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Full Length Article

Accumulation of Viral Coat Protein in Chloroplasts of Lily Leaves Infected with *Lily Mottled Virus*

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Abstract

The symptoms of *Lily mottled virus* (LMoV) disease are thought to be caused by metabolic changes in leaf chloroplasts. To observe variations in the ultrastructure of cells and the accumulation of viral coat protein (CP) in lily leaves, we examined ultrathin sections of lily leaves infected by LMoV. Immunogold labeling analysis demonstrated that LMoV-CP was localized to the chloroplasts. The chlorophyll fluorescence parameters of LMoV-infected lily, which indicating that the accumulation of LMoV-CP in chloroplasts inhibits PSII activity. We investigated the transmembrane transport of LMoV CP by incubating a gradient of this protein. The lowest concentration of LMoV-CP detected was 30 μg mL^{-1} and the optimal incubation time was 1 min. High levels of LMoV-CP that accumulate inside chloroplasts may affect photosynthesis in virus-infected lily by inhibiting photosystem activity. © 2017 Friends Science Publishers

Keywords: Virus infection; Cell ultrastructure; Chloroplasts; Coat protein

Introduction

Lily mottled virus (LMoV) is one of the main viruses that seriously affect the growth of lily. Leaves infected by this *Potyvirus* exhibit green streaking, deformity and other symptoms (Dekker *et al.*, 1993; Yamaji *et al.*, 2001). Most plant viruses can cause plants to exhibit mosaic symptoms, which mainly damage chloroplast structure and affect photosynthesis in the plant host (Benetti and Tomassoli, 1988). Many hypotheses have been put forward about the pathogenesis of mosaic virus the most popular of which is that the interaction between mosaic viral coat protein (CP) and chloroplasts is one of the root causes of the mottled symptoms of the host (Reinero and Beachy, 1989; Li *et al.*, 2005). Large amounts of CMV-CP are present in the chloroplasts of cucumber infected by cucumber mosaic virus (CMV): the concentration of CP is positively correlated with the severity of symptoms (Zhu and Francki, 1992). Shintaku found that CP determines the mottled symptoms after examining the CMV nucleic acid compositions from different strains (Shintaku, 1991). Chun *et al.* (2008) investigated the transport of TMV-CP and RSV-CP in isolated chloroplasts. Subsequently Ren *et al.* (2012) investigated the chloroplast transmembrane transport system of three aphid CPs, finding that CPs could be transported into the host chloroplast and that the transport efficiency is related to CP concentration and time). However, the molecular mechanism underlying how LMoV causing LMoV symptoms remains unclear. Thus, in order to establish a new system for virus prevention and control the molecular mechanism underlying this disease must be elucidated.

In the current study, to clarify how LMoV destroys lily chloroplasts and affects photosynthesis, we examined the ultrastructure of diseased cells and performed immunogold labeling experiments. The results show that LMoV CP destroys lily chloroplasts and help reveal the virus particle interaction sites. Analysis of chlorophyll fluorescence induction kinetics parameters demonstrated that LMoV can affect the photosynthetic physiology of chloroplasts. Analysis of transmembrane transport of LMoV CP in lily chloroplasts shed light on the interaction between LMoV CP and chloroplast proteins providing a theoretical basis for elucidating the molecular mechanism of LMoV infection.

Materials and Methods

Materials

Test plants: The plants used in this study were healthy control and LMoV-infected lily plants of the Oriental Lily variety 'Siberia', which were provided by the College of Life.

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Reagents

Chloroplast extraction kit purchased from BestBio Company; Premix Ex Taq, DL2000 DNA Markers, Premixed Protein Markers (Low MW), RealB and Pink Blue Protein Markers purchased from Takara; Peroxidase-conjugated AffiniPure Mouse Anti-Rabbit IgG (H+L) Gold-conjugated Rabbit anti-mouse IgG, Coomassie brilliant blue R-250 ammonium persulfate purchased from Sangon Biotech.

Observation of Chloroplast Ultrastructure, Electron Microscopy and Immunogold Labeling

Leaves of healthy control and LMoV infected plants were cut into 1 mm × 3 mm pieces and divided into two parts. One part was used to prepare ultrathin sections. The other part was fixed for 2–3 h in 3% poly formaldehyde and 1% glutaraldehyde mixed solution in 0.1 mol L^{-1} phosphate buffer (pH 6.8–7.2) washed in buffer at 4°C dehydrated for 30 min in a 30% and 50% ethanol series incubated in a -20°C freezer and dehydrated in an ethanol gradient to 100%. The tissue was embedded in K4M resin polymerized under UV light for 72 h in a -20°C freezer and polymerized for 24─48 h at room temperature which fully polymerized and cured the sample. Immunogold labeling of ultrathin sections was performed as described by Xu *et al.* (1998). The sections were observed under an electron microscope after 15 min of uranium acetate staining. The control samples were not labeled with LMoV-CP primary antibody.

