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Autophagy induction in tobacco leaves infected by potato virus Y^O and its putative roles

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ABSTRACT

Autophagy plays a critical role in the innate immune response of plants to pathogen infection. In the present study, we examined autophagy induced by potato virus Y ordinary strain (PVY^O) infection in tobacco (*Nicotiana benthamiana*). Enzyme-linked immunosorbent assays revealed that the number of virus particles in the plant peaked at 2 weeks post-inoculation and then gradually decreased.

Additionally, the amount of virus increased significantly in the 3rd and 4th leaves distal to the inoculated leaf and decreased slightly in the 5th leaf. Within 2 weeks of PVY^O inoculation, the tobacco leaves showed typical symptoms of *Potyvirus* inoculation, including mottling, yellowing, a mosaic pattern, and necrotic tissue changes at the inoculated site. Based on an ultrastructural analysis of the PVY^O-infected tobacco leaves, virus aggregates appeared as longitudinal and transverse arrays and pinwheels, which are typical of *Potyvirus* inoculation. Moreover, PVY^O infection caused changes in the number, size, and shape of chloroplasts, whereas the number of plastogranules increased markedly. Furthermore, double-membrane autophagosome-like vacuoles, including electron-dense materials, laminated structures, and cellular organelles, were found. The induction of autophagy after the PVY^O infection of tobacco leaves was further confirmed by the expression of lipidated microtubule-associated protein 1 light chain 3 (LC3)-II, an autophagy marker and p62, an autophagy adaptor protein. The LC3-II levels increased daily over the 4-week period. Although virus inoculation was performed systemically on the basal leaves of the plants, LC3-II was expressed throughout the leaves and the expression was higher in leaves distal to the inoculated leaf. Moreover, PVY^O infection caused the activation of stress-activated protein kinases/c-Jun N-terminal kinases. Therefore, PVY^O infection-induced autophagy was positively correlated with the virus content, suggesting that autophagy induction following PVY^O infection is involved in the anti-pathogen response of the host.

1. Introduction

Potato virus Y (PVY), a single-stranded RNA virus, is a member of the genus *Potyvirus* (family *Potyviridae*) [1]. PVY spreads *via* aphids and infects several crops in the family *Solanaceae*. Reportedly, PVY is a serious threat to potato tubers and decreases potato yields [2,3]. PVY is highly diverse genetically and has a number of strains and variants [3–5]. PVY^O, the ordinary strain of PVY, causes yellowing, mottling, and mosaic pattern symptoms in tobacco and induces necrosis and severe stunting in potato cultivars [6]. The virus spreads rapidly throughout the stem at approximately the same time [7,8]. Upon entering the phloem tissues of infected leaves, the virus moves rapidly toward the apex or young parts of the plant [8,9].

Ultrastructural changes resulting from *Potyvirus* infection in various plants have been reported [10]. A common feature appearing in the cytoplasm of cells infected with *Potyvirus*, and a critical feature for the diagnosis of *Potyvirus* infection, is the induction of characteristic pinwheel-shaped inclusion bodies [11], which are composed of the virus-encoded cylindrical inclusion (CI) protein.

Autophagy, a lysosomal degradation process, is a highly conserved pathway from yeast to mammals and is involved in

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protection *via* the recycling of cytoplasmic constituents, including long-lived proteins and damaged cellular organelles [12]. Autophagy in animals is induced by various stresses and is involved in physiological or pathophysiological conditions [13,14]. Numerous studies indicate that autophagy in plants plays a role in growth, development, and various stress responses such as carbon and nitrogen starvation [15–17]. Plant *atg* genes with significant homology to yeast *atg* genes have been found in *Arabidopsis* [18]. Furthermore, autophagy plays a role in cell protection against virus infection in plants. Reportedly, autophagy in plants also acts as a regulator of programmed cell death (PCD); autophagy restricts virus-induced PCD to sites of infection [19]. The knockdown of autophagy genes, including *PI3K/VPS34*, *atg3*, and *atg7*, resulted in delocalized PCD upon tobacco mosaic virus (TMV) infection [19]. Thus, autophagy in plants appears to be involved in innate immunity. Furthermore, Beclin1/ATG6, an autophagy-related protein, can facilitate the autophagic degradation of a vacuolar-processing enzyme exhibiting caspase-1 like activity, thereby inhibiting the spread of PCD [20], indicating crosstalk between autophagy and apoptosis in plants. However, whether PVY infection can induce autophagy has not been determined.

In this study, we investigated autophagy induced by PVY^O inoculated into tobacco leaves. Based on the presence of lipidated microtubule-associated protein 1 light chain 3 (LC3)-II and autophagosome-like vacuoles, autophagy was shown to occur.

