Eur J Plant Pathol
https://doi.org/10.1007/s10658-023-02802-z

Proteomic insights into the effect of Broad bean wilt virus-1 infection in *Nicotiana benthamiana* plants

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Accepted: 6 December 2023

Abstract Broad bean wilt virus 1 (BBWV-1, genus *Fabavirus*, family *Secoviridae*) infects many plants species, including important horticultural crops. Since some tobacco plants such as *Nicotiana benthamiana,* are susceptible to many viruses, they are used as experimental hosts and therefore abundant information about these species is available in databases. Here, the protein differential expression in *N. benthamiana* plants infected with BBWV-1 was studied using Liquid Chromatography coupled with Mass Spectrometry in tandem analysis (LC–MS/MS). Also, we studied the role of viral VP37 protein which is a BBWV-1 determinant of pathogenicity in the accumulation of the host proteins. For this purpose, we agroinfiltrated *N. benthamiana* plants with two BBWV-1 cDNA infectious clones: pBBWV1-Wt wilt type and the pBBWV1-G492C mutant knocking out for the viral VP37 protein. Virus infection induced the differential expression of 44 host proteins: 22 were overexpressed and the other 22 were underexpressed. These proteins were involved in important plant processes and located in different cell organelles, mainly in chloroplasts. Finally, 24 of these proteins were expressed differentially according to the presence of VP37 protein. Relation among host proteins that were differentially expressed, plant symptoms, and subcellular alterations are discussed.

Keywords BBWV-1 · LC–MS/MS · Proteomic · *Nicotiana benthamiana* · Viral VP37 protein

Introduction

The impact of infectious diseases has been increasing on crops worldwide due to the proliferation of extensive monocultures, globalization with intense trade of plant material and global climatic change (Sanfaçon, 2017). Viruses are considered as one of the major threats reaching up to 50% of the infectious diseases in crops (Jones, 2021). Also, viruses have usually a wide range of host species: for example, cucumber mosaic virus (CMV) infects over 1200 species in more than 100 plant families including vegetables, ornamentals, and woody plants (Palukaitis & García-Arenal, 2003). Reducing damage produced by viruses in crops is a difficult task since viral diseases have no cure and their control is limited to the application of prophylactic measures to prevent the infection and the use of resistant plants. Losses caused by plant viruses are difficult to evaluate, but in some tropical and subtropical regions, they can reach up to 98% of the total crop production (Nazarov et al., 2020). Before the use of transgenic plants resistant to cassava brown

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Published online: 13 December 2023

streak disease (CBSD), which is caused by two ipomoviruses (family *Potyviridae*), the annual cassava production losses in east and central Africa were estimated in 1.9—2.7 billion US$ (Wagaba et al., 2017).

Another important problem for the control of plant diseases is the large number of vector species involved in virus transmission. Viruses from the genus *Fabavirus* (family *Secoviridae*) are transmitted by more than 20 species of aphids in a non-persistent manner and are distributed worldwide producing important losses in horticultural, fruit, and ornamental crops such as bean (*Vicia faba*), pepper (*Capsicum annuum*) tomato (*Solanum lycopersicum*), sour cherry (*Prunus cerasus*) and petunia (*Petunia sp.*) (D. Ferriol et al., 2013; King et al., 2011; Šafářová et al., 2017). The genus *Fabavirus* is composed of eight species: broad bean wilt virus 1 (BBWV-1), broad bean wilt virus 2 (BBWV-2), cucurbit mild mosaic virus, peach leaf pitting-associated virus (PLPaV), gentian mosaic virus (GEMV), grapevine fabavirus (GFabV), lamium mosaic virus (LMMV) and prunus virus F (PrVF) (International Committee on Taxonomy of Viruses, ICTV). BBWV-1 infects important horticultural crops such as pepper, broad bean, and tomato, and its genome is composed of two positive-sense single-stranded RNAs (RNA1 and RNA2) that are separately coated into icosahedral virions (Sanfaçon, 2015). RNA 1 (5.8 kb) has an open reading frame (ORF) encoding for a polyprotein which is processed by proteolytic cleavage to form a protease cofactor (PRO-CO), an helicase (HEL), a viral genome-like protein (VPg), a protease (PRO) and an RNA-dependent RNA polymerase (POL). RNA 2 (3.4 kb) has two "in frame" successive translation initiation codons encoding for the alternative polyproteins that differ only in their N-terminal extension length (Chen & Bruening, 1992; Lekkerkerker et al., 1996; Qi et al., 2002; Xie et al., 2016). Both polyproteins are processed by proteolytic cleavage rendering the large and the small coat protein (LCP and SCP, respectively) and two putative movement proteins (MPs) of different sizes but with overlapping C-terminal regions, with 47 and 37 kDa (VP47 and short VP37, respectively) (Carpino et al., 2020a).

Advances in the different "omic" techniques have contributed to understand the physiological processes underlying viral infections and plant symptom development. Proteomics is a powerful technique for identifying massive changes in the protein accumulation patterns produced by virus infections. Plant proteins usually affected are related to cellular cytoskeleton, photosynthesis, photorespiration, chlorophyll metabolism, and RNA binding (Brizard et al., 2006; Carli et al., 2012; Souza et al., 2019). Classical two-dimensional electrophoresis (2-DE) coupled to mass spectrometry (MS) has been a platform commonly used for plant proteomic analysis (Carli et al., 2012). However, the application of a quantitative "second generation" of proteomic techniques such as the Liquid Chromatography-Mass Spectrometry (LC–MS) has been growing in the last years (Gomaa & Boye, 2015). This technique combines the physical separation of liquid chromatography (LC) with mass spectrometry (MS) analysis improving the sensitivity of the protein detection and reducing the background noise produced by contaminations. In addition, the analytical capabilities of MS can be improved by performing a tandem analysis MS/MS (Cui et al., 2018; Mittapelly & Rajarapu, 2020). Proteomic analysis has been performed in plants infected by different viruses showing important changes in the patterns of the host protein accumulation. For example, it has been detected that the infection of maize plants with maize chlorotic mottle virus (MCMV) induces the alteration of more than 600 proteins, including structural, metabolic, storage, processing, and cell signaling proteins. Some of these proteins such as the heat shock protein 70 (Hsp70) and the lipoxygenase were overexpressed, while others such as ferredoxin and calcium-dependent protein kinases were underexpressed (Dang et al., 2020). In the *Secoviridae* family, the infection with cowpea severe mosaic virus (CPSMV) leaded to the alteration in the accumulation of 2033 proteins in cowpea plants (*Vigna unguiculata*), most involved in processes such as metabolism, photosynthesis and photorespiration, cellular homeostasis, and intercellular transport (Lidia et al., 2017).