Determination of Fluorescence Kinetic Parameters in Lily Leaves

Leaves from five healthy and five LMoV-infected lily plants were subjected to F_0, F_V, Fm, q_N, q_P and ΦPSII measurements after 0.5─1 h of dark treatment. F_0/Fv and Fv/Fm values were calculated for each sample. The fourth leaf from the top of each plant was measured and the temperature was controlled at 25°C. The relative humidity was 65% and the light intensity was 600 μmol m^{-2} s^{-1}.

Extraction and Detection of Total Chloroplast Proteins

Intact chloroplasts of healthy lily leaves and lily leaves infected by LMoV were extracted using a Chloroplast Extraction Kit according to the method of Cline *et al.* (1984). Chloroplast suspensions were combined with trypsin solution (10 mg mL^{-1}) to a final concentration of 0.1 mg mL^{-1} and incubated at 23°C for 30 min. Trypsin inhibitor (10 mg mL^{-1}) was then added to a final concentration of 0.1 mg mL^{-1} and the sample was centrifuged at 4,000 g to recover intact chloroplasts. The chloroplasts were combined with extraction buffer solution and an equal volume of chloroform (to dislodge the pigments) followed by the addition of acetone to precipitate the proteins. The proteins were suspended in extraction buffer containing only 0.1% SDS and boiled for 2 min to obtain total chloroplast proteins. The protein samples were subjected to SDS-PAGE and the gel was divided into two pieces: one piece was subjected to Coomassie brilliant blue R-250 staining and the other was subjected to western blot analysis.

Transport and Detection of LMoV CP in Chloroplasts *in Vitro*

According to the method of Banerjee 300 μg mL^{-1} LMoV CP produced from prokaryotic expression was combined with a 1,000 μL intact chloroplast suspension from healthy lily. After the addition of 10 μL RNase A (10 mg mL^{-1}) the suspension was incubated at 23°C for 30 min. LMoV CP that did not enter the chloroplast was digested with 10 μL RNase A (10 mg mL^{-1}) for 30 min at 25°C followed by the addition of 10 μL trypsin inhibitor (10 mg mL^{-1}). The sample was centrifuged at 4,000 g to collect the intact chloroplasts. Using the above method various incubation time gradients were set up (1 min, 5 min and 60 min) using different concentrations of LMoV CP (7.5 μg mL^{-1}, 15 μg mL^{-1}, 30 μg mL^{-1}, 100 μg mL^{-1} and 300 μg mL^{-1}) to investigate chloroplast transmembrane transport followed by western blot analysis.

Results

Chloroplast Ultrastructure of Lily Infected by LMoV

The chloroplasts of healthy lily leaves were oval the integrity of the outer membrane was good and the basal lamellae and stroma lamellae were highly developed and arranged in a neat and orderly manner. Lily leaves infected by LMoV exhibited a large degree of damage. Some chloroplast starch granules were markedly swollen or even disintegrated and some parts of the chloroplast outer membrane appeared to be seriously disintegrated. The grana lamella was arranged in a disorderly loose manner. The shapes of a large number of chloroplasts were altered: the nucleus was squeezed to the side of the cell and the cell wall was partially ruptured (Fig. 1).

Immunogold Labeling of LMoV CP

Microscopy analysis of ultrathin sections subjected to low-temperature immunogold labeling showed that gold particles were mainly distributed in the cytosol and chloroplasts of diseased lily leaves. The thylakoid membranes of chloroplasts contained some gold particles and a small number of columnar inclusions (Fig. 2B and Fig. 3). No gold particles were present in the cytosol and chloroplasts of healthy control plants and the negative control (not incubated with LMoV CP antiserum; Fig. 2A and Fig. 4) indicating that LMoV-CP antigen was only present in the chloroplast membranes of diseased lily leaves.

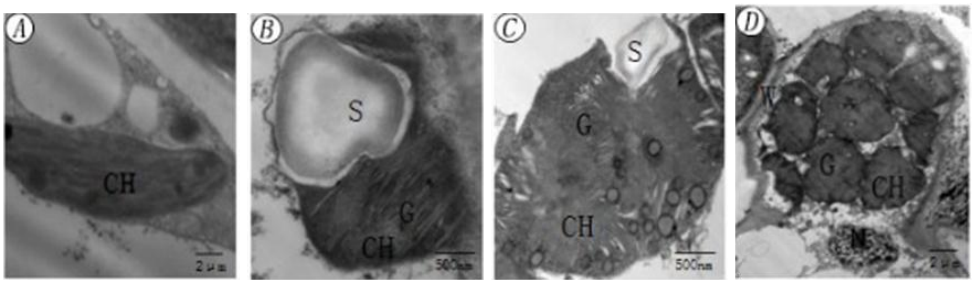

Fig. 1: Transmission electron micrographs of lily chloroplast structure
A: Normal chloroplast (20,000×); B: Inflated starch grain (30,000×); C: The outer membrane is disintegrated and the granum lamella is disordered and loose (25,000×); D: The chloroplast shape changes and the cell wall ruptures (6,000×); CH: Chloroplast; G: Granum; S: Starch grain; W: Cell wall; N: Nucleus

