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NANOTECHNOLOGY, NANOPOLLUTION, NANOTOXICOLOGY AND NANOMEDICINE (NNNN)

Systemic resistance induced by *Phoma* sp. GS8-3 and nanosilica against *Cucumber mosaic virus*

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Abstract

Cucumber mosaic virus (CMV) is a very serious hazard to vegetable production worldwide. This study is focused on evaluation of resistance stimulated by the plant growth-promoting fungus, *Phoma* sp. GS8-3, or nanosilica against CMV under pot and field conditions. The specific aim was to illustrate the mechanism of resistance stimulated by GS8-3 against CMV using microarray technology. Treatments with GS8-3 as well as nanosilica significantly decreased CMV severity and titer in tobacco and cucumber under pot and field conditions, respectively. Growth characters of tobacco and cucumber were significantly increased due to GS8-3 inoculation followed by nanosilica compared with control and BTH treatments. Microarray results showed highly upregulation of defense-related genes expression specially those related to heat shock proteins. Therefore, GS8-3 as well as nanosilica is suitable to serve as effective inducers against CMV in cucumber plants.

Keywords *Phoma* sp. · *Cucumber mosaic virus* · Nanosilica · Microarray · Induced systemic resistance · Tobacco

Introduction

Several sophisticated mechanisms are activated by plants against pathogen infection for effective innate immune response (Hassan et al. 2014b; El-Kazzaz et al. 2015a, b; El-Naggar et al. 2015). *Cucumber mosaic virus* (genus Cucumovirus), a widespread plant pathogen, considers the most economically damaging viral pathogen worldwide (Palukaitis et al. 1992; Roossinck 1999). It is impossible to control *Cucumber mosaic virus* (CMV) directly by chemical applications. Hence, an applicable and effective management strategy is needed for CMV infection. Induced resistance occurs due to increased levels of resistance to plant pathogens after applied stimulations (Van Wees et al. 2000; Postma et al. 2003; Elsharkawy et al. 2014). Induced resistance involves the use of beneficial microorganisms to control undesirable pests and pathogens. Induced systemic resistance (ISR) was initially reported with *Pseudomonads fluorescens* (Van Peer et al. 1991). Several elicitor substances were successfully used to enhance resistance in plants through activation of varied defense responses to protect them from invasion by pathogens. The most essential defense response is the underlying signaling pathways. Distinct signal pathways are activated by different stimuli.

Nanotechnology has offered novel solutions to some obstacles in crop production. Nano particles of aluminosilicates, zinc, silver, carbon, and silica have been introduced in the field of controlling different insects and diseases (Mousa et al. 2014; Elsharkawy et al. 2015). Nanosilica was successfully applied for controlling of *Tomato yellow leaf curl virus* (El-Sawy et al. 2018). Disease management by biological agents has attracted attention because it is ecologically mild and environmentally safe. Plant growth-promoting fungi (PGPF) are beneficial root-associated fungi that are positively affecting plant growth or health. Root colonization with PGPF was found to suppress several plant diseases (Hyakumachi 1994; Hossain et al. 2007; Elsharkawy et al. 2013; Hassan et al. 2014a). The mechanisms included in management strategies by PGPF could be

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antibiosis, predation, niche exclusion, mycoparasitism, and ISR (Shivanna et al. 1994, 1996; Whipps 2001). PGPF exhibited the ability to promote ISR in a variety of different plants. *Phoma* sp. GS8-3 was originally identified as endophytic PGPF on *Arabidopsis*. To perform a molecular dissection of physiological and biochemical alterations occurring in the host triggered by GS8-3 colonization, microarray analysis was done on *Arabidopsis thaliana* plants. Recently, systemic resistance was activated in cucumber plants against anthracnose disease by barley grain inoculum (BGI) and culture filtrate (CF) of GS8-3 (Chandanie et al. 2006, 2009). Although ISR is often associated with host resistance by rhizosphere colonization, CF treatment of *Penicillium simplicissimum* GP17-2 showed the same protection levels as BGI (Elsharkawy and Mousa 2015). This result proposes that certain factors produced in fungal metabolites might be responsible for increased resistance in tobacco plants. Host resistance was activated in different plant species after application of CFs to their leaves or roots (Koike et al. 2001; Hossain et al. 2007; Sultana et al. 2009). Although the signaling pathways mediated by PGPF against fungal and bacterial pathogens were studied extensively, few studies concentrated on the resistance stimulated by PGPF against plant viruses. Microarrays are a quintessential tool in functional genomics (Schenk et al. 2000). The recent develop of microarray-based profiling techniques has played the key role in understanding the plant pathogenesis-related responses. To get a general view on transcript modification during the tobacco-GS8-3 interaction, we implemented microarray analysis using Agilent Genechip probe arrays representing approximately 22,500 genes. The effects of treating the roots of tobacco with GS8-3 on foliar disease development by CMV were examined, and the defense reactions that are activated in CF-treated and pathogen-challenged plants were evaluated.