Experimental herbaceous hosts are important tools for the study of plant-virus interactions and abundant information on nucleotide sequences and proteins of some species are available in databases. Several tobacco species such as *Nicotiana benthamiana* and *N. tabacum* are highly susceptible to virus infections and they have been widely used for different studies. Recently, the effect of BBWV-1 infection in *N. benthamiana* was studied by Transmission Electron Microscopy (TEM), reporting many subcellular changes in chloroplasts, plasmatic membrane, and

mitochondria, and the presence of virus-like particles aggregates in inclusion bodies into the vacuoles (Medina et al., 2022). Furthermore, some of these ultrastructural alterations could be associated with the expression of BBWV-1 VP37 protein, which is a host specificity and pathogenicity determinant that was previously identified by using 35S-driven viral full-length cDNA infectious clone (pBBWV1-G492C) knocking out the viral VP37 protein (Carpino et al., 2020a). In these experiments, *N. benthamiana* and broad bean plants agroinfiltrated with pBBWV1-G492C showed milder leaf mosaic symptoms compared with those agroinfiltrated with the wilt type cDNA infectious clone (pBBWV1-Wt). In addition, pBBWV1-G492C were unable to infect systemically tomato and pepper whereas pBBWV1-Wt did it (Carpino et al., 2020a). Transient expression in *N. benthamiana* 16C plants revealed that VP37 and LCP are viral suppressors (VSR) of the post-transcriptional gene silencing pathway (PTGS) (Carpino et al., 2020b).

In the current work, the effect of BBWV-1 infection on protein accumulation in *N. benthamiana* was studied by LC–MS/MS. Moreover, the role played by BBWV-1 VP37 protein in the differential accumulation of some host proteins was determined by using the BBWV-1 cDNA infectious clone pBBWV1-G492C. The relationship of changes in the accumulation of some host proteins with plant symptoms and subcellular alterations is discussed.

Materials and methods

BBWV-1 cDNA infectious clones, *Agrobacterium tumefaciens* transformation and plant agroinfiltration

BBWV-1 cDNA infectious clones pBBWV1-Wt and pBBWV1-G492C, that were engineered as described in Carpino et al., 2020a, were used in this work. pBBWV1-Wt contained the full-length RNA1 and RNA2 of BBWV-1 isolate Ben into the binary plasmid pJL89 between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase terminator (t-Nos). pBBWV1-G492C contained the full-length RNA1 of pBBWV1-Wt but the full-length RNA2 included a single mutation at nucleotide position 492 (AUG > AUC, Met > Ile) knocking out the viral VP37 protein.

BBWV-1 cDNA infectious clones were introduced into *Agrobacterium tumefaciens* COR308 cells by electroporation following standard procedures (Martínez et al., 2014). *N. benthamiana* plants at four- to six-leaf stage were agroinfiltrated with transformed *A. tumefaciens* cells in infiltration buffer (10 mM MgCl2, 10 mM MES, pH 5.6). For each BBWV-1 cDNA infectious clone, 12 plants were agroinfiltrated. Also 12 *N. benthamiana* plants were mock agroinfiltrated (with only infiltration buffer not containing transformed *A. tumefaciens* cells) and used as healthy controls. *N. benthamiana* plants were kept in a growth chamber under conditions of 16 h light at 24 °C and 8 h darkness at 20 °C until sample collection. Periodically, plants were checked for symptoms.

Plant material sampling and protein extraction

To obtain protein extracts, about 100 mg of symptomatic apical leaves from *N. benthamiana* plants agroinfiltrated with BBWV-1 cDNA infectious clones or asymptomatic apical leaves from control mock-infiltrated plants were collected and homogenized by using a TissueLyser (Quiagen, Germany). Plant homogenates were resuspended in 350 µl of buffer Tris–HCL 1 M pH 7.5, EDTA 0.5 M pH 8, DTT 1 M, NaCl 5 M, and Triton X-100 and centrifugated 10.000 × g for 10 min at 4ºC. The supernatant phase was collected in a new tube and stored at -20ºC until its use. Protein concentration was estimated by Spectrophotometer Nanodrop 2000 (ThermoFisher Scientific, USA). Protein extracts of three different plants were pooled to form an individual biological replicate, obtaining four biological replicates for each condition: i) pBBWV1-Wt- agroinfiltrated, ii) pBBWV1-G492C-agroinfiltrated, and iii) mock-infiltrated.

LC–MS/MS analysis

Spectral libraries were obtained by mixing equivalent amounts of protein extracts from all the individual *N. benthamiana* plants. Protein extract pool (100 µg) was resolved in 1D SDS-PAGE gel, and proteins were visualized by gel staining with Coomassie blue solution and digested with 500 ng of trypsin (Promega, USA). The trypsin digestion was stopped with 10% trifluoroacetic acid (TFA), and the peptide solution was dried in a speed vacuum and resuspended in 0.1%

TFA and 2% acetonitrile (ACN). For the analysis of biological replicates, the same procedure was carried out but with 20 µg of protein extract.

LC–MS/MS analysis was performed with 5 µl of trypsin-digested samples, which were loaded onto a C18 NanoLC Column (Eksigent Technologies, USA) and desalted with 0.1% TFA at 3 µl/min during 5 min. The peptides were loaded onto a C18 LC Column (Nikkyo Technos, Japan) equilibrated in 5% ACN and 0.1% formic acid (FA). Peptide elution was carried out with a linear gradient of 5% to 35% B (ACN, 0.1% FA) for 90 min at a flow rate of 300 nl/min. Peptides were analyzed in a 5600 TripleTOF mass spectrometer nanoESI qQTOF (Sciex, Canada).

