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Full Length Article

Early ROS Accumulation in Chloroplasts of *Nicotiana glutinosa* Infected by *Cucumber mosaic virus*

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Abstract

Reactive oxygen species (ROS) are considered to play an important role on the plant disease resistance. The accumulation of ROS was detected in chloroplasts (not in soluble fractions) from *Nicotiana glutinosa* leaves inoculated with C*ucumber mosaic virus* (CMV) in the early stage of infection (3 days post-inoculation (dpi)); though the accumulation of CMV was lower than that at 9 dpi. During the late stage of infection (9 dpi, disease development), ROS accumulation was detected both in cytoplasm and chloroplasts. At 3 dpi, a significant decrease of the activity of chloroplastic ascorbic acid peroxidase (APX) was observed involved in its elimination that correlated with ROS and TBARS accumulation. The decreased activity of chloroplastic APX was considered as a quick response for *N. glutinosa* to CMV. At the same time, the activity of cytoplasmic APX only slightly decreased. ROS as signaling molecules of infected cells originated from the chloroplast but not the cytoplasm. The damage of membrane by lipid peroxidation increased as ROS generation and the photosynthetic efficiency continues to decline in systemically-infected leaves. It suggested that an alteration of the chloroplastic structure and function was produced in early responses to CMV. © 2019 Friends Science Publishers

Keywords: ROS; *Cucumber mosaic virus*; Chloroplast; Oxidative stress

Introduction

Virus as biotic stress leads to a large number of Reactive oxygen species (ROS) accumulation in infected plants (Riedle-Bauer, 2000; Clarke *et al.,* 2002; Hernández *et al.,* 2006; Torres, 2010; Scheler *et al.,* 2013). Reactive oxygen species caused damage to cell components that resulted in oxidative stress (Tuteja *et al.,* 2009; Wi *et al.,* 2012). Hydrogen peroxide (H_2O_2) is a molecule involved in signaling trigger tolerance to various biotic stresses (Sofo *et al.,* 2015). During virus infection, defense responses are related to the ROS accumulation.

The antioxidase system protects plants against oxidative stress according to scavenging of ROS (Asada, 1999; Alscher *et al.,* 2002; Guo *et al.,* 2004; Gapi´nska *et al.,* 2008; Yang *et al.,* 2008; Pukacki and Kamińska-Rożek, 2013). The antioxidase contained superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione peroxidase (GPX) and glutathione-S- transferase (GST). Some chemical compound produced by plant (such as ascorbic acid, ASA; glutathione, GSH; phenolic compounds) were also related to oxidative stress. Reactive oxygen species also influence the gene expression which regulate and control the plant response to pathogen and systemic signal transduction. Reactive oxygen species as a systemic signal induces antioxidant and defense responses in non-infected plant cells. The rapid accumulation of ROS at virus infection sites was very important (Hernández *et al.,* 2016). *Cucumber mosaic virus* caused oxidative stress, hydrogen-peroxide accumulation, and changes in the activity of antioxidants enzymes in infected plants (Song *et al.,* 2009). The responses to CMV infection in host plant were due to different factors, the host varieties, the ways of inoculation and the time when the growth of the new upper leaves appear. *Nicotiana glutinosa* showed a slow hypersensitive response (HR) which resulted in oxidative stress and programmed death (PCD) of infected leaves. That is order to elicit an imbalance of antioxidase system due to plant resistance to virus (Shang *et al.,* 2010).

The subcellular level of antioxidant systems may be imbalance, which affects the accumulation of early ROS. In the present work, in order to clarify the accumulation of the early active oxygen species in the sub cellular level, changes of the antioxidant systems at the subcellular level were analyzed. The physiological

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parameters of infected plants were also detected. In addition, the virus accumulation was analyzed at different stage of infection so that we can learn more about the interaction between plant and pathogen.

Materials and Methods

Plant Material

N. glutinosa seedlings were grown in greenhouse with 16 h-light and 8 h-dark cycles at 20-25°C. The plants were inoculated by CMV according to Zhao *et al*. (2013). After one day of inoculation, 5 m*M* ascorbic acid (AsA) and 5 m*M* dimethylthiourea (DMTU) was sprayed to the whole seedlings every day (Luo *et al.*, 2009).

