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Application of 24-epibrassinolide decreases the susceptibility to cucumber mosaic virus in zucchini (*Cucurbita pepo* L)

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ABSTRACT

Brassinosteroids (BRs) are involved in a wide range of developmental processes and response to biotic and abiotic stresses. However, the roles of BRs in defense against virus in economically important crops have not been studied. Here, we showed that application of 24-brassinolide (EBR), a biologically active BR, at 0.2 μM was effective in decreasing the incidence of infection by cucumber mosaic virus (CMV) in susceptible zucchini (*Cucurbita pepo* L) plants. EBR reduced the accumulation of CMV in systemic leaves but not in inoculated leaves. CMV infection caused oxidative stress, abnormal chloroplast structure and damages to the photosynthetic apparatus. However, EBR treatment ameliorated the symptoms caused by CMV. EBR-induced defense to CMV is not accompanied by accumulation of salicylic acid. In contrast, EBR treatment alone induced a transient accumulation of H_2O_2. Subsequently, EBR treatment led to an increased accumulation of H_2O_2 during the early phase of CMV infection. The results suggest that application of EBR could be a promising approach to control virus disease in susceptible crops and EBR-induced oxidative burst may play a role in defense to virus disease.

1. Introduction

Cucumber mosaic virus (CMV) is the type member of the genus Cucumo virus, family Bromoviridae (Palukaitis and Garcia-Arenal 2003) and is one of the most important viruses in the world due to its wide range of hosts. The most common symptom induced by CMV is mosaic, which is associated with reduced expression of chloroplast and photosynthesis related genes and abnormal chloroplasts structure (Mochizuki et al., 2014). In the model plant *Arabidopsis thaliana*, the resistance (*R*) gene *RCY1* confers resistance to CMV, which is accompanied by the development of hypersensitive response (HR) as characterized by the necrotic local lesions at the primary infection sites (Takahashi et al., 2002). Unfortunately, the germplasm of most crops lack the *R* genes against CMV (Morroni et al., 2008). Considering the large economic impacts of CMV in the production of cucurbit crops, transgenic approach expressing the CMV coat proteins has been used to increase the CMV resistance in crops (Tomassoli et al., 1999; Shin et al., 2002). However, the resistance of the transgenic lines varied to a large extent, due to the lack of understanding of the mechanism of CMV resistance (Morroni et al., 2008). Therefore, novel strategies are needed for decreasing susceptibility of crops to CMV, with knowledge regarding the mechanism of basal defense against CMV in the absence of *R* genes.

Hormones are signals produced by plant in response to developmental and environmental clues. Among the plant hormones, salicylic acid (SA) plays a central role in both incompatible and compatible plant-virus interaction (Alazem and Lin, 2015). Low SA level in *NahG* transgenic plants leads to negation of *R*-gene-mediated resistance against virus (Baebler et al., 2014). More importantly, SA protects susceptible plants from virus infection by inhibiting virus accumulation and/or systemic movements (Mayers et al., 2005). SA may act upstream of the small interfering RNA (siRNA) silencing system, thereby limiting the accumulation of viral RNA (Yu et al., 2003; Alamillo et al., 2006). SA also affects the virus infection in susceptible plants by regulating the mitochondria alternative oxidase (AOX) (Lee et al., 2011).

In addition to SA, plant *R*-gene-mediated resistance and basal defense to virus is accompanied by accumulation of reactive oxy-

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gen species (ROS). An oxidative burst has long been known to play a critical role in HR response during early phase of virus infection in resistant plants (Torres et al., 2006). However, the role of ROS in compatible plant-virus interaction is unclear. CMV infection leads to accumulation of ROS with concomitant inductions of antioxidant enzymes and mitochondria AOX activity in cucumber (Clarke et al., 2002; Song et al., 2009). Consistently, chemical activation of AOX decreases the viral RNA accumulation of tobacco mosaic virus (TMV) in susceptible tomato, which is associated with reduced accumulation of ROS (Fu et al., 2010; Liao et al., 2012). In contrast, overexpression of AOX did not enhance the basal immunity of susceptible plants (Ordog et al., 2002). Therefore, the timing and/or location of ROS accumulation may be important for the outcome of compatible plant-virus interaction.

The interactions between different hormones determine the plant resistance or susceptibility to virus. Interestingly, brassinosteroids (BRs) enhance the resistance to TMV in *N*-gene containing *Nicotiana tabacum* independent of the SA signaling (Nakashita et al., 2003). BRs are a type of steroid hormones and play roles in a wide range of developmental processes, including cell division, cell elongation, vascular differentiation, photomorphogenesis and flowering (Vriet et al., 2012). BR binds and activates the receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) and the coreceptor BRI1-associated kinase 1 (BAK1), which is followed by the inactivation of BRASSINOSTEROID INSENSITIVE2 (BIN2), a negative regulator of BR signaling. Subsequently, the transcription factors, BRASSINAZOLE-RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1), are activated, leading to expression of BR responsive genes and plant growth (Clouse, 2011). However, the roles of BR in plant-virus interaction are still unknown, largely due to the lack of a link between BR-mediated development pathway and the plant antiviral machinery. Furthermore, only a few studies report the role of BR in basal immunity to virus in *Arabidopsis* (Korner et al., 2013; Zhang et al., 2015), whereas the effects of BR on the virus infection in susceptible crops have not been reported.