Materials and methods

Plants and pathogens

Cucumber mosaic virus (CMV-Y) was preserved in *Nicotiana tabacum* cv. Xanthi-nc. *Phoma* sp. GS8-3 was isolated from zoysiagrass (*Zoysia tenuifolia*) rhizosphere and stored as a PGPF collection. BGI and CF treatments of GS8-3 were prepared following the method described by Elsharkawy et al. (2012). GS8-3 was grown on PDA medium for 6 days. Twenty five mycelial disks (5 mm) of GS8-3 culture were relocated to a 500-mL Erlenmeyer flask of potato dextrose broth (PDB) and incubated for 12 days at 24 °C. Culture filtrate was filtered through a 0.22-μm Millipore filter. Nanosilica (purity, 99.9%) was provided by Egypt Nanotech Company Limited, Cairo, Egypt.

Plant growth conditions and fungal pre-treatment

For the BGI experiment, tobacco seeds were grown in sterilized paper pots (6 cm × 8 cm) filled with sterilized peat-based medium (Kyodohiryo Co. Ltd., Aichi, Japan) amended with the powdered BGI (0.5% *w*/*w*) of GS8-3. Similarly, the control plants were treated with sterilized barley grains. For a positive control, plants were soil-drenched with 0.3 mM of BTH (Novartis Agro, Tokyo, Japan) or nanosilica (100 μg L^{-1}) at 1 day prior to the challenge inoculation as described by (Elsharkawy et al. 2012; Elsharkawy and Mousa 2015). After germination, plants were transferred to growth chamber under a 12/12-h day/night cycle at 24 °C.

For CF experiment, tobacco seeds were grown in sterilized rock wool and transferred to the growth chambers as described above. Four-week-old tobacco plants were immersed in 50% diluted CF of GS8-3 for 1 h. The control plants were immersed with 50% diluted PDB. Similarly, BTH solution (5 mM) and nanosilica (100 μg L^{-1}) were treated a day before inoculation.

Evaluation of plant growth

Growth characters of tobacco plants including shoot fresh and dry weights and number of leaves from the BGI or nanosilica-treated and non-treated plants were assessed at 6 weeks after planting. The experiment was repeated three times with five plants per replicate.

Cucumber mosaic virus inoculations and disease assessment

CMV was mechanically inoculated following inoculation technique described by Elsharkawy et al. (2012). Disease severity was assessed by counting symptomatic leaves. The results of the disease severity represent the mean values of 30 samples in each treatment. Enzyme-linked immunosorbent assay (ELISA) was utilized to measure CMV titer as explained by Elsharkawy et al. (2013).

Gene expression (microarray analysis)

Four comparisons were analyzed in this time course analysis. In all comparisons, each array was hybridized at the same time with cRNA from (i) GS8-3-protected plants at 1-day post inoculation (DPI) (2 days after CF treatment), (ii) CMV-challenged plants at 1 DPI, (iii) GS8-3/CMV-treated plants at 1 DPI, and (iv) GS8-3/CMV-treated plants at 2 DPI versus control leaves. The harvested leaves were stored at − 80 °C. Extraction of RNA was executed as elucidated by Elsharkawy et al. (2012).

Microarray analysis

Dye-flip method was used for Agilent microarray system (Agilent Technologies, Palo Alto, CA, USA). The experiment was performed according to Agilent manufacturer's protocols version 6.5. Agilent DNA microarray scanner (software version 6.1) was used for scanning the hybridized slides, and data points were extracted using Agilent Feature Extraction software (version 8.1). GeneSpring GX 7.3 (Silicon Genetics, Redwood City, CA, USA) was used to illustrate scatter plots and analyze microarray data. Data points based on less than three measurements and spots with low fluorescence intensity (i.e., < 80) were ignored from the analysis, and also, duplicated genes or flags were deleted from the analysis. After the quality control, 22,500 spotted genes were chosen from the tobacco microarray slides. The highly upregulated genes (2.5%) were selected in each treatment group according to FC values. GeneSpring GX 7.3 was used for calculating the means and fold changes. Venn diagrams were used for identifying the upregulated and unique genes responding to each treatment. Microarray data were presented by the MapMan software (http://gabi.rzpd.de/projects/MapMan).

