We have observed similar results in this study for the AC2 protein of ToCMoV, which regulated the expression of different chloroplast and mitochondria proteins. The accumulation of the mitochondrial protein voltage-dependent anion channel, succinate dehydrogenase, and mitochondrial formate dehydrogenase were higher in PVX-AC2 infected plants, which showed a higher severity in the symptoms. Several studies have shown that the expression of these proteins is altered under stress conditions as a defense response [51, 52] and may be associated to symptom development.

On the other hand, α-MPP was downregulated in PVX-AC2 infected plants. This subunit is one of the components that forms the mitochondrial processing peptidase [53] and under stress conditions, the levels of this protein can be altered, resulting in the increase of protein synthesis and energy production in the mitochondria of infected plants [54].

In general, it is interesting to observe that the ToCMoV AC2 protein was able to affect important proteins involved in cellular respiration, revealing a possible function in modulating the mitochondrial proteome. These results give some insight as to the processes affected by begomovirus-host interactions.

Besides proteins associated to photosynthesis and energy production, we also identified a callose synthase (spot 78; 1.59-fold), a cellular metabolism protein. An increased accumulation of callose has been previously observed around TMV-induced lesions in β-1,3-glucanase-deficient tobacco plants, which resulted in decreased susceptibility to TMV [55]. In soybean, repression of callose synthase at 7 days after inoculation and later upregulation at 14 days postinoculation was observed, which may be associated with a delayed resistance response [56]. The molecular mass observed for the identified callose synthase (23 kDa) presented a significant difference to the theoretical mass (226 kDa). It has been suggested that callose synthase represents a multisubunit enzyme complex, with polypeptides varying between 25 and 92 kDa [57].

Taken together, these results suggest that the presence of the AC2 protein was able to cause an imbalance in cellular homeostasis, interfering in chloroplast and mitochondria functioning. The up- or downregulation of several genes and proteins is dependent on sampling time (days after infection) and many of them may be involved in symptom development. In the present study, leaves of different sampling points were pooled together and therefore this fluctuation in protein detection was probably minimized.

Previous studies in virus-infected plants have shown that disease symptoms are correlated with increased glycolysis, respiration, and starch accumulation, decreased photosynthesis and reduced protein synthesis [56, 58]. In this study, we show that ToCMoV AC2 expressed from a PVX viral vector is capable of interfering in important cellular processes in the host plant, indicating that a similar situation may occur in ToCMoV infection, highlighting the importance of this protein in viral infection.

3.3 AC2 modulates defense-related proteins and oxidative stress in the host plant

Another group of proteins induced by ToCMoV AC2 was the pathogenesis-related proteins (PRs). These proteins are induced by the plant defense system in an attempt to avoid pathogen development and infection [59]. In this study, three PRs were upregulated in PVX-AC2 inoculated plants, including spots identified as glucan endo-1,3-beta-D-glucosidase (spots 465, 187, and 440) with different isoelectric points (Table 1), acidic chitinase (spot 117; 3.63-fold), and 27 kDa pathogenesis-related protein (NtPRp27) (spot 487; exclusive to PVX-AC2). It has been reported that plants infected with begomovirus increase the levels of PR proteins [60]. The results obtained herein, showed the induction of these proteins in response to PVX-AC2 in the absence of other ToCMoV proteins. Soitamo et al. [44] have likewise shown similar findings in transgenic tobacco plants expressing ACMV AC2, which presented upregulation of defense and oxidative stress associated genes, comprising transcripts involved in the systemic acquired resistance, ROS scavenging, and respiratory burst oxidation.

Interestingly, a suppressor of G2 allele of Skp1, SGT1 (spot 306; 2.22-fold), was also identified in this study, with a higher expression level in plants inoculated with PVX-AC2. This protein is involved in several biological processes such as plant development, defense response [61], protein folding [62], and degradation of target proteins during the interaction with the SCF complex (SKP1, Cullin, F box), which is a multiprotein E3 ubiquitin ligase complex that catalyzes protein ubiquitination and is important for the elimination of misfolded proteins [63]. These results indicate that AC2 may affect the regulatory centre of the ubiquitination process [12] to avoid cytotoxicity, and consequently interfere with the expression of other proteins to favor viral infection. Lozano-Durán et al. [64] used reverse genetics to identify several host genes involved in geminivirus infection, which included an SKP1-like 2 gene. These authors show that AC2 is capable of hindering the activity of SKP1 and other SCF complex proteins, probably conferring a biological advantage for the viral infection, in particular related to the ubiquitination processes of plant proteins.

We have also identified several upregulated proteins involved directly or indirectly in oxidative stress, such as epoxide hydrolase (spot 492; exclusive to PVX-AC2), superoxide dismutase (spot 31; 1.58-fold), annexin (spot 201; 1.88-fold), phosphomannomutase (spot 455; 1.85-fold) and a putative chloroplast thiazole biosynthetic protein (spot 466; 8.83-fold). It is well known that epoxide hydrolase and superoxide dismutase are directly involved in antioxidant activity and are induced as a defense response in order to reduce the effects of oxidative stress [65]. Two metabolism proteins, an annexin and a phosphomannomutase, and one associated to vitamin biosynthesis, the putative chloroplast thiazole biosynthetic protein, have been reported to be indirectly involved with the oxidative process [66, 67]. Annexin has also been implicated in the modulation of callose synthase activity in cotton [68], which may be related to wound healing and stress situations [69]. Callose synthase was also identified in this study, as discussed above, and like annexin, was upregulated in response to AC2.

