Expression of Cucumber Green Mottle Mosaic Virus Movement Protein in Cucumber Leads to the Expression Changes of Endogenous Gene

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Abstract

Cucumber green mottle mosaic virus (CGMMV) is one of the most important diseases of cucurbit crops. To date the only method available to control this devastating disease is the use of resistant varieties or disease-resistant rootstocks. However, the development of transgenic technology offers the potential to create resistant varieties through the expression of foreign genes. Such approaches are not without risk, and it has been noted that introduction of transgenes can have wide ranging effects, often affecting non-target processes. The current study was therefore initiated to investigate the effect of genetic modification on 12 related genes in transgenic cucumber seedlings expressing the CGMMV movement protein (CGMMV-MP) at the two-true-leaf stage. Compared with non-transgenic cucumbers (cv. Zhongnong 16), the results of quantitative PCR (qPCR) indicated that six of the genes had significant altered expression in the transgenic plants, four that were up-regulated including the cucumber peeling cupredoxin, Histone H4, Cytochrome oxidase and Thaumatin-like protein and two that were down-regulated, cytochrome b6-f complex and disulfide isomerase. The data collected therefore provide greater understanding of the impact of introduced exogenous genes in cucumber, as well as highlighting resistance genes that have the potential to prevent CGMMV infection.

Keywords

Cucumber green mottle mosaic virus, Yeast two-hybrid system, Transgenic cucumber, qPCR

Abbreviations

PCR: Polymerase china reaction; qPCR: Quantitative real-time polymerase china reaction; CGMMV: Cucumber green mottle mosaic virus; MP: Movement proteins; Bt: Bacillus thuringiensis; SEM: Scanning electron microscope; YTHS: Yeast two-hybrid system; iTRAQ: Isobaric tags for relative and absolute quantitation; BA: 6-Benzyladenine; MS: Murashige and Skoog; IAA: Indole-3-acetic acid; CB: Carbenicillin; NAA: Neomycin phosphotransferase; Kan+: Kanamycin

Introduction

Cucumber green mottle mosaic virus (CGMMV), which belongs to the Tobamovirus genus of the Virgaviridae family, was first reported in Cucumis sativus from Great Britain [1], but has quickly spread to most regions of the world [1-9]. As well as being soil borne, the disease can be spread by contaminated plant materials, including seeds, pollen and vegetative propagation stock, and is easily transmitted to healthy cucumber plants [7,10,11]. Although precautions can be taken to avoid the spread of CGMMV between crops and different geographic regions, once CGMMV has been introduced to fields or nurseries, all infected plants, as well as suspect plants from the surrounding area, must be removed and destroyed [12]. In the absence of effective methods of control, CGMMV, which has a wide host range, has become one of the most devastating pathogens of cucurbitaceous crops. However, recent developments using transgenic plants have shown that expressing components of CGMMV genome, including the coat protein (CP), movement protein (MP) and RNA replicate, can induce CGMMV resistance in cucumber plants via post-transcriptional gene silencing [13,14]. Such CGMMV-resistant varieties could be an invaluable tool for control CGMMV during seed production or the preparation of vegetative propagation stocks by grafting.

The genomes of most plant viruses contain genes that encode movement proteins (MP), which facilitate the movement of virus...
The PCR protocols was as following: 50°C for 30 min, 95 °C for 2
Finally, added DEPC treated water to make 25 µL reaction volumes.
sequence contained in the NCBI data base (Accession No. D12505).
5 µM primers PMU, 5'-actgagctcctaggtgtgatcggattgta-3' and PMD,
Platium® taq high fidelity enzyme mix (Invitrogen, U.S.), 0.5 µL of
12.5 µL of 2 × reaction mix and 0.5 µL of SuperScript® III RT/
on MyCycler thermo cycler (Bio-Rad). For the PCR reaction, 1 µL of
EASYspin Kit (Biomed, Beijing, China). The RT-PCR was performed
procedures for SEM were followed as for the previous studies [24].
cucumber leaves were ground in 0.1 M phosphate buffer with pestle
and mortar [25]. Then, adopted the scanning electron microscope
(SEM) to observe the CGMMV particles in 5 µL plant extracts, the
procedures for SEM were followed as for the previous studies [24].