CMV-CP Gene Detection

Total RNAs were isolated plant tissues by a Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA). The quantitative real-time PCR analysis of *CMV-CP* gene expression was performed on a Bio-Rad iCycler according to Shang *et al.* (2009, 2011).

ROS Staining

Superoxide and H_2O_2 were detected by nitroblue tetrazolium (NBT) and 3, 3-diaminobenzidine (DAB), respectively (Yang *et al.*, 2004).

Determination of H_2O_2 and Oxidative Stress Parameters

The soluble fractions (the cytoplasm) and chloroplasts were divided for detection of H_2O_2 content, thiobarbituric acid reactive substances (TBARS) content and electrolyte leakage according to Shi *et al.* (2006). Lipid peroxidation and electrolyte leakage was measured according to Shang *et al.* (2009).

Measurement of Chlorophyll Fluorescence

Fluorescence parameters were detected by using a fluorometer (PAM-2100, Walz, Germany) according Yuan *et al.* (2007). The following parameters were calculated using the following equations: *F*v/*F*m = (*F*m - *F*o)/*F*m, where *F*o is the minimum fluorescence; the changes in the apparent PSII quantum yield Φ_{PSII} = (*F*m' - *F*s)/*F*m', where *F*m' is maximum fluorescence yield after light adaptation; and non-photochemical quenching NPQ = *F*m/*F*m' - 1.

Analysis of Gas Exchange

Photosynthetic gas exchange was detected using the instrument (HCM-1000, Walz, Effeltrich, Germany) according to Yuan *et al.* (2007). Leaf net photosynthetic rate (Pn) and stomatal conductance (Gs) were determined at a temperature of 25°C, CO_2 concentration of 350 μmol mol^{-1}, 45% relative humidity, and photon flux density of 800 μmol m^{-1} s^{-1}. Leaf temperature was controlled using a leaf cuvette with a 1010-M system (TPS-1, PP system).

Antioxidants Enzymatic Assays

The soluble fractions and chloroplasts were separated for measurement of the enzyme activity. Infected plants at 3 dpi and 9 dpi were used for this operation. All operations were performed at 4°C according to Hernández *et al.* (2006). The activity of SOD and CAT were detected according to Shi *et al.* (2006). The POD activity was analyzed according to Rossum *et al.* (1997). The glucose-6P-dehydrogenase (G6PDH) activity was measured as described in Hernández *et al*. (2000). APX activity was detected by the method of Hernández *et al.* (2004). The activity of GPX and GR was determined according to Overbaugh and Fall (1985). GST activity was analyzed according to Habig and Jakoby (1981).

Statistical Analysis

An independent (unpaired) Student's t-test was chosen to test the significance of differences among means of small 'n' sample sets. A difference was considered to be statistically significant when $P < 0.05$.

Results

Symptom and CMV Replication

Slight mosaic symptom appeared at 3 dpi. More severe symptom was displayed over time. The expression of *CMV-CP* gene was detected by qPCR at 3 - 9 dpi. The accumulation of *CMV-CP* was tested in the different stage of disease development. The expression level of *CMV-CP* of systemically-infected leaves at 9 dpi was higher than that at 3 dpi. There was a slight increase of the *CMV-CP* level of in systemically-infected leaves at 9 dpi compared with that at 6 dpi (Fig. 1).

DAB and NBT Staining

It was shown that the highest H_2O_2 and superoxide accumulation occurred at systemically-infected leaves (Fig. 2). The results suggested that the accumulation of ROS was much higher in systemically-infected leaves at 9 dpi than at 3 dpi (Fig. 2).

ROS elimination was imitated in seedlings of *N. glutinosa* by exogenous AsA and dimethylthiourea (DMTU, a trap for H_2O_2) treatment (Fig. 3). AsA treatment can reduce the accumulation of ROS compared with systemically-infected leaves. But solo eliminator could neither alleviate the symptom, nor inhibit CMV replication at 9 dpi (Fig. 3).