Management of CMV by GS8-3 and nanosilica in cucumber under field conditions

The experiments were performed twice under plastic greenhouse (45 m × 10 m) during the seasons 2017–2018 using cucumber cultivar DP-164. Rectangular plastic trays (27 × 14 × 10 cm) were filled with sterilized peat-based potting medium and planted with cucumber seeds. Each experimental unit was divided to equal replicates (13 plants for each replicate). Two weeks later, cucumber seedlings were transplanted at spacing of 55 cm between plants on two sides of the ridge within the row. Seedlings were allocated in three rows (0.8 × 2 m). BGI treatment was applied by mixing BGI of GS8-3 with potting medium to a concentration of 0.5% (*w*/*w*) just before sowing the seeds, while CF treatment was carried out 1 day before virus inoculation by treating 4-week-old cucumber plants with 50% diluted CF of GS8-3 as soil drench. Similarly, nanosilica treatment was done by treating cucumber plants with 100 μg L^{-1} solution a day prior to the virus challenge inoculation. Disease severity and virus concentration were determined as mentioned above. Moreover, plant growth and yield parameters were determined in the experimental plants.

Table 1 Effect of *Phoma* sp. GS8-3 colonization, nanosilica, and BTH treatment on growth of *Nicotiana tabacum*

Treatment	Fresh weight	Dry weight	Number of leaves/plant
GS8-3	9.70 ± 0.20[a]	1.23 ± 0.06[a]	7.12 ± 0.44[a]
Nanosilica	6.59 ± 0.12[b]	0.78 ± 0.04[b]	7.03 ± 0.41[a]
BTH	2.87 ± 0.09[d]	0.27 ± 0.02[d]	6.84 ± 0.31[a]
Control	4.34 ± 0.13[c]	0.44 ± 0.01[c]	6.90 ± 0.36[a]

Values are means ± SEM ($n = 15$). Different letters mean significant difference Fisher's LSD at $P \leq 0.05$

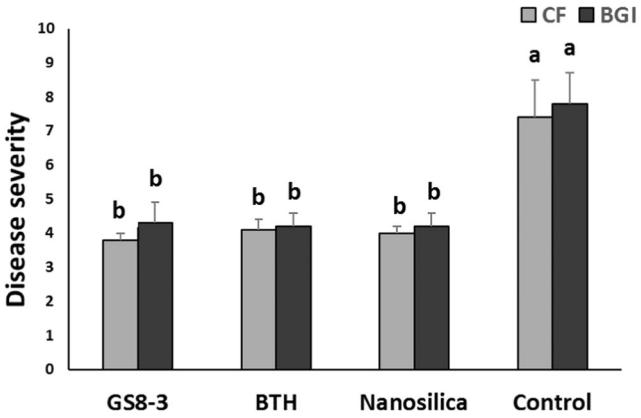

Fig. 1 Disease severity of *Cucumber mosaic virus* in *Nicotiana tabacum* treated with *Phoma* sp. GS8-3 (as BGI or CF), nanosilica, or BTH relative to non-treated control plants at 14 days post-inoculation

Data analysis

The experiments were repeated at least thrice. Statistical analysis was performed using Fisher's least significant difference (LSD) test and Steel-Dwass test by Ekuseru-Toukei 2010 (Social Survey Research Information CO., Ltd) at $P \leq 0.05$.

Results

Effect of BGI of GS8-3 and nanosilica on the growth of tobacco plants

BGI of GS8-3 promoted the growth of tobacco plants and increased shoot fresh and dry weights relative to the control.