2. Materials and methods

2.1. Plant preparation and growth conditions

Tobacco plants were grown from seed in a growth room at 23 ± 1 °C under a 16 h photoperiod and an 8 h dark period. Four-week-old plants were used in our experiments. The plants were seeded in autoclaved soil.

2.2. PVY^O inoculation and sample collection

PVY^O virus was purchased from the American Type Culture Collection (PV-575). PVY^O was mechanically inoculated into tobacco plants. Leaves of 4-week-old tobacco plants were dusted with silicon carbide powder (Sigma-Aldrich, St. Louis, MO, USA) and inoculated with homogenized PVY^O-inoculated plant material diluted (1:10) in 0.1 M sodium phosphate buffer, pH 7.6. After inoculation, the plants were grown under 16 h of daylight at 22 °C. Non-inoculated tobacco plants were included in all experiments as healthy controls. PVY^O-inoculated leaves were collected every 7 days over a 4-week period. Each sample was obtained by pooling the leaf samples from two or three pots and stored at −80 °C until use.

2.3. Enzyme-linked immunosorbent assay (ELISA)

To measure the number of virus particles, a direct double-antibody sandwich ELISA (Agdia, Inc., Elkhart, IN, USA) was used according to the manufacturer's instructions. Total proteins were extracted from the leaves of each plant with a general extraction buffer (Agdia, Inc.). Briefly, protein (50 μg) was plated onto anti-PVY^O-coated 96-well plates (Agdia, Inc.), incubated for 2 h at room temperature (RT), and washed several times with phosphate-buffered saline with Tween-20 (PBST). The enzyme conjugates (100 μL) were then added to each well and the plates were incubated for 2 h at RT in the dark under humid conditions. After washing with PBST, the substrate p-nitro phenyl phosphate was added to each well and further incubated for 1 h at RT in the dark under humid conditions. The absorbance was read at 405 nm using an ELISA Reader (BioTek, Winooski, VT, USA).

2.4. Immunoblot analysis

Immunoblot analysis was performed as described previously [31]. The leaf tissues were frozen in liquid nitrogen and ground by using pestle and daunce homogenizer. The lysate was then resuspended in 500 μl lysis buffer. The lysis buffer (RIPA, pH 8.0) includes 50 mM Tris-HCl, 1% NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Sigma Aldrich). The lysates were then centrifuged, and the protein content was quantified. Equal amounts of protein were separated via SDS-polyacrylamide gel electrophoresis (12–15%), transferred to a PVDF membrane, and immunoblotted with the corresponding antibodies. Antibodies were as follows: anti-phospho-JNK, LC3B, and p62 were obtained from Cell Signaling (Beverly, MA, USA). Anti-β-actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.5. Statistical analysis

Statistical data were analyzed by the student *t*-test. Differences of means at $P < 0.05$ or $P < 0.01$ were considered statistically significant. Statistical analyses of data were performed using SPSS software version 20.0 (IBM Corporation, Chicago, IL, USA).

3. Results

3.1. Amount of virus in PVY^O-infected tobacco plants

The amount of virus in PVY^O-infected plants was quantified using ELISAs. The value showed a peak at 2 weeks post-inoculation and then gradually decreased, but it was higher than in the non-inoculated controls (Fig. 1A). Next, the virus particles were analyzed based on the leaf position from the inoculated leaf (1st leaf) to the distal 5th leaf of 2-week-old plants post-inoculation. The number of particles increased significantly in the 2nd leaves, and further increased in the 3rd and 4th leaves from the inoculated leaf and decreased slightly in the 5th leaves, but it was higher than in the controls (Fig. 1B). These results indicate that virus replication was the highest at 2 weeks post-inoculation and higher in leaves distal to the inoculated leaf, indicating that the virus moved to young leaves.

3.2. Phenotypic changes in the tobacco leaves after PVY^O infection

To examine whether infection with the virus caused the observed morphological changes, the phenotype was examined. The 3rd and 4th leaves of young tobacco seedlings were mechanically inoculated with PVY^O (1 month; Fig. 2A, arrow). The inset of Fig. 2B shows the leaf state immediately after inoculation (dotted circle). Approximately 7 days post-inoculation, the tobacco leaves began to exhibit a variety of infection symptoms, including a mosaic pattern, mottling, lesions, and necrotic changes, which became more severe over time (Fig. 2C–F). Necrotic changes were observed at the infection site (Fig. 2C and D, dotted circle) and the leaves finally etiolated (Fig. 2F, dotted circle). The insets in Fig. 2E and F shows the mosaic pattern and yellowing of the leaves.

3.3. Cytological changes in PVY^O-infected leaves

To examine the cytological changes in PVY^O-infected leaves using an electron microscope, samples were obtained from the upper leaf of inoculated leaves at 2 weeks post-inoculation. Healthy tissue samples were obtained from the leaves of non-inoculated plants localized at the same node as the inoculated