Mass spectrometer was operated in information-dependent acquisition mode with a 250-ms TOF MS scan from 350–1250 m/z, followed by a 150-ms product ion scans from 350–1500 m/z on the 25 most intense 2-5 charged ions. The rolling collision energies equations were set for all ions as for 2 + ions according to the following equations: |CE| = (slope) x (m/z) + (intercept). ProteinPilot v 5.0 (Sciex) default parameters were used to generate a peak list directly from 5600 TripleTof wiff files. The Paragon algorithm (Shilov et al., 2007) of ProteinPilot was used to search the Uniprot database (https://www.uniprot.org/help/uniprotkb) with the following parameters: trypsin specificity, cysteine alkylation, without taxonomy restriction, and the search effort set to through and FDR (p-value adjusted < 0.01) correction for proteins. The protein grouping was done by Pro group algorithm (a protein group in a Pro Group Report is a set of proteins that share some physical evidence). The protein groups in Pro Group were guided only by observed peptides.

Analysis of individual biological replicates was done with SWATH TripleTOF mode with a 0.050-s TOF MS scan from 350–1250 m/z followed by 0.080-s product ion scans from 350–1250 m/z on 32 defined windows (3.05 s/cycle). The individual SWATH injections were randomized, and SWATH windows parameters were 15 Da window widths from 450 to 1000 Da.

Protein quantitation and analysis of protein differential expression

Wiff files obtained from SWATH TripleTOF analysis were studied by Peak View 2.10, and the protein peak areas were analyzed with Marker View (Sciex). First, the protein areas were normalized by the total sum of the areas of all the quantified proteins. Then, Principal Component Analysis (PCA) and Discriminate Analysis (DA) were carried out, both with Pareto scaling for reduction of the dimensionality. Data were analyzed with the Elastic net penalization to obtain the proteins with significant differential expression (FDR < 0.01). Information on annotated proteins such as functions, organisms, and subcellular localization were obtained with Uniprot and Gen ontology (GO) (http://geneontology.org/) databases.

Results and discussion

Protein expression analysis in *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt or pBBWV1-G492C compared with non-infected plants.

The effect of BBWV-1 infection on protein expression of *N. benthamiana* plants was analyzed by LC–MS/MS in plants agroinfiltrated with *A. tumefaciens* cells harboring the 35S-driven full-length cDNA infectious clone corresponding to BBWV-1 RNA1 and RNA2 wild type (pBBWV1-Wt). The role of BBWV-1 protein VP37 in the accumulation of *N. benthamiana* proteins was also studied by agroinfiltration of a BBWV-1 cDNA infectious clone mutant knocking out VP37 (pBBWV1-G492C) (Carpino et al., 2020a). As healthy controls, *N. benthamiana* plants were mock-infiltrated by using infiltration medium without *A. tumefaciens* cells. All *N. benthamiana* plants agroinfiltrated with pBBWV-Wt developed identical symptoms to those induced by BBWV-1 such as plant stunting and severe leaf mosaic symptoms in the new emergent leaves at 10–21 days post infiltration (dpi), whereas the plants agroinfiltrated with pBBWV1-G492C showed only slight mosaic symptoms. The healthy mock-infiltrated control plants showed no symptoms in the new emergent leaves and remained symptomless after the 21 dpi (Fig. 1). All plants showed some necrotic spots in the infiltrated leaves due to the mechanical injuries produced during the infiltration process. These results are identical to those previously reported that described severe symptoms in *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt or mechanically infected with BBWV-1 and slight symptoms in equivalent plants

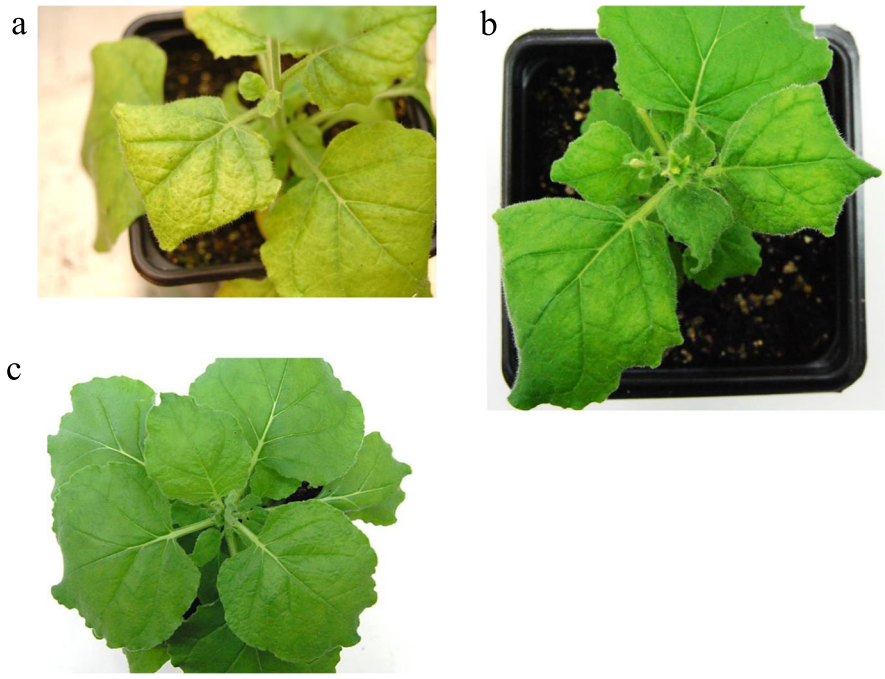

Fig. 1 Symptoms in *Nicotiana benthamiana* plants agroinfiltrated with pBBWV1-Wt (**a**) or pBBWV1-G492C (**b**) and mock-infiltrated used as healthy controls (**c**) after 15 days post infiltration

agroinfiltrated with pBBWV1-G492C (Carpino et al., 2020a; Medina et al., 2022).

