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Research article

Comparison of a compatible and an incompatible pepper-tobamovirus interaction by biochemical and non-invasive techniques: Chlorophyll *a* fluorescence, isothermal calorimetry and FT-Raman spectroscopy

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ABSTRACT

Leaves of a pepper cultivar harboring the L^3 resistance gene were inoculated with *Obuda pepper virus* (ObPV), which led to the appearance of hypersensitive necrotic lesions approx. 72 h post-inoculation (hpi) (incompatible interaction), or with *Pepper mild mottle virus* (PMMoV) that caused no visible symptoms on the inoculated leaves (compatible interaction). ObPV inoculation of leaves resulted in ion leakage already 18 hpi, up-regulation of a pepper carotenoid cleavage dioxygenase (*CCD*) gene from 24 hpi, heat emission and declining chlorophyll *a* content from 48 hpi, and partial desiccation from 72 hpi. After the appearance of necrotic lesions a strong inhibition of photochemical energy conversion was observed, which led to photochemically inactive leaf areas 96 hpi. However, leaf tissues adjacent to these inactive areas showed elevated ΦPSII and Fv/Fm values proving the advantage of chlorophyll *a* imaging technique. PMMoV inoculation also led to a significant rise of ion leakage and heat emission, to the up-regulation of the pepper *CCD* gene as well as to decreased PSII efficiency, but these responses were much weaker than in the case of ObPV inoculation. Chlorophyll *b* and total carotenoid contents as measured by spectrophotometric methods were not significantly influenced by any virus inoculations when these pigment contents were calculated on leaf surface basis. On the other hand, near-infrared FT-Raman spectroscopy showed an increase of carotenoid content in ObPV-inoculated leaves suggesting that the two techniques detect different sets of compounds.

1. Introduction

Several viruses belonging to the genus *Tobamovirus* are major pathogens of pepper plants. In *Capsicum* species the resistance against tobamoviruses is conferred by four different alleles of a resistance gene (L^1, L^2, L^3 and L^4, numbered in order of increasing effectiveness) at the locus L of chromosome 11. In resistant pepper leaves the infection results in the formation of necrotic local lesions and virus confinement to the primary infection site (Berzal-Herranz et al., 1995; Elvira et al., 2008). Resistance conferred by the L^3 gene is very efficient against most tobamoviruses except for some closely related *Pepper mild mottle virus* (PMMoV) isolates, which are able to overcome this type of resistance and to cause systemic infection (Tóbiás et al., 1989; Velasco et al., 2002; Elvira et al., 2008). PMMoV is a positive-sense, single-stranded RNA virus with a relatively small (approx. 6400 bp) monopartite genome that encodes four proteins including two replication proteins, a movement protein, and a coat protein (Ishibashi et al., 2010). PMMoV can cause serious economic losses in both field and greenhouse-grown peppers (Berzal-Herranz et al., 1995; Beczner et al., 1997). Visible disease symptoms on PMMoV-infected leaves can be mild chlorotic spots

Abbreviations: ABS/CSm, energy absorbed by photosynthetic antennae; CCD, carotenoid cleavage dioxygenase; DIo/CSm, energy dissipated as heat; ETo/CSm, energy transfer on electron transport chain; FT, Fourier-transformation; Fv/Fm, maximal quantum yield of PSII; hpi, hours post-inoculation; HR, hypersensitive response; LOX, lipoxygenase; NPQ, non-photochemical quenching; ObPV, *Obuda pepper virus*; OEC, oxygen-evolving complex; PEA, Plant Efficiency Analyzer; PMMoV, *Pepper mild mottle virus*; PS II, photosystem II; qP, photochemical quenching coefficient; ΦPSII, efficiency of PSII; ROS, reactive oxygen species; TRo/CSm, energy trapped in reaction centers.

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but often no symptom appears. However, mottling, mosaic or curling symptoms can appear on those leaves that develop after inoculation. Infected plants can be stunted and the fruits are usually severely deformed, mottled or blotched (Beczner et al., 1997; Velasco et al., 2002; Elvira et al., 2008).