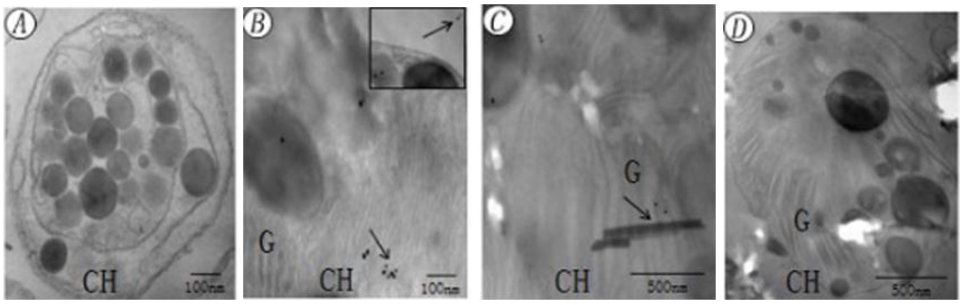

Fig. 2: Immunogold labeling of LMoV CP in chloroplast of lily leaf cell infected with LMoV
A: Healthy lily (60,000×); B–C: Lily infected with LMoV (60,000× 40.000×); D: Lily infected with LMoV, without anti-LMoV CP serum (40,000×); CH: Chloroplast; G: Granum

Effects of LMoV on Chlorophyll Fluorescence Parameters

Compared with healthy plants the Fv/F_0, Fv/Fm, PSII and qp values of lily leaves infected by LMoV exhibited varying degrees of reduction. The reduction in Fv/F_0 and PSII was quite obvious decreasing by 31% and 42% respectively, compared to the control. The decline in Fv/Fm and q_P was less severe but that of q_N was significantly higher. These results indicate that LMoV inhibits photosynthetic activity in lily (Table 1).

Analysis of Total Chloroplast Proteins

SDS-PAGE analysis showed that the total chloroplast protein fractions of healthy control plants and plants infected by LMoV were similar. The only difference was that the LMoV-infected plants produced an extra lightly stained protein band of approximately 30 kDa, which is in accordance with the predicted molecular weight of LMoV CP (Fig. 3). Western blot analysis showed that the protein band showed significant binding to LMoV CP antiserum (Fig. 4) confirming that the extra protein band in lily chloroplasts infected by LMoV was LMoV CP. However, the molecular weight of this protein band was slightly larger than the theoretical value.

Cross-membrane Transport of LMoV CP in the Chloroplast

To investigate the transmembrane transport of LMoV CP, we set up five gradient concentrations of LMoV CP, which we incubated with lily chloroplasts. Western blot analysis showed that LMoV CP could not be detected when the treatment concentration was below 15 μg mL^{-1} at a concentration of 30 μg mL^{-1} and LMoV-CP was detected in chloroplasts the amount of LMoV CP detected in chloroplasts increased with increasing concentration of LMoV-CP (Fig. 5). We incubated lily chloroplasts with 30 μg mL^{-1} LMoV CP for various amounts of time. LMoV-CP was detected in chloroplasts after incubation for only 1 min with the amount of LMoV-CP detected in chloroplasts increasing with increasing incubation time (Fig. 6).

Discussion

Numerous studies have investigated the effects of plant virus infection on the chloroplasts of the host plant. After viral infection the number of chloroplasts in the host is significantly reduced and chloroplast structure is seriously distorted (Korbin and Kaminska, 1998). In the current study, we found that there was a significant change in the chloroplasts of lily infected by LMoV. The internal structure of the chloroplast was destroyed to varying degrees indicating that the mottled symptoms of

Table 1: Effects of LMoV on chlorophyll fluorescence parameters

Samples	Fv/F_0	Fv/Fm	PS□	q_P	q_N
Healthy control	5.19	0.82	0.19	0.44	0.85
LMoVinfected	3.58	0.77	0.11	0.36	0.95

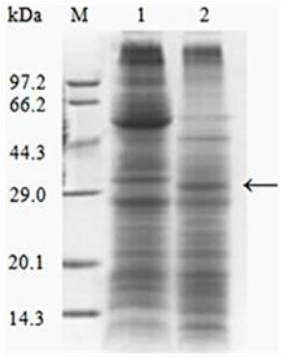

Fig. 3: SDS-PAGE analysis of total lily chloroplast proteins
M: Premixed Protein Markers (Low MW); 1: Healthy lily; 2: Lily infected with LMoV