Samples of symptomatic apical leaves of *N. benthamiana* plants agroinfiltrated with BBWV-Wt or pBBWV1-G492C were collected at 15 dpi, when symptoms were completely developed. At the same time, samples of asymptomatic apical leaves of mock-infiltrated plants were collected and used as healthy controls. All the samples were processed, and the protein extracts corresponding to three individual plants per each group of plants (pBBWV1-Wt, pBBWV1-G492C, or mock-infiltrated) were pooled to obtain one biological replicate. Then, four biological replicates for each group of plants were analyzed by LC–MS/MS. Statistical PCA and DA analysis of dimensionality reduction were performed to assess the homogeneity of results showing an acceptable aggrupation of the biological replicates in each plant group (Fig. 2). The LC–MS/MS output of spectral library identified 1186 proteins (FDR < 0.01). Analysis by using an Elastic-Net regression model of the individual biological replicates revealed a total of 46 proteins (including the two viral polyproteins) with differential expression (FDR < 0.01) and accumulation ratio between + 1.5 and -2 that could be related to BBWV-1 infection (Fig. 3). In the *N. benthamiana* plants agroinfiltrated with pBBWV-Wt, 46 proteins (including the two viral polyproteins) were differentially expressed respect the healthy control plants (22 host proteins were overexpressed and 22 host proteins were underexpressed). Twenty out of 22 overexpressed proteins were also overexpressed in the *N. bentamiana* plants agroinfiltrated with pBBWV1-G492C; one protein was overexpressed only in plants agroinfiltrated with pBBWV1-Wt, and other protein had high expression level in plants agroinfiltrated with pBBWV1-wt and healthy control plants.

Finally, 24 proteins were differentially expressed in *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt with respect to those agroinfiltrated with BBWV1-G492C (not expressing BBWV-1 VP37 protein): two proteins were overexpressed, and 22 were underexpressed. The differential expression of host proteins in the absence of VP37 could be related with the function of this viral protein that is a determinant of pathogenicity exacerbating symptoms, acting as suppressor of gene silencing and producing important subcellular alterations (Carpino et al., 2020a, 2020b; Medina et al., 2022).

Proteomic analysis employing different platforms has been successfully used for the study of plant-virus interactions for several viruses (Jain et al., 2021; Souza et al., 2019). However, the detection of host proteins showing differential expression depended on many

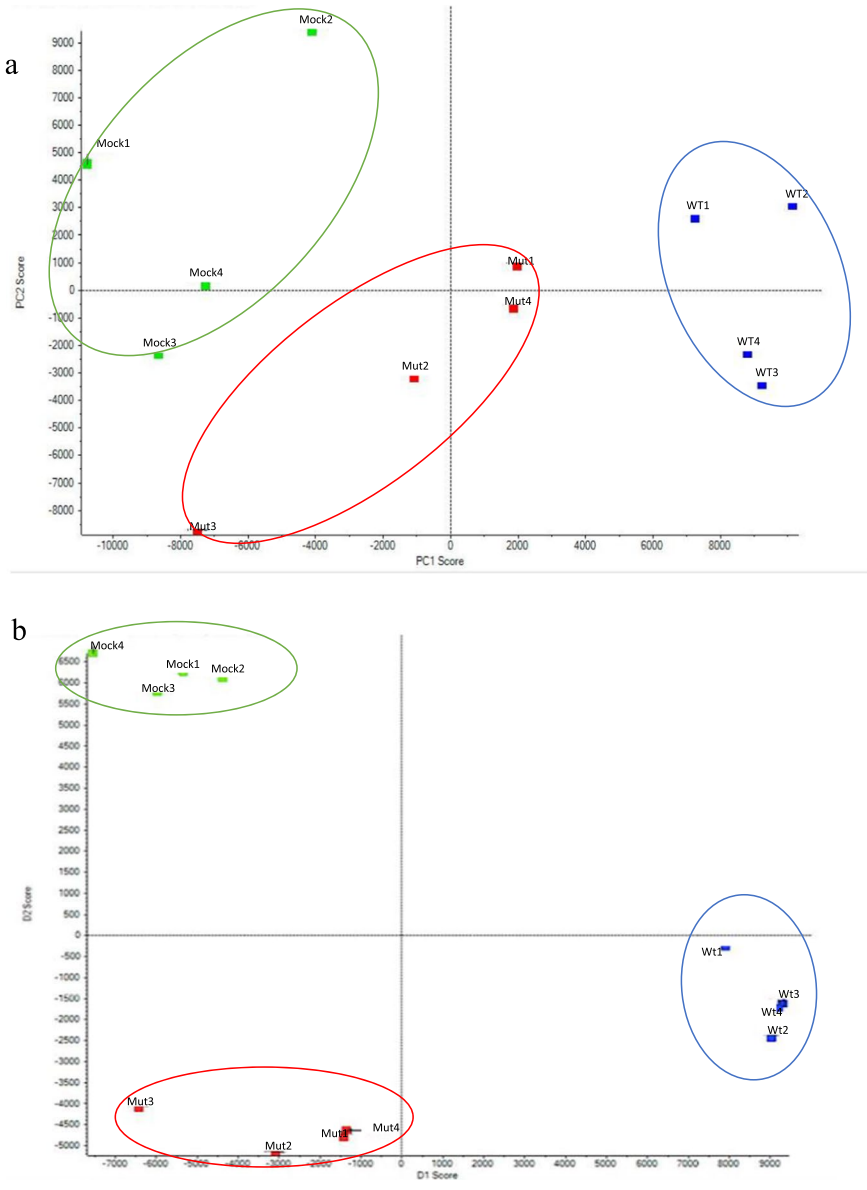

Fig. 2 Principal Component Analysis (PCA) and Discriminant Analysis (DA) of LC–MS/MS data (a and b, respectively) obtained from the four biological replicates (represented as squares) corresponding to *Nicotiana benthamiana* plants agroinfiltrated with pBBWV1-Wt (blue) or pBBWV1-G492C (red) cDNA infectious clones and mock-infiltrated used as healthy controls (green). X and Y axes represent the two-dimensional scores. Each square from the same color is a biological replicate and circles correspond to sample aggrupation

factors such as the virus-plant model studied, the proteomic technique employed (including restriction levels implemented by statistical analysis) and the infection period. It was reported that CPSMV infection, from the family *Secoviridae*, induced the differential expression of 2033 proteins in cowpea. Over these 2033 proteins, 1078 were detected at 2 dpi, whereas the number decreased to 955 proteins at 6 dpi (Varela et al., 2017). Pea (*Pisum sativum*) plants infected with pea seed-borne mosaic virus (PSbMV) showed 116 proteins with differential expression in comparison to healthy plants (Cerna et al., 2017). Tobacco mosaic virus (TMV) infection rendered different results, in different tobacco (*Nicotiana tabacum*) cultivars: the NC89 hypersensitive cultivar showed 260 proteins with differential expression, whereas in the Yuyan8 tolerant cultivar, the number decreased to 183 proteins (Wang et al., 2016).