In this study, we studied the effects of BR on the susceptibility of zucchini plants to CMV through foliar application of 24-epibrassinolide (EBR), a biologically active BR. We find that EBR was effective in ameliorating the disease symptom by inhibiting the accumulation of CMV in systemic leaves. BR-induced defense to CMV is independent of SA accumulation, and is not related to an enhanced antioxidant capacity. We detected stronger accumulation of H_2O_2 during early phase of CMV infection after EBR treatment. The role of BR-induced oxidative burst in basal defense to virus in susceptible crops is discussed.

2. Materials and methods

2.1. Plant materials and treatments

Zucchini seeds (*Cucurbita pepo* L. var. Xinzaoqing No.1) were germinated and grown in vermiculite for 10 days. Plants were then grown hydroponically in a 10-L plastic tank containing Enshi nutrient solution in a naturally illuminated greenhouse. The average temperature in the greenhouse is 25/20 °C (day/night). The nutrient solution was aerated with an air pump and changed every 7 days. When the 1st true leaf fully expanded, all the leaves were sprayed with 0.2 μM 24-epibrassinolide (EBR) using a 1-L portable garden sprayer until the leaves were wet. Plants were treated with EBR every 6 days for a total of five times. One day after the first EBR treatment, plants were inoculated with cucumber mosaic virus (CMV). The EBR stock was prepared by solving EBR powder (Sigma–Aldrich, St. Louis, MO, USA) in 100% ethanol and stored at 4 °C. Aliquots of EBR stock was diluted by 1000 folds and applied to the plants. Water with equivalent concentration of ethanol served as control. CMV was prepared from virus-infected leaf tissues by grounding in an inoculation buffer containing 0.1 M sodium phosphate (pH 7.5), 2% (w/v) polyvinylpyrrolidone (PVP), and 0.2% (w/v) Na_2SO_3 at ratio of 1:100. The extract was used for inoculation of the cucumber leaves. For inoculation, the plants were sprayed with Carborundum and then inoculated by rubbing viral inoculum onto the leaves with cotton swabs. Mock-inoculation controls were prepared using the same procedure with buffer. The rate of infection and disease index was evaluated after CMV infection.

2.2. Determination of the CMV content

The DAS-ELISA (double antibody sandwich enzyme-linked immunosorbant assay) method is used for the analysis of CMV content. Leaf tissue of 0.1 g was collected, immediately frozen at −80 °C, and used for DAS-ELISA which was carried out in 96-well plates using commercially available alkaline phosphate compound ELISA test kits (Agdia Inc., Elkhart, USA) for CMV according to the manufacturer's instructions. The optical density (OD) was measured at 405 nm with a spectrophotometer (Spectra Max Plus384; Molecular Devices, Sunnyvale, USA).

2.3. SA quantification

Quantification of SA was performed using a biosensor method according to DeFraia et al. (2008). Leaf tissue of 0.1 g was grounded into powder with liquid nitrogen, and then 200 μL of acetate buffer (0.1 M, pH 5.6) was added. Samples were then mixed thoroughly and centrifuged for 15 min at 16,000 × *g*. Half of the supernatant was stored on ice for free SA measurement and half was incubated at 37 °C for 90 min with 4 U of β-glucosidase (Sigma–Aldrich, St. Louis, MO) for conjugated SA measurement. Culture of *Acinetobacter* sp. ADPWH_*lux* was diluted in 37 °C LB (1:20) and grown for 3 h at 200 rpm to an OD_{600} of 0.4. Leaf extract (20 μL), LB (60 μL) and biosensor culture (50 μL) were added to the wells of a black 96-well black plate. The plate was incubated at 37 °C for 1 h without shaking and then the luminescence was read with a PerkinEllmer EnSpire Multilabel Plate Reader (PerkinElmer, Waltham, Massachusetts).

2.4. Gas exchange, chlorophyll fluorescence and pigment contents measurements

The net photosynthetic rate (Pn) was determined by a portable photosynthesis system (LI-COR 6400, Lincoln, NE, USA). The air temperature, air relative humidity, CO_2 concentration, and PPFD for measurement of Pn were 25 °C, 85%, 400 μmol mol^{-1}, and 1000 μmol m^{-2} s^{-1}, respectively.

The chlorophyll fluorescence parameters were determined after 30 min of dark adaptation using Dual-PAM-100 system (Walz, Germany). The initial fluorescence (Fo) was obtained after switching on the measuring beam, and then the maximum fluorescence (Fm) was obtained after applying a 0.8s saturating pulse (>10000 μmol m^{-2} s^{-1}). After the fluorescence signal decayed for 20 s, the actinic light (280 μmol m^{-2} s^{-1}) was switched on for 300 s, during which the saturating pulse was applied every 20 s. The steady-state fluorescence (Fs) and maximum fluorescence under illumination (Fm') were recorded. The minimal fluorescence under illumination (Fo') was calculated according to Baker (2008). Actual quantum yield of PSII (Φ_{PSII}), photochemical quenching coefficient (qP) and antenna excitation transfer efficiency (Fv′/Fm′) were calculated as (Fm′−Fs)/Fm′, (Fm′−Fs)/(Fm′−Fo′), and (Fm′−Fo′)/Fm′, respectively.

Chlorophylls (Chl*a* and Chl*b*) were extracted in 80% acetone and analyzed spectrophotometrically as described by Yu et al. (2004).