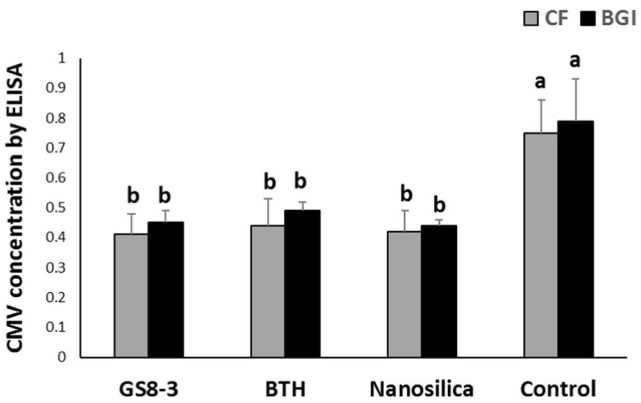

Fig. 2 *Cucumber mosaic virus* accumulation in leaves of *Nicotiana tabacum* treated with either *Phoma* sp. GS8-3, nanosilica, or BTH at 14 days post-inoculation

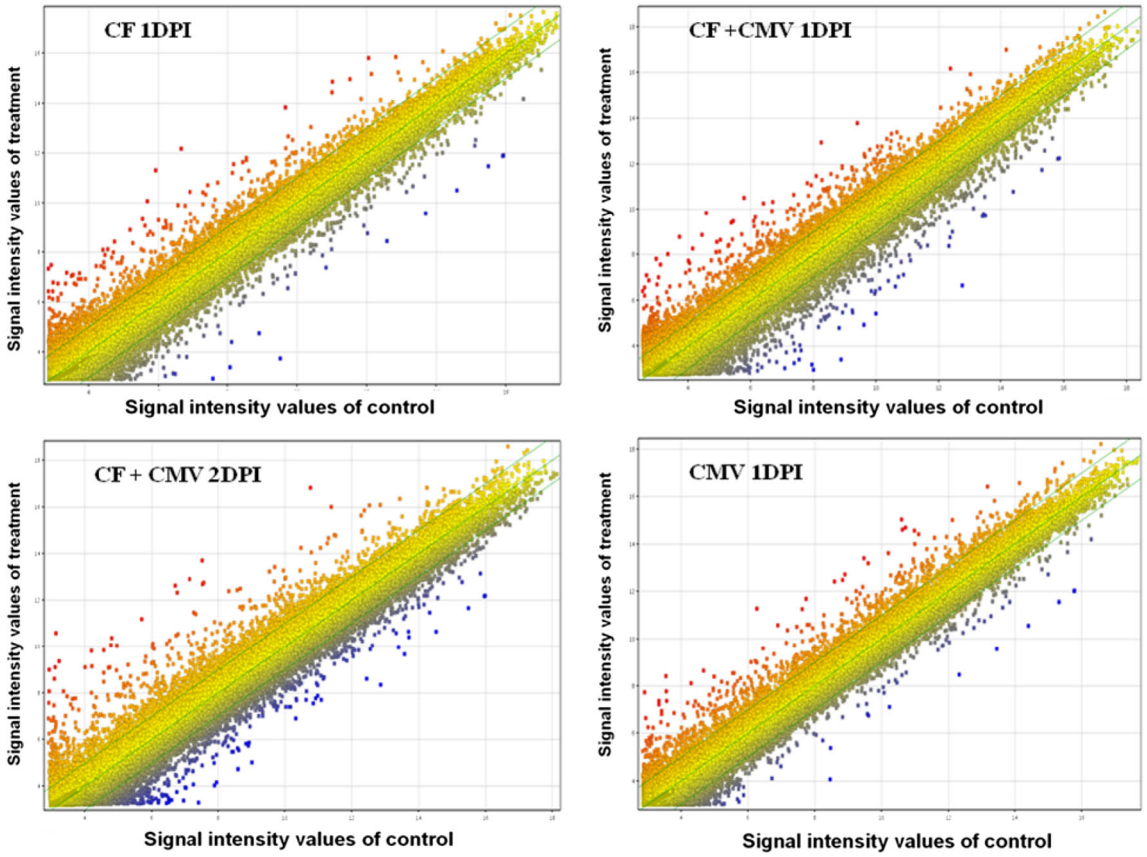

Fig. 3 Scatter plot of competitive microarray data from leaves of tobacco subjected to CF 1 DPI, CF + CMV 1 DPI, CF + CMV 2 DPI, and CMV 1 DPI. Total RNA was extracted and microarray analysis was performed using the Agilent tobacco 2 Oligo Microarray system. X and Y axes indicate signal intensities in control and CF treatments, respectively. Mean of signal intensities from three biologically independent replications is plotted

Nanosilica exhibited also significant increase in the average of fresh and dry weights in comparison with the control, but no significant increase was found in the number of leaves (Table 1). However, across all plants, the BTH treatments showed a significantly reduced growth relative to the control.