Identification of the host proteins showing differential expression in *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt or pBBWV1-G492C

The host proteins showing differential expression among the three groups of *N. benthamiana*

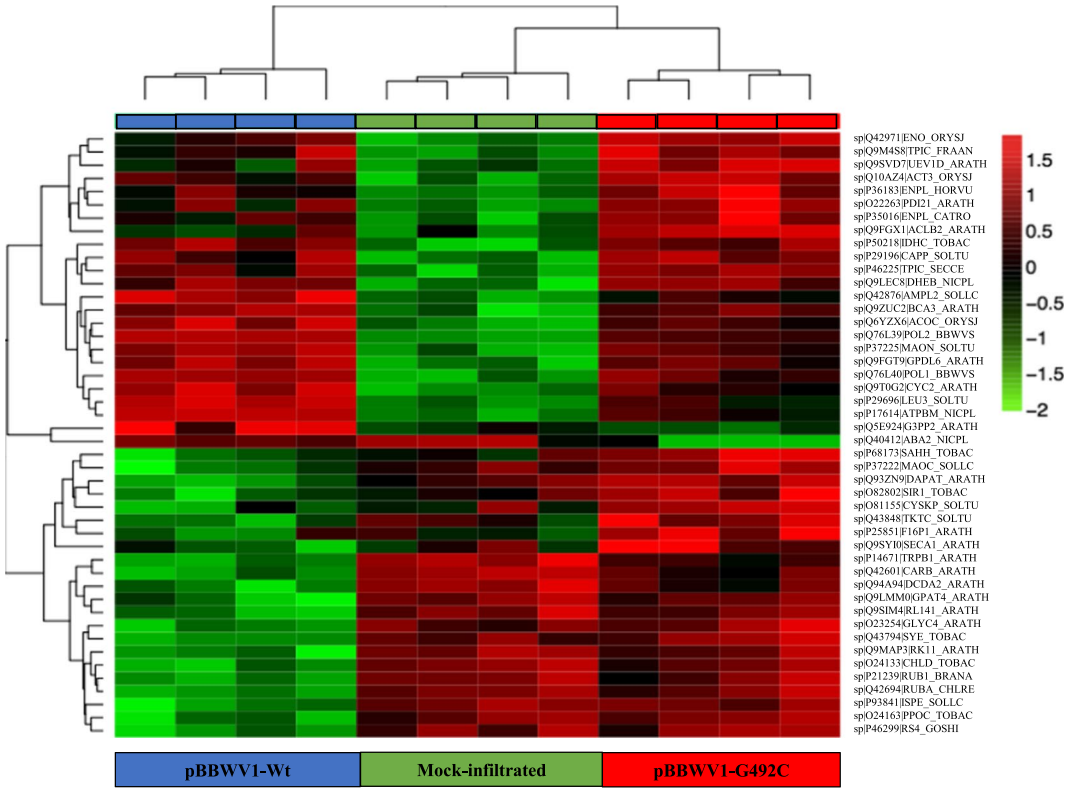

Fig. 3 Heat map of 46 proteins (44 of the *Nicotiana benthamiana* host and two of BBWV-1) showing significant differential expression levels (FDR < 0.01) related to BBWV-1 infection. The columns correspond to four biological replicates corresponding to each group of *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt (blue), pBBWV1-G492C (red) and mock-infiltrated used as healthy controls (green). The color intensity represents the different levels of expression ranging from overexpression + 1.5 (red) to the underexpression -2 (green) as shown at the upper right part of the figure. Protein accession numbers available in UniprotKB database (https://www.uniprot.org/) are at the right of the figure

(agroinfiltrated with pBBWV1-Wt or BBWV1-G492C and mock-infiltrated control plants) were analyzed by using the UniprotKB (https://www.uniprot.org/) and Gen Ontology (GO, http://geneontology.org/) databases. All information obtained in these analyses such as the detection of homologous proteins in different hosts, potential functions, and subcellular localizations are shown in Table 1.

The 44 host proteins showing differential expression between *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt and healthy controls were related with a broad spectrum of biochemical and physiological processes such as cytoskeleton formation, amino acid, carbohydrate or fatty metabolism, photosynthesis, and photorespiration, synthesis of proteins and plant hormones, ubiquitination, intercellular communication, and plant development. Each protein is also involved in different biochemical and biological pathways, showing a complex interaction of BBWV-1 with its host. These proteins were mainly located in the chloroplast (24 proteins), whereas the rest of them had a cytoplasmic (10 proteins), mitochondrial (four proteins), reticulum endoplasmic (three proteins), membranous (two proteins), ribosomal (one protein), chromoplast (one protein) and nuclear (one protein) localization. Two chloroplastic proteins, the glutamate-tRNA ligase and the cysteine synthase, are also in the mitochondria and chromoplast, respectively.

Following the UniprotKB and Gen Ontology database, the 20 overexpressed proteins in both *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt or BBWV1-G492C respect to the healthy control plants were related with: i) amino acid metabolism (isocitrate dehydrogenase, 3-isopropylmalate dehydrogenase, glutamate dehydrogenase B, leucine aminopeptidase 2 and NAD-dependent malic enzyme

Table 1 Database analysis (UniprotKB and Gen ontology (GO) of the 44 *Nicotiana benthamiana* proteins showing significant differences (FDR < 0.01) in expression level among plants agroinfiltrated with pBBWV1-Wt, pBBWV1-G492C and mock-infiltrated used as healthy controls