The total RNA was extracted from 100 mg leaf using the
EASYspin Kit (BioMed, Beijing, China). The RT-PCR was performed
on MyCycler thermo cycler (Bio-Rad). For the PCR reaction, 1 µL of
RNA was added into 13 µL of the PCR reaction mixture containing
12.5 µL of 2 × reaction mix and 0.5 µL of SuperScript® III RT/
Platium* taq high fidelity enzyme mix (Invitrogen, U.S.), 0.5 µL of
5 µM primers PMU, 5'-actgagctcctaggtgtgatcggattgta-3' and PMD,
5'-gactagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagctagc
The results indicated that six of the genes evaluated had significantly
expression levels of the six interaction proteins identified by the
transgenic and wild-type (cv. Zhongnong 16) seedlings at the two-
the total RNA from the leaf of different seedlings and then evaluated
the leaf samples were confirmed to be infected with CGMMV by SEM
identified using iTRAQ analysis [28]. The YTHS analysis identified
two proteins, cytochrome b6-f complex and a thaumatin-like protein,
which had been among the eight pathogenesis-related proteins (Table
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Table 1: Twelve related proteins identified by YTHS and iTRAQ analysis of cucumber plants infected with CGMMV.

| No. | Protein name                  | Accession / Homology (Length) | Protein / expressing change | Associated Function                                |
|-----|------------------------------|--------------------------------|----------------------------|--------------------------------------------------|
| 1   | Cytochrome b6-f complex      | AF527536 / 86% (841 bp)        | -                          | Electron carrier activity and metal ion binding   |
| 2   | Cysteine synthase            | -                              | -                          | Transport proteins, located in the chloroplast    |
| 3   | Dsulfide isomerase           | -                              | -                          | Respiratory electron-transport chain               |
| 4   | Catalase                     | QJ420912 / -0.7                 | -                          | Pathogenesis-related proteins                     |
| 5   | Cucumber peeling cupredoxin  | AF627536 / -0.8                 | -                          | Pathogenesis-related proteins                     |
| 6   | NADH-quinone oxidoreductase subunit K | -                              | -                          | Pathogenesis-related proteins                     |
| 7   | Histone H4                   | -                              | -                          | Pathogenesis-related proteins                     |
| 8   | Pathogen regulatory proteins | U93586 / 74% (568 bp)          | -                          | Pathogenesis-related proteins                     |
| 9   | NADH-quinone oxidoreductase subunit J | -                              | -                          | Pathogenesis-related proteins                     |
| 10  | Phloem protein (PP2)         | AF527536 / 86% (841 bp)        | -                          | Pathogenesis-related proteins                     |
| 11  | CuOxidase                    | Q4VZK4 / -1.4                  | -                          | Pathogenesis-related proteins                     |
| 12  | Thaumatin-like protein       | JF694925 / 96% (750 bp)        | -                          | Pathogenesis-related proteins                     |

1Interaction proteins identified by YTHS.
2Proteins with significantly altered abundance in CGMMV-infected cucumber plants identified by iTRAQ analysis, the change value were assessed to estimate between the CGMMV-infected and CGMMV-free cucumber [28].
altered expression compared to healthy cucumber (Figure 2), four that were up-regulated including cucumber peeling cupredoxin, histone H4, cytochrome oxidase and the thaumatin-like protein and two that were down-regulated including cytochrome b6-f complex and disulfide isomerase.

**Discussion**

Twelve related proteins were identified in the current YTHS analysis and previous iTRAQ study, were selected to investigate how the transgenic expression of the CGMMV-MP in cucumber seedlings affected the expression levels of endogenous genes. Only six of the genes assessed were found to have significant altered expression in the genetically modified cucumber seedlings. The thaumatin-like protein (TLP, Q5DJS5), which was identified in both the iTRAQ and YTHS analyses was the most affected being 2.3-fold up-regulation [28]. This protein is known to be an important pathogenesis-related (PR) protein belonging to the PR-1 to PR-17 family that is involved in host defense and developmental processes in plants [29,30].