Effect of BGI and CF of *Phoma* sp. GS8-3 and nanosilica on systemic protection against CMV in tobacco

Disease severity was decreased in BGI and CF pre-treated plants relative to the control which displayed serious

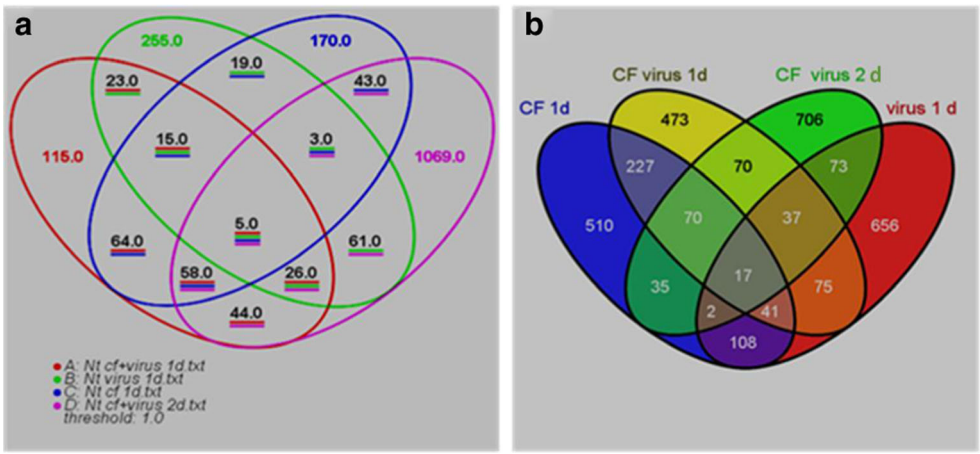

Fig. 4 Venn diagrams of **a** upregulated transcripts and **b** 2.5% of highly upregulated transcripts under CF 1 days post-inoculation (DPI), CF + CMV 1DPI, CF + CMV 2 DPI, CMV 1 DPI treatments

symptoms (Fig. 1). Consistent with this observation, the treatment with BGI or CF of GS8-3 resulted in reduction of virus accumulation in comparison with the control (Fig. 2). Similarly, CMV severity and titer were decreased in nanosilica or BTH-treated plants relative to the controls (Figs. 1 and 2). These results indicated that both BGI and CF of GS8-3 and nanosilica had the potential to enhance systemic resistance that was effective against challenge infection with CMV.

Gene expression in tobacco after treatment with CF of GS8-3

The microarray data were analyzed using GeneSpring GX 7.3 (http://www.agilent.com/chem/genespring/Agilent Technologies, USA), and the signal intensity of the genes analyzed by the microarray under the four treatments CF 1 DPI, CF + CMV 1 DPI, CF + CMV 2 DPI, and CMV 1 DPI is shown visually in the scatter diagrams (Fig. 3). Microarray data showed that 19,417 genes of the 43,760 genes tested displayed upregulation of their expression in response to CF of GS8-3 at 1 DPI. Among all the GS8-3-responsive genes, a gene coding for heat shock protein (A_95_P180267) was observed as the most highly upregulated gene. The common and unique genes induced by each treatment and the overlapping between treatment were visualized using Venn diagram (Fig. 4). Among the highly upregulated genes, 473 genes were unique to CF + CMV 1 DPI increased to 706 genes in CF + CMV 2 DPI treatment. The cellular and metabolism responses of tobacco presented using Mapman software. Several genes responsive to biotic stress were highly upregulated (Figs. 5 and 6). The expression levels were raised sharply in GS8-3-treated plants. The increased expression levels in CF-treated plants proved the role of these genes in increased resistance against CMV.

Management of CMV by GS8-3 and nanosilica in cucumber under field conditions

All treatments decreased CMV severity and concentration relative to the control (Table 2). BGI of GS8-3 achieved the best