Accession number	Name	Subcellular localization	Organism	Function
sp|Q42971|ENO_ORYSJ	Enolase	Cytoplasmic	*Oryza sativa*	Glycolysis
sp|Q9M4S8|TPIC_FRAAN	Triosephosphate isomerase	Chloroplastic	*Fragaria ananassa*	Calvin cycle
sp|Q9SVD7|UEV1D_ARATH	Ubiquitin-conjugating enzyme E2 variant 1D	Nucleus	*Arabidopsis thaliana*	Ubiquitination
sp|Q10AZ4|ACT3_ORYSJ	Actin-3	Cytoplasmic	*Oryza sativa*	Cell cytoskeleton, cytoplasmic streaming, cell shape and division, organelle movement
sp|P36183|ENPL_HORVU	Endoplasmin homolog	Endoplasmic reticulum	*Hordeum vulgare*	Chaperone (protein folding)
sp|O22263|PDI21_ARATH	Protein disulfide-isomerase like 2–1	Endoplasmic reticulum	*Arabidopsis thaliana*	Rearrangement of -S–S- bonds in proteins: Plant fertility and embryo development
sp|P35016|ENPL_CATRO	Endoplasmin homolog	Endoplasmic reticulum	*Catharanthus roseus*	Chaperone (protein folding)
sp|Q9FGX1|ACLB2_ARATH	ATP-citrate synthase beta chain protein 2	Cytoplasmic	*Arabidopsis thaliana*	Lipid synthesis
sp|P50218|IDHC_TOBAC	Isocitrate dehydrogenase [NADP]	Cytoplasmic	*Nicotiana tabacum*	Glutamate synthase pathway
sp|P29196|CAPP_SOLTU	Phosphoenolpyruvate carboxylase	Chloroplastic	*Solanum tuberosum*	Calvin cycle
sp|P46225|TPIC_SECCE	Triosephosphate isomerase	Chloroplastic	*Secale cereale*	Calvin cycle
sp|Q9LEC8|DHEB_NICPL	Glutamate dehydrogenase B	Cytoplasmic	*Nicotiana plumbaginifolia*	Glutamate pathway
sp|Q42876|AMPL2_SOLLC	Leucine aminopeptidase 2	Chloroplastic	*Solanum lycopersicum*	N-terminal amino acid release of proteins
sp|Q9ZUC2|BCA3_ARATH	Beta carbonic anhydrase 3	Cytoplasmic	*Arabidopsis thaliana*	Reversible hydration of carbon dioxide
sp|Q6YZX6|ACOC_ORYSJ	Putative aconitate hydratase	Cytoplasmic	*Oryza sativa*	Glyoxylate and dicarboxylate metabolism
sp|P37225|MAON_SOLTU	NAD-dependent malic enzyme 59 kDa isoform	Mitochondrial	*Solanum tuberosum*	Malate metabolism an amino acid biosynthesis
sp|Q9FGT9|GPDL6_ARATH	Glycerophosphodiester phosphodiesterase GDPDL6	Membrane	*Arabidopsis thaliana*	Lipid and glicerol metabolism
sp|Q9T0G2|CYC2_ARATH	Cytochrome c-2	Mitochondrial	*Arabidopsis thaliana*	Electron transfer activity, ATP synthesis
sp|P29696|LEU3_SOLTU	3-isopropylmalate dehydrogenase	Chloroplastic	*Solanum tuberosum*	L-leucine biosynthesis
sp|P17614|ATPBM_NICPL	ATP synthase subunit beta	Mitochondrial	*Nicotiana plumbaginifolia*	ATP synthesis
sp|Q5E924|G3PP2_ARATH	Glyceraldehyde-3-phosphate dehydrogenase GAPCP2	Chloroplastic	*Arabidopsis thaliana*	Glycolysis

Table 1 (continued)

Accession number	Name	Subcellular localization	Organism	Function
sp|Q40412|ABA2_NICPL	Zeaxanthin epoxidase	Chloroplastic	*Nicotiana plumbaginifolia*	Abscisate biosynthesis
sp|P68173|SAHH_TOBAC	Adenosylhomocysteinase	Cytoplasmic	*Nicotiana tabacum*	L-homocysteine biosynthesis
sp|P37222|MAOC_SOLLC	NADP-dependent malic enzyme	Chloroplastic	*Solanum lycopersicum*	C4 acid pathway
sp|Q93ZN9|DAPAT_ARATH	LL-diaminopimelate aminotransferase	Chloroplastic	*Arabidopsis thaliana*	Lysine biosynthesis
sp|O82802|SIR1_TOBAC	Sulfite reductase 1 [ferredoxin]	Chloroplastic	*Nicotiana tabacum*	Assimilatory sulphate reduction pathway involved in development and growth
sp|O81155|CYSKP_SOLTU	Cysteine synthase	Chloroplastic/Chromoplastic	*Solanum tuberosum*	L-cysteine biosynthesis
sp|Q43848|TKTC_SOLTU	Transketolase	Chloroplastic	*Solanum tuberosum*	Calvin cycle
sp|P25851|F16P1_ARATH	Fructose-1,6-bisphosphatase	Chloroplastic	*Arabidopsis thaliana*	Calvin cycle
sp|Q9SYI0|SECA1_ARATH	Protein translocase subunit SECA1	Chloroplastic	*Arabidopsis thaliana*	Protein signalling and translocation
sp|P14671|TRPB1_ARATH	Tryptophan synthase beta chain 1	Chloroplastic	*Arabidopsis thaliana*	L-tryptophan biosynthesis
sp|Q42601|CARB_ARATH	Carbamoyl-phosphate synthase large chain	Chloroplastic	*Arabidopsis thaliana*	L-arginine biosynthesis
sp|Q94A94|DCDA2_ARATH	Diaminopimelate decarboxylase 2	Chloroplastic	*Arabidopsis thaliana*	L-lysine biosynthesis
sp|Q9LMM0|GPAT4_ARATH	Glycerol-3-phosphate 2-O-acyltransferase 4	Membrane	*Arabidopsis thaliana*	Esterifies acyl-group
sp|Q9SIM4|RL141_ARATH	60S ribosomal protein L14-1	Ribosomal	*Arabidopsis thaliana*	Ribosomal large subunit biogenesis
sp|O23254|GLYC4_ARATH	Serine hydroxymethyltransferase 4	Cytoplasmic	*Arabidopsis thaliana*	one-carbon (C1) pathways by catalyzing the reversible conversions of L-serine to glycine and tetrahydrofolate (THF) to 5,10-methylene THF
sp|Q43794|SYE_TOBAC	Glutamate–tRNA ligase	Chloroplastic/Mitochondrial	*Nicotiana tabacum*	Attachment of glutamate to tRNA
sp|Q9MAP3|RK11_ARATH	50S ribosomal protein L11	Chloroplastic	*Arabidopsis thaliana*	Ribosomal component
sp|O24133|CHLD_TOBAC	Magnesium-chelatase subunit ChlD	Chloroplastic	*Nicotiana tabacum*	Chlorophyll biosynthesis
sp|P21239|RUB1_BRANA	RuBisCO large subunit-binding protein subunit alpha	Chloroplastic	*Brassica napus*	RuBisCO small and large subunits binding and assembly of the enzyme oligomer
sp|Q42694|RUBA_CHLRE	RuBisCO large subunit-binding protein subunit alpha	Chloroplastic	*Chlamydomonas reinhardtii*	RuBisCO small and large subunits binding and assembly of the enzyme oligomer