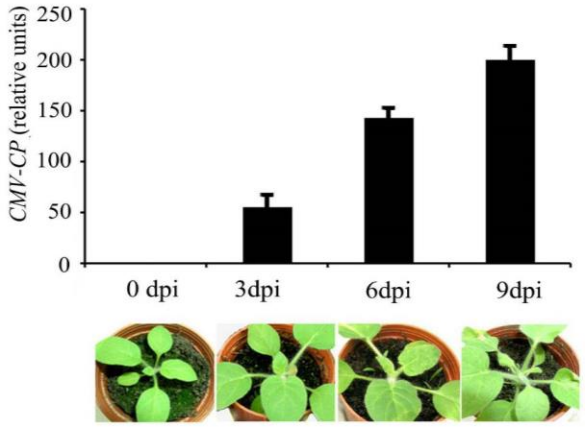

Fig. 1: The symptom and the expression of *CMV-CP* gene in systemically-infected leaves of *N. glutinosa* at 0, 3, 6 and 9 days post-inoculation (dpi)

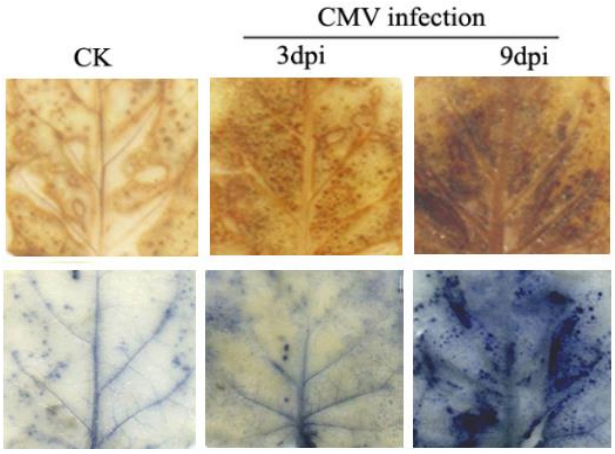

Fig. 2: DAB and NBT staining of systemically-infected leaves of *N. glutinosa*

The alleviation of symptom is crucial for plants. AsA and DMTU simultaneous co-pretreatment could alleviate the symptoms of systemically-infected leaves more effectively than AsA or DMTU single pretreatment. The co-pretreated plant was close to the healthy plant (Fig. 3).

H_2O_2 Content in Different Fractions

At the early stage of infection (3 dpi), the H_2O_2 levels was increased in the chloroplasts from inoculated plants compared with the control. In soluble fraction, the H_2O_2 levels did not showed a significant change compared with soluble fraction from healthy leaves (Fig. 4A). At the late stage (9 dpi), a severe oxidative stress was observed in leaves because of the increase in lipid peroxidation and electrolyte leakage, while the H_2O_2 levels greatly increased in soluble fractions and chloroplasts in systemically-infected leaves (Fig. 4B).

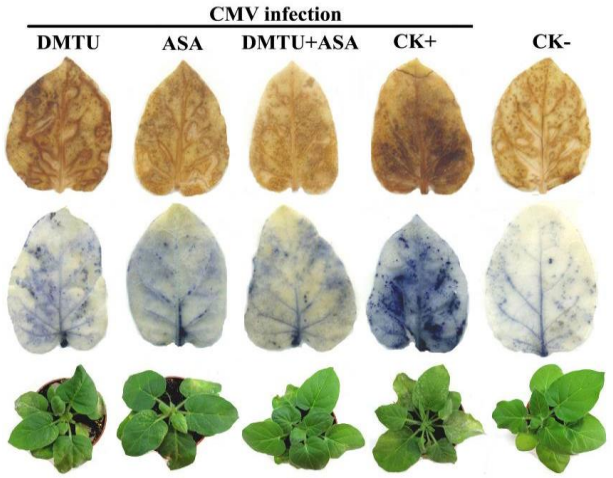

Fig. 3: Symptoms and ROS (at 9 dpi) of 5 m*M* ascorbic acid (AsA), 5 m*M* dimethylthiourea (DMTU)-treated *N. glutinosa* seedlings infected with CMV. AsA and DMTU were sprayed to the whole seedlings every day after virus inoculation; CK+ means virus infected control and CK- means control without virus. The figure shows H_2O_2 accumulation after DAB-staining (the first row), superoxide accumulation after NBT-staining (the second row) and symptoms of *N. glutinosa* infected with CMV (the third row)