**Table 2:** Primers used to evaluate the expression levels of 12 related genes in transgenic cucumber seedlings expressing the CGMMV-MP.

| Primer name                        | Sequence (5’ to 3’)                        | Product length |
|------------------------------------|--------------------------------------------|----------------|
| Tubulin (reference gene)           | Forward, GCGTTTGTCGTTGACTATG               | 232 bp         |
|                                    | Reverse, GGATACAAGGCGGTTGAGG               |                |
| Cytochrome b6-f complex            | Forward, GCCACCACTTCATCATCG               | 238 bp         |
|                                    | Reverse, GGAAGAGAACACCAAAATG               |                |
| Cysteine synthase                  | Forward, GCCATCTTTTGAGAAGACTAG            | 222 bp         |
|                                    | Reverse, GAAACATGAGGTTTGAGCCG             |                |
| Disulfide isomerase                | Forward, GAGCAAGCCTTTTGTAAG               | 213 bp         |
|                                    | Reverse, GATTCCTGTGTTGCG                  |                |
| Catalase (CAT)                     | Forward, GATAGATGCGAGGAGGATTG             | 231 bp         |
|                                    | Reverse, GGAGTAACAGGCAACTG                |                |
| Cucumber peeling cupredoxin        | Forward, GACTTGGATTCTGCGAAAG              | 215 bp         |
|                                    | Reverse, GCAAGAGAAGATCACCGTG              |                |
| NADH-quinone oxidoreductase subunit K | Forward, GTTCGACTCTTGCCTATG                | 226 bp         |
|                                    | Reverse, GTCGTATTTTCGTTGCGTG              |                |
| NADH-quinone oxidoreductase subunit J | Forward, GAAATGTCGTTGTAAGGG              | 229 bp         |
|                                    | Reverse, GGTGTAGATAGTATGCG                |                |
| Histone H4                         | Forward, GAAAGCAGCGGACACCA                | 220 bp         |
|                                    | Reverse, GAGGTAACACACGAAACG               |                |
| Pathogen regulatory proteins CuPi1 | Forward, GCTCAAGCTACCTCAAG                | 201 bp         |
|                                    | Reverse, GCGTGATAAGCTGCGTGGT              |                |
| Phloem protein (PP2)               | Forward, GAAATGAGCGTGGCCAC                | 235 bp         |
|                                    | Reverse, GATCCGAAAACACATCCTCG             |                |
| Cytochrome oxidase                 | Forward, GTCATTTGTGTTGTAAGTCG             | 222 bp         |
|                                    | Reverse, GACGAATGGGTAACGGAGA              |                |
| Thaumatin-like protein             | Forward, GCCTATTGTGTTGATGTTCG             | 172 bp         |
|                                    | Reverse, GATCTGGAGCTGACGAGCATG            |                |

**Figure 2:** Expression level of 12 related genes in non-transgenic (ZN) and transgenic (GM) cucumber seedlings expressing the CGMMV-MP gene.

Vertical axis, take an average value of expression level from twenty-five independent transgenic seedlings; Horizontal axis, the detection of 12 genes as follow list. 1: Tubulin control, 2: Cytochrome b6-f complex, 3: Cucumber peeling cupredoxin, 4: Cysteine synthase, 5: Catalase (CAT), 6: NADH-quinone oxidoreductase subunit K 7: Histone H4 8: Pathogen regulatory proteins CuPi1 9: Cytochrome oxidase 10: NADH-quinone oxidoreductase subunit J, 11: Phloem protein (PP2) 12: Thaumatin-like protein 13: Disulfide isomerase, GM: Genetically modified cucumber seedlings; ZN: ‘Zhongnong 16’ cucumber seedlings; **, t test significant at $P < 0.01$. 

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Many TLP genes have been validated via empirical experiments as being associated with increased resistance to pathogen infections in transgenic plants [29]. For example it has been found that TLPs can be induced during the hypersensitive response to cucumber mosaic virus (CMV) and they specifically interact with the CMV-MP and -CP in transgenic yeast models [31]. It is therefore interesting to note that the current study found that TLPs could also be up-regulated in cucumber plants expressing the CGMMV-MP, and those previous studies have shown that TLPs are candidate genes with the potential to create cucumber varieties resistant to CGMMV infection. The most significantly down-regulated protein (1.4-fold) in the current study was cytochrome b6-f (cyt-b6-f), which is in agreement with the iTRAQ study that found this protein was also down-regulated in response to CGMMV infection. The cyt-b6-f complex is an important protein in chloroplasts having a critical function in PS I and II and ATP synthase during photosynthesis. In addition, it has also been found to be an important component of the plant pathogen interaction, with one study finding that cyt-b6-f was inhibited in rice (Oryza sativa) plants infected by rice stripe virus (RSV) [32] causing reduced energy production and reduced synthesis of structural components of the chloroplast, which were linked to the various symptoms of infection. Furthermore, it was also found that the accumulation of RSV altered the expression of 9788 genes affecting many aspects of the host’s cellular system including protein synthesis systems, organelle function, cell structure and defense systems. These studies might therefore suggest that the down-regulation of cyt-b6f could negatively affect the chloroplasts of the transgenic cucumbers and lead to reduced resistance to CGMMV.