Interestingly, the upregulation in ROS scavenging proteins show that the effect of AC2 occurs toward creating a suitable environment for virus infection by containing stress to avoid cellular death, which would be unfavorable for viral spread. Indeed, such an antioxidant protein profile could not only ameliorate disease symptoms but also facilitate viral survival. In this way, AC2 seems to prolong cell lifespan facilitating virus multiplication and virus movement in host cells.

3.4 Transcription and translation factors affected by AC2

The modulation of transcription and translation factors by begomovirus AC2 proteins has been previously reported. In this study, we identified a cytosolic malate dehydrogenase (cyMDH) that was downregulated in response to the ToCMoV AC2. Similar results were obtained by Soitamo et al. [44], when analyzing the effect of a silencing suppressor from ACMV in tobacco. cyMDH is an important enzyme in metabolism, which affects the stability and transactivation of a transcriptional factor that controls cellular cycle (arrest), cell death, and DNA repair [70]. Under stress conditions, the level of cyMDH can be altered, favoring plant growth and conferring tolerance to stress [71].

Evidence has shown that the AC2 protein is also able to affect the expression of proteins involved in the transcriptional process, such as the Scarecrow transcription regulators-like SCL6-II and SCL8 [9]. Interestingly, other proteins involved in the transcription and translation processes were also identified in this work, such as the ribosomal protein L12–1a (spot 59; 1.49-fold), the basic transcription factor BTF3 (spot 58; 2.0-fold), and maturase K (spot 111; –3.17-fold).

Maturase K, which was downregulated in PVX-AC2 infected plants, is an enzyme that acts as a posttranslational splicing factor in the chloroplast and the generated proteins are important for the correct functioning of the chloroplast, including photosynthesis [72]. It has been reported that higher levels of maturase K are associated to increased photosynthetic activity [73], which is not usually observed upon pathogen infection.

Our study also revealed the accumulation of the transcription factor BTF3 in PVX-AC2 infected plants. BTF3 was first studied in mammalian cells as one of the basal transcription factors involved in initiation of transcription from class II promoters [74] and have been implicated in apoptosis [75]. In plants, the function of this protein is still not clear. It was observed that BTF3 deficiency in *N. benthamiana* plants preferentially affects chloroplasts and mitochondria [76]. Subunits of BTF3 have also been associated with HR-mediated cell death in *Capsicum annuum* against TMV in a resistant

interaction but not in a susceptible one [77]. The authors suggest that BTF3 regulates the transcription of defense-related genes upon TMV infection [77]. In our model system, the BTF3 could play a similar role triggered by AC2 expression.

3.5 Chaperone accumulation in PVX-AC2 infected plants

Three proteins involved in folding were identified in this study and were upregulated in PVX-AC2 infected plants: disulfide isomerase (spot 379), chaperonin 20 (spot 451), and 78 kDa glucose-regulated protein homolog 5 (BiP 5/GRP 78; spot 406). Interestingly, it has been proposed that BiP might regulate the cytotoxic effects of PVX proteins during virus infection in order to reduce cytotoxicity and consequently necrosis to favor viral multiplication [78]. This response may be important to avoid cellular disturbance and even death in cases in which viral proteins reach high levels. Similarly, our results indicate that AC2 is also able to enhance BiP expression and may contribute to facilitate virus infection and movement in host cells.

It is well known that chaperones are upregulated under stress conditions [79–81] and may form complexes with viral proteins during infection. Krenz et al. [82] reported that the *Arabidopsis thaliana* heat shock protein 70 kDa (HSP70) interacts with the movement protein of the Abutilon mosaic virus, and propose that this interaction is important for viral transport and symptom induction. Lozano-Durán et al. [64] also showed the importance of an HSP70 in Tomato yellow leaf curl Sardinia virus infection. These authors propose that heat shock proteins may be important for rapid protein maturation and turnover during a short virus multiplication cycle. HSP70 has also been reported to be a major interactor of SGT1 [83], which is essential for resistance to viruses [84] and was also identified in our study. The possible interaction between the AC2 protein of ToCMoV with host plant chaperones was not reported previously, however, it may occur and could play a key role in favoring successful viral infection by providing the host protein modification machinery for the virus to replicate in the plant.

4 Concluding remarks

As mentioned earlier in our study, a successful viral infection relies on the host plant machinery for viral replication and spread. Although several studies have been performed to investigate host proteins involved in these processes, the identification of the exact role of the host genes and proteins is still a challenging task. In this work, we have used proteomic techniques to better understand the effects of the ToCMoV AC2 pathogenicity factor in the protein expression pattern of the host plant. The AC2 protein was able to significantly alter the global protein profile in *N. benthamiana* leaves, and consequently affect several critical biological processes. This is the first report of the global effect of the virulence factor ToCMoV AC2 in the protein profile of a host plant. This study revealed that more than 50% of the differentially expressed proteins were upregulated in *N. benthamiana* plants. Moreover, this study highlights the effect of AC2 in the modulation of plant defense processes, by increasing the expression of several oxidative stress-related and PR proteins, as well as its role in modulating the proteome of the photosynthesis and energy production systems. Interestingly, the protein that showed the most pronounced upregulation in response to PVX-AC2 was the putative thiazole biosynthetic protein (spot 466; 8.8-fold change). This protein is associated to thiamine biosynthesis, which is a very important cofactor in several metabolic pathways and has also been associated with enhanced tolerance to oxidative stress [67] and resistance to plant diseases [85]. Based on the results obtained, this new information on the effect of the AC2 protein in the host reveals that this pathogenicity factor has an extremely important role in the interaction between the virus and the plant, which compromises the homeostatic equilibrium of the cell. It is clear that the AC2 protein plays a key role in viral infection and is essential for pathogenicity in tobacco. Based on the results obtained, target proteins can now be selected to direct future studies aiming at the generation of plant lineages tolerant or even resistant to ToCMoV.

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