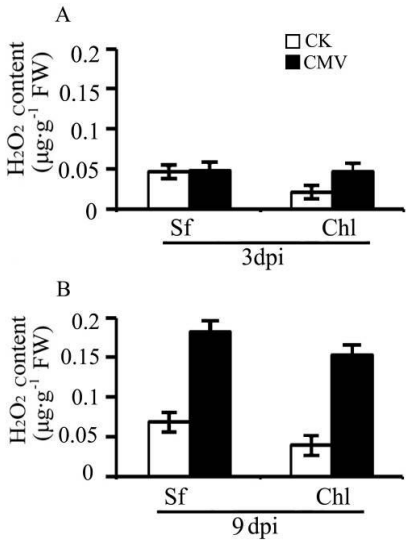

Fig. 4: Effect of CMV infection on H_2O_2 contents in different cell fractions from *N. glutinosa* leaves during (A) the initiation (3 dpi, early response) and (B) the elaboration (9 dpi, disease development) phases of disease development Sf, soluble fraction from leaves; Chl, chloroplast from leaves. Bars represent standard deviations of 3 independent replicates (n = 3) CK, control

TBARS Content in Different Fractions

TBARS content of the chloroplasts are significantly elevated at 3 dpi. At 9 dpi, due to a ROS rise in cytoplasm, TBARS content of cytoplasm began to rise.

This is a relatively slow process of TBARS accumulation and therefore TBARS content of cytoplasm is still less than of the chloroplast. After 3 dpi, the change of TBARS content of chloroplast was not significant (Fig. 5).

Changes in Antioxidant Enzymes Activity

The activities of antioxidant enzymes were changed in the soluble fractions and chloroplasts in infected cell. H_2O_2 is removed primarily by CAT enzyme. In soluble fraction from inoculated leaves, the CAT activity at 3 dpi and 9 dpi was remarkable higher than those from healthy leaves (Fig. 6B), which is consistent with the H_2O_2 contents. At 3 dpi, APX was declined while an increase in GST activity in the soluble fractions was found (Fig. 6A and F). In chloroplasts, H_2O_2 content increased while APX and G6PDH declined (Fig. 6A and H). The levels of antioxidant enzymes changed more at 9 dpi than 3 dpi. In soluble fractions, APX, and GR increased while GST GPX, GR and SOD decreased (Fig. 6A, E and F). Activities of POD and SOD reflected a similar trend of CAT (Fig. 6B, C, D). It could be inferred that after virus infection, the enzymes activities were enhanced by ROS accumulation, and highest in chloroplasts from inoculated leaves.

Degrees of Membrane Injury of the Whole Leaf

TBARS is the product of lipid peroxidation, whose content showed the degree of membrane injury. The content of TBARS in systemically-infected leaves at 9 dpi was significantly higher than that at 3 and 6 dpi, and higher than to the healthy leaves (Fig. 7A). H_2O_2 levels showed a similar trend with the electrolyte leakage and the contents at 9 dpi were much higher (Fig. 7A and C). The stability of the plasma membrane was influenced by virus infection.

Chlorophyll Fluorescence

At 3dpi, NPQ increased which was the energy consumption involved in CO_2 assimilation. Fv/Fm, Fv'/Fm' slightly decreased in systemically-infected leaves at 3-9 dpi (Fig. 7F and G). At 3dpi, PSII quantum yield, Leaf net photosynthetic rate (Pn) was declined significantly. The stomatal conductance (Gs) was seen as a steady decline since leaves infected by virus (Fig. 7D, H and I).