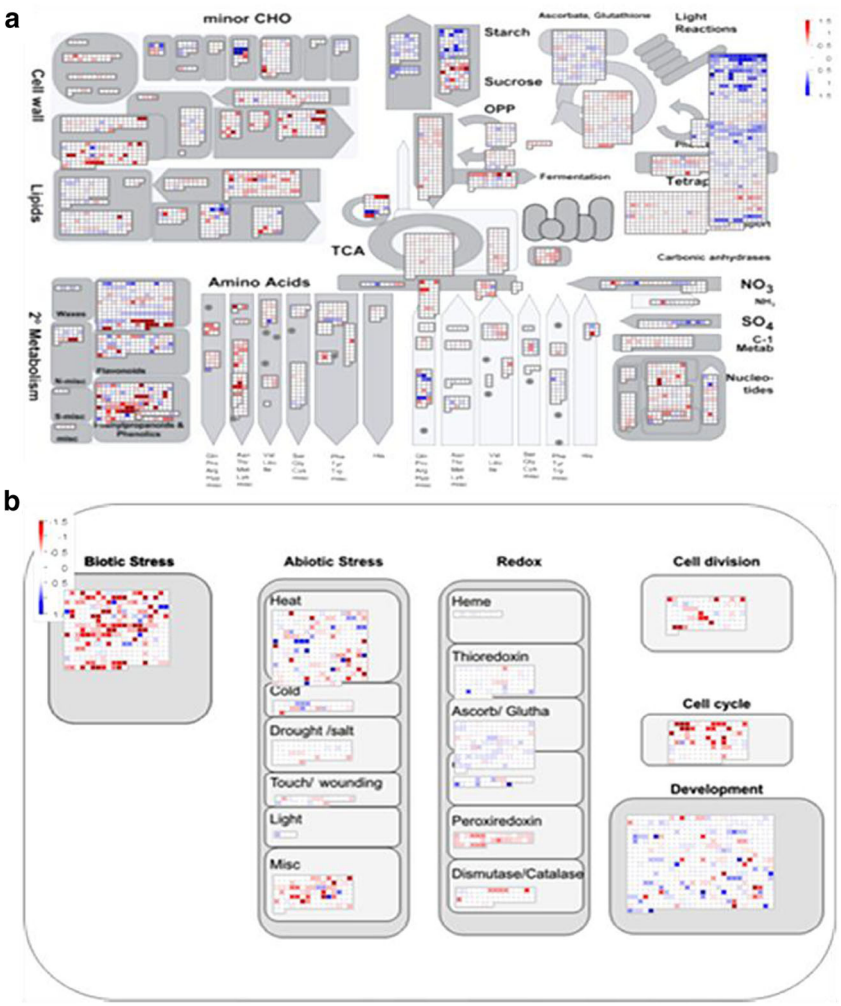

Fig. 5 Impact of *Phoma* sp. GS8-3 on its host metabolism after 1 day from CF treatment (1 DPI). **a** The MapMan metabolism overview display created using the CF of *Phoma* sp. GS8-3-regulated genes identified from the plant growth-promoting fungi versus control comparison. **b** The MapMan cellular response overview display created using the same pool of genes. The fold change is displayed as illustrated in the fold change color bar in the upper left of each panel (red is repressed, blue is induced)