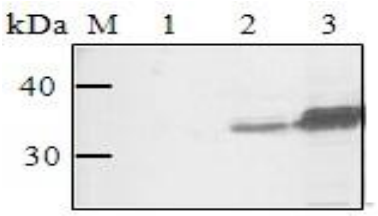

Fig. 4: Western blot analysis of total lily chloroplast proteins
M: Prestained Protein Ladder; 1: Healthy lily; 2: Lily infected with LMoV; 3: LMoV CP expressed from Prokaryotic

lily leaves are related to chloroplast damage. Electron microscopy observations after immunogold labeling showed that gold particles combined with the LMoV CP antigen were mainly localized to the chloroplast thylakoid membrane. However, few gold particles were detected suggesting that the concentration of virus in diseased leaves might have been low. Damage to the chloroplast structure also directly affects the accumulation of chlorophyll and the activity of various enzymes (Xu *et al.*, 2014). Our fluorescence induction kinetic experiments showed that the Fv/F_0, Fv/Fm, PSII and q_p of lily leaves infected by LMoV were reduced to varying degrees suggesting that LMoV inhibits the photosystems and reduces chlorophyll accumulation in lily.

CPs are multifunctional proteins whose functions are related to each stage of the cycle of infection. CPs can adjust the host response during the processes of reproduction, translation and movement (Ivanov and Mäkinen, 2012). In recent years a model of the interaction between the CP and the chloroplast has helped elucidate the mechanism

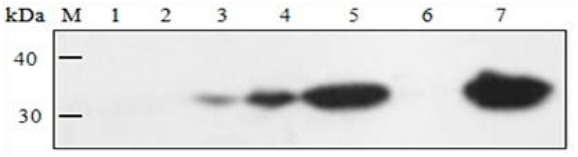

Fig. 5: Western blot analysis of import of LMoV CP at different concentration gradients into intact lily chloroplasts *in vitro*
M: Prestained Protein Ladder; 1-5: concentration gradient of LMoV CP: 7.5 μg mL^{-1}, 15 μg mL^{-1}, 30 μg mL^{-1}, 100 μg mL^{-1}, 300 μg mL^{-1}; 6: Chloroplast from healthy lily; 7: LMoV CP expressed from Prokaryotic

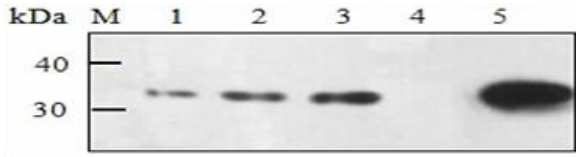

Fig. 6: Western blot analysis of import of LMoV CP at different incubation times into intact lily chloroplasts *in vitro*
M: Prestained Protein Ladder; 1-3: incubation time: 1 min, 30 min and 60 min; 4: chloroplast from healthy lily; 5: LMoV CP expressed from Prokaryotic

underlying the pathogenesis of mosaic plant viruses. In the current study, SDS-PAGE and western blot analysis demonstrated that CP was present in the chloroplasts of lily infected by LMoV. This result is consistent with the results of immunogold labeling. To help confirm that LMoV CP indeed enters the chloroplast, we used trypsin to remove any CP that might have adhered to the outer membrane of the chloroplast. However, the molecular weight of the extra protein band detected in LMoV-infected samples was slightly larger than the theoretical molecular weight of LMoV CP perhaps due to the following: (1) the extraction methods used in different studies alter the structure or conformation of LMoV CP, which alters its mobility during SDS-PAGE; (2) LMoV CP is modified in the chloroplast for example, by combining with sugars, amino acids or small peptides in lily chloroplasts and (3) the proteins encoded by the entire LMoV genome are broken down in the cytoplasm, which leads to the mixing of CP with other non-structural protein fractions during entrance into the chloroplast (Fu *et al.*, 2004).

Conclusion

The occurrence of mosaic symptoms is related to the accumulation of CPs in the chloroplasts as was observed for TMV and CMV (Shintaku, 1991). In the current study, the severity of symptoms in lily leaves was also positively correlated with the accumulation of LMoV CP in the chloroplast. CP might accumulate in the chloroplast via

chloroplast transmembrane transport. We therefore investigated the transmembrane transport of LMoV CP by incubating various concentrations of this protein with lily chloroplasts followed by western blot analysis. We found that 30 μg mL^{-1} LMoV CP could be transported into the chloroplast after incubation for 1 min. This result suggests that the transport of LMoV CP into chloroplast from the cell membrane specifically occurs via transmembrane transport. However, the mechanism of chloroplast transmembrane transport of viral CP requires further analysis.

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