Discussion

N. glutinosa is very susceptible to CMV (Shang *et al.*, 2009), as shown by the strong mosaic symptoms observed in present study. There was an obviously positive correlation between the accumulation of ROS and mosaic symptoms (Hernández *et al.*, 2006; Shang *et al.*, 2011). In CMV-infected plants, ROS generation associated with the accumulation of *CMV-CP* resulted in severe mosaic symptoms. Although slight symptoms were observed,

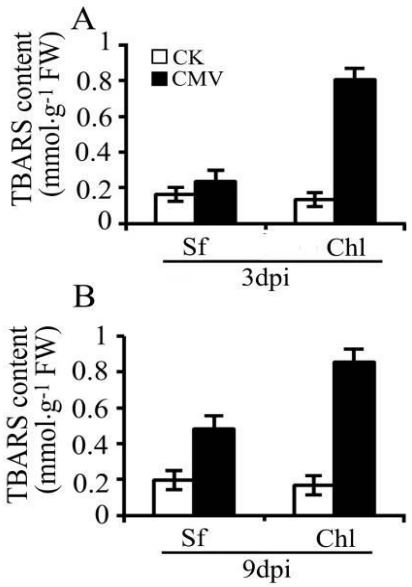

Fig. 5: Effect of CMV infection on TBARS contents in different cell fractions from in systemically-infected leaves of *N. glutinosa* during (A) the initiation (3 dpi, early response) and (B) the late stage (9 dpi, disease development) phases of disease development. Sf, soluble fraction from leaves; Chl, chloroplast from leaves. Bars represent standard deviations of 3 independent replicates (n = 3). CK, control

ROS was accumulated in chloroplasts of *N. glutinosa* in the initiation phase of infection (3 dpi). At the same time, ROS content in the cytoplasm of infected cells were similar to the control. It suggested that ROS originated in the chloroplast of infected cells at the early stage of infection. The extent of membrane injury was shown by TBARS and electricity permeability (Nanjo *et al.*, 1999; Luo *et al.*, 2011). At the early stage of infection, the oxidative stress parameters were changed due to the ROS accumulation in the chloroplast of infected cells.

Symptom formation is a time-consuming process and early symptoms were alleviated by AsA treatment. Solo ROS eliminators could not alleviate symptom. Salicylic acid (SA) was strictly correlated to H_2O_2 content (Mateo *et al.*, 2006; Shang *et al.*, 2009; Vlot *et al.*, 2009). Salicylic acid burst and SA-related gene expression was inhibited by AsA treatment in virus infected plants (Wang *et al.*, 2011), resulting in declined of ROS accumulation (Mittler *et al.*, 2004; Wang *et al.*, 2011).

ROS accumulation also made the endomembrane system of the chloroplast be destroyed (Moore *et al.*, 2001). The increasing energy dissipation in systemically-infected leaves, which lost as thermal energy, could not be used in photosynthesis (Shangguan *et al.*, 2000). That was the reason why net photosynthetic rate declined along with the decrease in the stomatal conductance. Finally, biomass declined in systemically-infected leaves leading to the formation of mosaic symptoms.