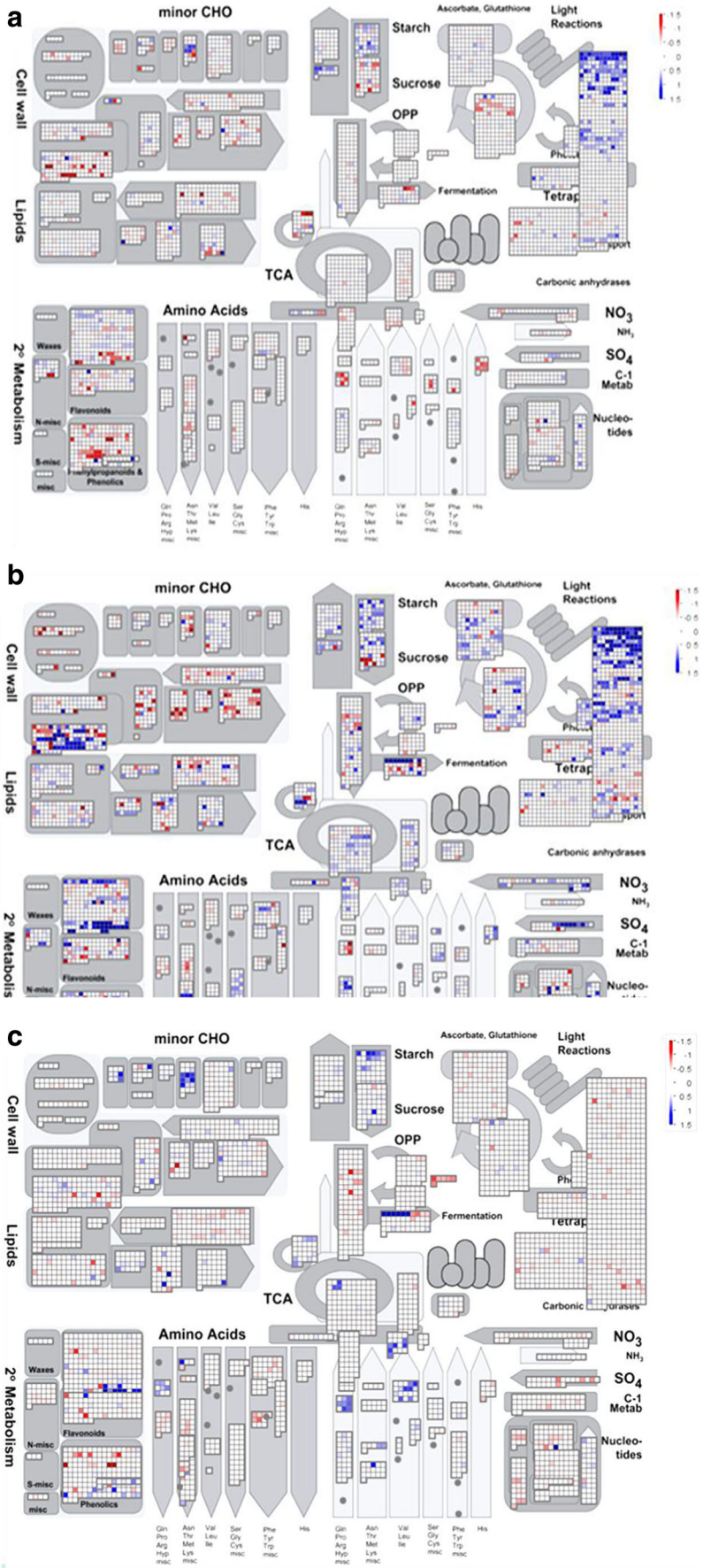

Fig. 6 *Phoma* sp. GS 8-3 counterbalances the impact of CMV pathogenesis on host metabolism. **a** The MapMan metabolism overview display created using the *Phoma* sp. GS 8-3-regulated genes identified from the CF of *Phoma* sp. GS 8-3-treated plants at 2 days after CF treatment (1 DPI) versus control comparison. **b** The MapMan cellular response overview display created using the induced systemic resistance (ISR)-regulated genes identified from the CF-treated and CMV-challenged plants (1 DPI) versus control comparison. **c** The MapMan metabolism overview display created using the CMV-regulated genes identified from the CMV-challenged plants (1 DPI) versus control comparison. The fold change is displayed as illustrated in the fold change color bar in the upper left of each panel (red is repressed, blue is induced)

Table 2 Effect of *Phoma* sp. GS8-3 or nanosilica on disease severity, CMV concentration, and growth characters of cucumber plants

Treatment	Disease severity	Virus con.	Plant height (cm)	Leaves number/ plant	Fresh weight/ plant (g)	Dry weight/ plant (g)	Fruit number/ plant	Product/plant (kg)
BGI of GS8-3	2.9^{c*}	0.4^{c}	268.2^{a}	33.8^{a}	43.3^{a}	5.2^{a}	35.6^{a}	3.8^{a}
CF of GS8-3	3.1^{c}	0.4^{c}	240.2^{c}	32.1^{a}	33.3^{c}	4.1^{c}	30.8^{b}	2.9^{b}
Nanosilica	3.9^{b}	0.6^{b}	249.5^{b}	32.6^{a}	37.5^{b}	4.5^{b}	31.8^{b}	3.1^{b}
Control	5.8^{a}	1.2^{a}	198.1^{d}	27.5^{b}	21.9^{d}	2.9^{d}	16.9^{c}	1.5^{c}

*Different letters mean significant difference with Fisher's LSD at $P \leq 0.05$

results in this respect followed by CF and nanosilica. Plant growth parameters are presented in Table 2 and showed improvement in the means of two seasons in plant height and fresh and dry weights in treated plants compared with the control especially in BGI-treated plants. Significant differences were not found in number of leaves between BGI, CF, and nanosilica treatments (Table 2). Furthermore, the average weight of fruits/plant was significantly different between BGI treatment and CF and nanosilica treatments (Table 2).