The four other genes that had significantly altered expression in the transgenic cucumber seedlings included disulfide isomerase (PDI), cucumber peeling cupredoxin, histone H4 and cytochrome oxidase. The PDI, which was down-regulated 0.6-fold, is known to be involved in the oxidative folding of cystine knot defense proteins [33]. These results are in contrast to a previous study that found that PDI was up-regulated in Nicotiana benthamiana plants infected with Potato virus X (PVX) [34]. Furthermore, positional cloning has confirmed that variants of PDI like 5-1 (HvPDI5-1) are linked to the Bymovirus resistance that occurs naturally in barley (Hordeum vulgare L.) [35]. Although the role of PDI is complicated, it is likely that its down-regulation in the transgenic cucumbers would have a negative effect overall, and reduce their resistance to infection. The three remaining genes that had altered expression in the transgenic cucumber seedlings were found to be up-regulated. The cucumber peeling cupredoxin, which is a common copper-binding protein, was found to be 0.8-fold up-regulated. Previous studies have shown that cupredoxin are an important factor contributing to symptoms of mottle and mosaic variegation during virus infections, which inevitably affects the photosynthesis of the host causing reduced yields [36]. It is therefore possible that the increased expression of the cucumber peeling cupredoxin in the transgenic cucumber seedlings could mitigate the symptom of CGMMV infection. Histone H4 was also found to be up-regulated in the transgenic cucumber plants. It is known that this protein can affect many developmental processes including root growth [37], flowering time [38] and seed development [39], cell wall development and plant defense response [40]. In addition, research has shown that infections of plant pathogens can lead to histone acetylation and methylation [41], and that mutations in histones can facilitate disease resistance in plants [42], which suggests that the up-regulation of histone H4 in the transgenic cucumber plants could enhance their resistance to infection. Cytochrome oxidase, which is located in the plant mitochondria and found to interact with the CGMMV-mp in the YTHS analysis, was also up-regulated in the transgenic cucumber plants. This protein has previously been shown to have a role in RNA editing and can negatively affect the viral gene silencing process [43], which indicates that cytochrome oxidase might contribute to CGMMV resistance in the GM-cucumbers.

In summary, the current study found strong evidence the introduction of transgenes into the cucumber genome has the potential to affect the expression of endogenous genes. Perhaps the most interesting effect was the down-regulation of cyt-b6-f, which indicates that the CGMMV-MP transgene has the potential to interact with the PSII of cucumber and not only increase disease resistance to CGMMV, but also suppress the expression of some resistance genes. It is well known that the introduction of foreign genes into the genome of crop plants can affect their nutritive value or alter their resistance to virus infection [44,45]. Furthermore, previous research has also demonstrated that the interaction of multiple genes in complex biological networks [46], which indicate that a wide range of factors should be assessed when considering the development of transgenic cucumbers resistant to CGMMV infection. It is also interesting to note that six of the related genes assessed in the current study were unaffected by the expression of the CGMMV-MP in the transgenic cucumber seedlings, including cysteine synthase, NADH-quinone oxidoreductase subunit K, pathogen regulatory protein CuPil, NADH-quinone oxidoreductase subunit J, catalase and phloem protein (PP2), even though the iTRAQ and YTHS studies had suggested that they had altered expression in the response of cucumber plants to CGMMV infection. Although the current study provides important information regarding the effect of the CGMMV-MP on 12 related genes in cucumber seedlings, further research is required to characterize the effect in adult plants exposed to CGMMV and at different developmental stages to characterize the relationships between PR-genes and phenotypic changes that occur due to CGMMV infection, and also assess their genetic stability to the next generation. However, the data collected so far has provided a greater understanding of the role of pathogenesis-related proteins in transgenic cucumber seedlings, and highlighted resistance genes that have the potential to prevent CGMMV infection.

Acknowledgment

This work was supported by the National Science Foundation of China (NSFC) project (Grant No. 31371910), the Program for Changjiang Scholars and Innovative Research Team in University (Grant No. IRT1042) and the Special Fund for Agro-scientific Research in the Public Interest of China (Grant No. 201303028).

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