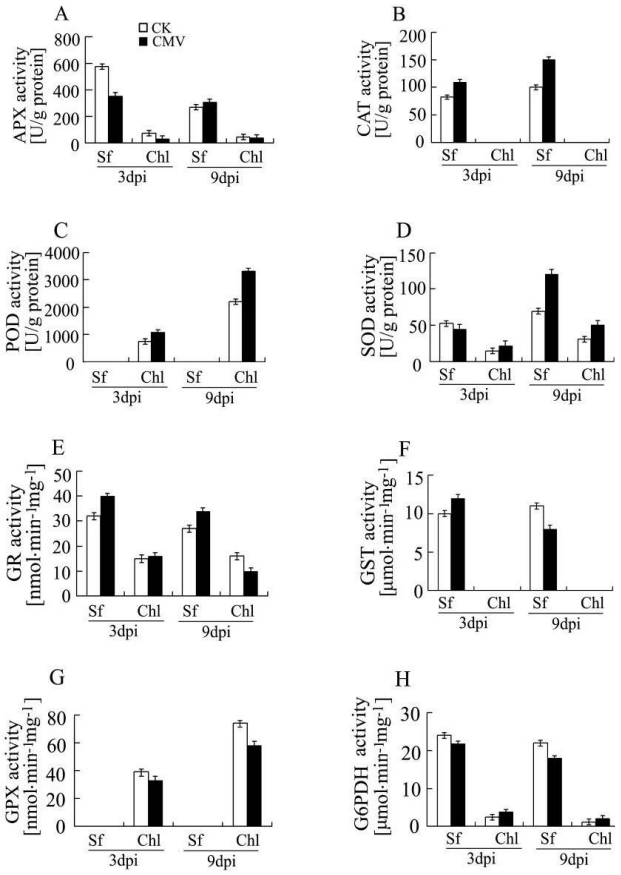

Fig. 6: Antioxidant enzyme activities in different cell fractions from systemically-infected leaves of *N. glutinosa* during the initiation (3 dpi, early response) and the late stage (9 dpi, disease development). Bars represent standard deviations of 3 independent replicates (n = 3). CK, control

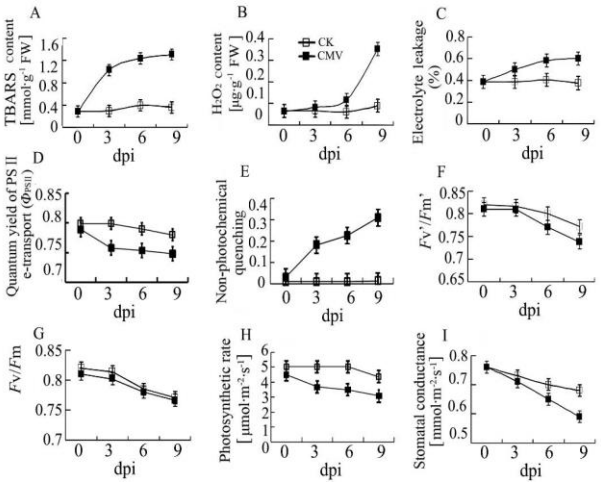

Fig. 7: (A) TBARS, (B) Hydrogen peroxide, (C) Electrolyte leakage, (D) PSII quantum yield, (E) Non-photochemical quenching capacity, (F) Fv'/Fm', (G) Fv/Fm, (H)Leaf net photosynthetic rate and (I) stomatal conductance in systemically-infected leaves of *N. glutinosa* at 3-9 days post-inoculation (dpi). Bars represent standard deviations of 3 independent replicates (n = 3). CK, control

H_2O_2 must be removed from cells to maintain healthy metabolic function in plant cell (Dat *et al.*, 2000). Under the light, the chloroplast is the main organ that produces ROS. Some H_2O_2 scavenging enzyme such as CAT was found in the cytoplasm but not in chloroplasts. Instead of that, APX was a key H_2O_2 eliminator in chloroplasts (Simonetti *et al.*, 2010). In the chloroplast of infected cells, APX decreased for increasing H_2O_2 elimination.

The chloroplastic APX was encoded by nuclear genes. APX was synthesized in the cytoplasm, but cannot be transferred to the chloroplast, which leaded to the accumulation of ROS in the chloroplast. The conclusion was proved by the decreases in cytoplasmic APX activity. Changes in cytoplasmic APX activity suggested that viral infection resulted in the disruption of chloroplast protein transport. That would explain why ROS burst occurring in the chloroplasts earlier than in the cytoplasm of infected cells. ROS as signaling molecules in cells originated from the chloroplast rather than the cytoplasm.

The accumulation of reactive oxygen species resulted in membrane injury of the chloroplast. The extent of membrane injury was shown by TBARS and electricity permeability (Nanjo *et al.*, 1999). Chloroplast degradation, resulting in the block for pigment-protein synthesis (Moore *et al.*, 2001), and consequently the chlorophylls reduced. The lack of chlorophyll led to a sharp decline in the photosynthetic rate. At the same time, the destruction of chloroplast membrane structure resulted in the damage of electron transport chain for photosynthesis. Light energy dissipated into heat energy.

Conclusion

The results of present study suggest that antioxidant enzyme activities changed in the chloroplasts of *N. glutinosa* in response to CMV infection. The antioxidant enzyme system in chloroplast prior to that in cytoplasm provides a rapid response to the early stages of disease development. Chloroplasts are important organs of plants and very sensitive to pathogenic invasion. The preferential activation of antioxidant enzyme system in chloroplast reflects the optimal allocation of defense resources. That was also the result of natural selection.

Acknowledgements

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