Discussion

The plant growth-promoting fungus (PGPF), *Phoma* sp. GS8-3, has already been established as an inducer of systemic resistance against foliar diseases in cucumber plants (Chandanie et al. 2005; Saldajeno and Hyakumachi 2011; Hassan et al. 2014a). Several CF isolated from PGPF showed antimicrobial activity against bacterial and fungal phytopathogens (Mur et al. 1996; Elsharkawy et al. 2014). Recently, CF isolated from *Penicillium simplicismum* GP17-2 has exhibited antimicrobial activity against CMV (Elsharkawy et al. 2012). Since plant growth-promoting ability of PGPR was accompanied by induced resistance against pathogens (Meera et al. 1994; Persello-Cartieaux et al. 2003), we evaluated the potential of GS8-3 and its CF to enhance systemic resistance against CMV in tobacco plants. Both of GS8-3 and its CF led to reduction of CMV severity and viral accumulation in tobacco and cucumber plants. Similarly, results revealed that nanosilica acted as a potential elicitor of resistance against CMV under pot and field conditions. Nanosilica treatment significantly reduced virus severity and accumulation in tobacco and cucumber plants. These results are in accordance with studies of Elsharkawy and Mousa (2015) and El-Sawy et al. (2018) in the management of *Papaya ring spot virus* (PRSV) in cucumber and *Tomato yellow leaf curl virus* (TYLCV) in tomato, respectively. Additionally, resistance against pathogens was stimulated by aqueous silicate solution (Brecht et al. 2003).

Growth characters of GS8-3 tobacco plants were increased relative to the control and BTH treatments. Shoot fresh and dry weights were higher in BGI treatment compared with those in the control. The results exhibited the additive effects of root colonization with GS8-3 on plant growth parameters at 6 weeks after planting. PGPF isolates keep minerals in more suitable form to the plant and suppress plant pathogens. Mechanisms of growth promotion could be the secretion of volatiles and hormones by the fungus or increasing the available minerals for plant uptake (Contreras-Cornejo et al. 2009). Moreover, aqueous silicate solution increased physiological activity and promoted the growth which could clarify the positive effect of nanosilica on tobacco growth (Kanto et al. 2004). In general, silicon is an essential element for the cell wall of leaves and stems and xylem vessels.

Although CF treatment of GS8-3 showed enhanced resistance against different pathogens, the mechanism involved in this antimicrobial activity is not well known. Thus, this report represents the first study on the mechanism of CF of GS8-3 against plant viruses. To explore gene expression and physiological processes involved in GS8-3 stimulated ISR, microarray system was exploited using tobacco as a modeling plant (Schenk et al. 2000). Using the Agilent tobacco gene chip probe array, we implemented a global analysis of transcript modification associated with CF treatment interaction with CMV infection. Since we would like to understand the signaling pathways involved in GS8-3-mediated ISR, it could be better to investigate in parallel changes in transcription induced by CF of GS8-3 in tobacco and those induced by CF of GS8-3 in CMV-challenged tobacco. We chose to compare four classes of samples: control leaves, CF-protected plant leaves, CMV-challenged plant leaves, and protected-challenged plant leaves. Four cross-hybridizations were performed: CF-protected plants versus control leaves at 2 days after CF treatment (1 day post inoculation), CMV-challenged plants versus control leaves at 1 DPI, protected-challenged plants versus control leaves at 1 DPI, and protected-challenged plants versus control leaves at 2 DPI.

The Agilent data study showed that 19,417 gene of the 43,760 genes tested displayed upregulation of their expression in CF of GS8-3 treatment. The identified upregulation of gene expression in leaves indicates that the treatment CF on tobacco roots could be perceived systemically in leaves. It is notable that most of 44 genes responding to ISR-inducing fungi were actually upregulated. The MapMan overview metabolism revealed that the alterations in gene expression induced by CF of GS8-3 were different from the pathogen signature. Treatment

of tobacco roots by CF of GS8-3 is illustrated by high impact on ISR-induced genes and that could elucidate the mechanism of CF-induced resistance in tobacco against CMV. Therefore, the ability of the GS8-3 to biologically control CMV is closely related to ISR induction in tobacco plants. In conclusion, CMV was suppressed by treatment with GS8-3 or nanosilica in tobacco and cucumber. Induced resistance against CMV using GS8-3 or nanosilica constitutes an alternative that has to be evaluated under different agro-climate conditions.

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