Table 1 (continued)

Accession number	Name	Subcellular localization	Organism	Function
sp|P93841|ISPE_SOLLC	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	Chloroplastic/Chromoplastic	*Solanum lycopersicum*	Isopentenyl diphosphate biosynthesis
sp|O24163|PPOC_TOBAC	Protoporphyrinogen oxidase	Chloroplastic	*Nicotiana tabacum*	Chlorophyll biosynthesis
sp|P46299|RS4_GOSHI	40S ribosomal protein S4	Cytoplasmic	*Gossypium hirsutum*	Ribosomal component

59 kDa isoform), ii) carbon fixation in the Calvin cycle during the photosynthesis (triosephosphate isomerase, phosphoenolpyruvate carboxylase and triosephosphate isomerase), iii) carbohydrate metabolism (enolase and putative aconitate hydratase), iv) fatty metabolism (ATP-citrate synthase beta chain protein and glycerophosphodiester phosphodiesterase GDPDL6), v) protein folding (two chaperone endoplasmic homologs), vi) ATP synthesis (cytochrome C-2 and ATP synthase subunit beta), vii) embryo development and plant fertility (protein -isomerase like 2–1), viii) component of the cellular cytoskeleton (actin-3), ix) reversible hydration of carbon dioxide (beta carbonic anhydrase 3), and multiple regulatory functions such as protein degradation, cellular growth, nucleic acid reparation, and stress response (ubiquitin-conjugating enzyme E2 variant 1D). These proteins seem to be related to the BBWV-1 infection independently of VP37 expression.

On the other hand, the 22 proteins underexpressed in the *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt respect to those agroinfiltrated with pBBWV1-G492C or healthy control plants were related with: i) amino acid metabolism (adenosylhomocysteinase, LL-diaminopimelate aminotransferase, cysteine synthase, tryptophan synthase beta chain 1, carbamoyl-phosphate synthase large chain and Diaminopimelate decarboxylase 2), ii) photosynthesis (NADP-dependent malic enzyme, transketolase, fructose-1,6-bisphosphatase, magnesium-chelatase subunit ChlD and protoporphyrinogen oxidase), iii) protein synthesis (60S ribosomal protein L14-1, glutamate–tRNA ligase, 50S ribosomal protein L11 and 40S ribosomal protein S4), iv) fatty metabolism (glycerol-3-phosphate 2-O-acyltransferase 4 and 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase), v) protein folding (two annotations of RuBisCO large subunit-binding protein subunit alpha, in different microorganisms), vi) one-carbon (C1) pathway involved in photorespiration and stress responses (serine hydroxymethyltransferase 4), vii) protein signalling and translocation (protein translocase subunit SECA1) and viii) development and growth (sulfite reductase 1).

In addition to the mentioned host proteins, there was one protein, the glyceraldehyde-3-phosphate dehydrogenase (GAPCP2), that was only overexpressed in *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt but not in those agroinfiltrated with pBBWV1-G492C or healthy control plants. Another host protein, the zeaxanthin epoxidase, was overexpressed in *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt and in control plants in comparison with those agroinfiltrated with BBWV1-G492C.

During infection, viruses produce different subcellular and molecular changes in the host to allow their replication, movement, and spread. These host changes can also include modifications in energy sources, development, and reproductive stage of infected plants (Souza et al., 2019). For example, rice stripe virus (RSV) alters the biosynthesis of the chlorophyll pigment (Wang et al., 2015), affecting the photosynthesis in infected rice (*Oryza sativa*) plants. In this work, it was found the alteration of the accumulation of eight *N. benthamiana* proteins involved in photosynthesis that could be associated with BBWV-1 infection (triosephosphate isomerase, phosphoenolpyruvate carboxylase, triosephosphate isomerase, NADP-dependent malic enzyme, transketolase, fructose-1,6-bisphosphatase, magnesium-chelatase subunit ChlD, and protoporphyrinogen oxidase). Five of these proteins were underexpressed in plants agroinfiltrated with pBBWV1-Wt compared with those agroinfiltrated with pBBWV1-G492C or healthy control plants, and therefore, they could be related to VP37 expression and the increase of virus

pathogenicity. Among these proteins is the fructose-1,6-biphosphatase, which modulates the CO_2 fixation in the Calvin Cycle. Cowpea infected with CPSMV and beet infected with beet curly top virus (BCTV), showed a deregulation of the photosynthesis associated with changes in the accumulations of this protein (Varela et al., 2017).

Other host proteins with altered expression during BBWV-1 infection are the actin-3 and cytochrome C-2. The first protein is an essential component of the cell cytoskeleton responsible for cytoplasmic streaming, cell shape determination, cell division, organelle movement, and growth. The actin can interact with some plant viruses such as TMV, potato virus X (PVX), or tomato bushy stunt virus (TBSV), allowing the viral movement to adjacent cells through plasmodesmata structures (Harries et al., 2009; Varela et al., 2017). Cytochrome C-2 is a protein with electron transfer activity and ATP synthesis and changes in its accumulation were reported for some plant viruses as mungbean yellow mosaic India virus (MYMIV) in *Vigna mungo* (Souza et al., 2019).

Amino acid biosynthesis and protein formation are also important metabolic pathways affected by plant virus infection (Zeier, 2013). Plant viruses optimize the translation of their own proteins modulating the tRNAs and the synthesis of host ribosomal proteins (Albers et al., 2016; Bahir et al., 2009). In this work, 14 host proteins related to the biosynthesis of different amino acids such as glutamate, leucine, or lysine, and the ribosomal proteins L14-1, L11, and S4 showed differential accumulation in *N. benthamiana* plants infected with BBWV-1. Nine of these proteins were related to BBWV-1 VP37 protein, including the glutamate-tRNA ligase and all the ribosomal proteins. Other important proteins that could be related to VP37 are the sulfite reductase 1, serine hydroxymethyltransferase 4, and GAPCP2. Sulfite reductase is coupled with the ferredoxin in the chloroplast photosystems, and it is also involved in plant development and growth. Some viral products such as the CP of ToMV or the HC-Pro of sugarcane mosaic virus (SCMV), can modulate the function of this protein (Yang et al., 2020). Serine hydroxymethyltransferase 4 is involved in one-carbon (C1) pathway, and changes in its accumulation can affect the photorespiratory pathway and stress responses (Zhang et al., 2019). GAPCP2 plays an important role in different pathways such as glycolysis, starch metabolism, generation of primary metabolites for fatty acid and amino acid synthesis and pollen formation. Also, this protein contributes to the generation of signalling molecules in the abscisic acid pathway (Agut et al., 2016; Cho & Yoo, 2011). The abscisic acid is a plant hormone with an important role in the development process including seed and bud dormancy, and its biosynthesis is usually activated for both abiotic and biotic stresses (Chan, 2012). In this work, it was determined that GAPCP2 was overexpressed in *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt in comparison with those agroinfiltrated with pBBWV1-G492C and the healthy control plants. Overexpression of GAPCP2 protein was reported in plants infected with different viruses such as RSV, TMV, and CPSMV (Varela et al., 2019; Wang et al., 2015, 2016). Finally, the zeaxanthin epoxidase, which is involved also in the abscisic acid biosynthesis, was underexpressed in *N. benthamiana* plants agroinfiltrated with pBBWV1-G492C in comparison with those agroinfiltrated with pBBWV1-Wt and healthy control plants, that showed similar levels of accumulation. Curiously, the lack of viral VP37 protein reduce the expression of zeaxanthin epoxidase.

BBWV-1 infection induces strong symptoms and subcellular alterations in *N. benthamiana* plants, and the role played by the viral VP37 protein has been studied (Carpino et al., 2020a; Medina et al., 2022). Severe leaf mosaic symptoms and subcellular changes in mesophilic cells such as large starch and plastoglobuli accumulation in chloroplasts, mitochondrial proliferation, cytoplasmic vesiculation, and the presence of plasmalemmasomes were reported in *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt. However, equivalent plants agroinfiltrated with pBBWV1-G492C knocking out VP37 showed slight leaf mosaic symptoms, and the subcellular alterations were restricted to the presence of plastoglobuli in chloroplasts and cytoplasmic vesiculation.

Plant symptoms and subcellular alterations observed in *N. benthamiana plants* agroinfiltrated with pBBWV1-Wt could be related to the differential expression of 44 plant proteins reported here. These proteins (up to 24) were localized in chloroplasts, and most of them (up to 19) were related to the viral VP37 protein. Therefore, deregulation of these chloroplastic proteins may be associated with the high accumulations of starch observed in *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt. One of these

proteins is the GAPCP2, that is directly involved in starch metabolism. GAPCP2 is overexpressed in *N. bentamiana* agroinfiltrated with pBBWV1-Wt in comparison with those agroinfiltrated with pBBWV1-G492C or healthy control plants. Starch is synthesized in the chloroplasts from ADP-Glucose (primary product of photosynthesis) and functions as a transient reserve of carbohydrates metabolized during night conditions (Zeeman et al., 2010). Abiotic and/or biotic stresses such as infection by plant viruses, alter the starch metabolism inducing its accumulation (Ali Fayez and Younis Mahmoud, 2011; Zechmann et al., 2021; Zhan et al., 2021).

Plasmalemmasomes or paramural bodies are membranous structures showing continuity of the plasmatic membrane. The function of these structures has been related to the virus movement between cells (Wan & Laliberté, 2015). We found two proteins with membranous localization: i) the glycerophosphodiester phosphodiesterase GDPDL6 overexpressed in both *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt and pBBWV1-G492C, and ii) the glycerol-3-phosphate 2-O-acyltransferase 4 that is underexpressed only in *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt. For these two host proteins, only the underexpression of the last one was associated with the viral VP37 protein expression and plasmalemmasomes formation. Other membranous alterations reported previously in *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt and pBBWV1-G492C were the increase of cytoplasmic vesicles (Medina et al., 2022). In this case, cytoplasmic vesiculation did not depend on viral VP37 protein, and could be consequence of overexpression of GDPDL6 in both groups of plants.

In conclusion, LC–MS/MS analysis revealed the differential expression of 44 host proteins related to BBWV-1 infection, plant symptom development, and subcellular alterations in mesophilic cells. Predicted functions and localizations of the proteins showing differential expression revealed an alteration in the principal metabolic and physiological processes of *N. benthamiana* plants infected with BBWV-1. These proteins were localized in different cellular organelles but mainly in chloroplast, which is affected by the virus infection inducing the accumulation of starch and plastoglobuli (Medina et al., 2022).

This work is the first analysis of the host protein expression profile during BBWV-1 infection. Results presented here complement previous studies conducted to understand the pathological process during BBWV-1 infection and virus-host interactions (Carpino et al., 2020a, 2020b; Medina et al, 2022). Moreover, provide new data about the putative role of BBWV-1 VP37 to modulate plant symptoms and subcellular alterations during BBWV-1 infection.

Acknowledgements We thank Dr. Leonardo Velasco for the critical review of the manuscript. Proteomic and biostatistical analysis were conducted by the proteomics unit of the Universitat de València.

Author contributions C. Carpino and L. Elvira-González prepared the plant material for proteomic analysis. L. Elvira-González is responsible of the experimental analysis and the manuscript writing. L. Galipienso conducted the experimental design, the manuscript revision and edition. Luis Rubio participated in the manuscript revision and edition.

Funding This research work was supported by funds of the Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain (PROY-IVIA-2013/14 n 5426). C. Carpino was a recipient of predoctoral fellowships from the Italian government.

Declarations

Ethics statements Not applicable.

Competing interests The authors declare no competing interests.

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