Obuda pepper virus (ObPV), which also belongs to the genus *Tobamovirus*, can not break the L^3 gene-mediated resistance, the defense mechanisms of pepper plants are effectively activated. Thus ObPV inoculation leads to the development of local necrotic lesions (hypersensitive response, HR) (Csilléry et al., 1983; Tóbiás et al., 1989). The genome organization of ObPV is similar to that of PMMoV (Padgett and Beachy, 1993). Earlier marked inductions of pathogenesis-related proteins and ethylene formation were observed in ObPV-infected pepper leaves (Tóbiás et al., 1989). ObPV inoculation brought about also a substantial induction of lipoxygenase (LOX) enzyme activity, and elevated transcription of several *LOX* genes (Gullner et al., 2010). In addition, the expression of a divinyl ether synthase gene was massively up-regulated in ObPV-inoculated pepper leaves. In contrast to ObPV, PMMoV exerted only negligible effects on these metabolite and gene expression levels (Tóbiás et al., 1989; Gullner et al., 2010).

Virus infections usually substantially damage the photosynthetic apparatus in plants. The amount of the major photosynthetic pigments chlorophyll *a* and *b* can be markedly diminished. The level of carotenoids, which also have a role in light harvesting and in protection from excess light by energy dissipation and singlet oxygen deactivation, can also be decreased by virus infections (Wilhelmová et al., 2005). The photosynthetic electron transport in photosystem II (PSII) is usually severely impaired in virus-infected leaves, which results in disturbed CO_2 fixation, reduced carbohydrate accumulation, and plant growth (Rahoutei et al., 2000; Almási et al., 2001). On the other hand, the marked accumulation of starch in the chloroplasts was also reported in virus infected susceptible leaves, which suggests that the sink-source relationships should also be considered (Técsi et al., 1994). Reactive oxygen species (ROS) can also accumulate in plant tissues leading to oxidative stress (Hakmaoui et al., 2012). The oxygen-evolving complex (OEC) of PSII was shown to be one of the main targets of PMMoV infection in thylakoid membranes of *Nicotiana benthamiana* leaves. The infected plants also showed a reduction in the efficiency of excitation capture in PSII by photoprotective thermal dissipation (Rahoutei et al., 2000). In recent years non-invasive chlorophyll fluorescence imaging techniques have been developed to continuously follow-up the physiological status and performance of plants, as well as to detect stress-induced deviations presymptomatically (Chaerle et al., 2007; Rolfe and Scholes, 2010). Modulation of photosynthetic activity can be revealed by measurements of chlorophyll *a* fluorescence emission. Chlorophyll *a* fluorescence imaging can reveal disease progress at early time points and with high contrast (Chaerle et al., 2004). PMMoV inoculations also resulted in alterations of fluorescence emission in infected leaves (Pineda et al., 2008a, b).

Heat production of leaf tissues reflects the general metabolic activity of plants, among others alterations in rate of growth, respiration and/or substrate carbon conversion efficiency (Skoczowski and Troc, 2013). The detection of foliar heat emission by isothermal calorimetry has also proved to be a valuable non-destructive tool to study the defense reactions of infected plants (Fodor et al., 2007; Skoczowski and Troc, 2013). In particular thermography is well suited to monitor infections, which often lead to changes in plant water status (Chaerle et al., 2004). However, there is no direct relationship between isothermal calorimetry and thermography. Isothermal calorimetry provides information about the global, average emission of metabolic heat whereas thermography shows local changes in leaf tissue temperature.

An other non-destructive technique, the Fourier-transformation Raman (FT-Raman) spectroscopy has also been applied to study the chemical composition and properties of plant tissues (Gierlinger and Schwanninger, 2007), including plants exposed to abiotic and biotic stress effects (Taddei et al., 2002; Skoczowski and Troc, 2013). This method was used for *in situ* analysis of primary and secondary metabolites in living plant tissues in two different ways: by point-measurements or by two dimensional Raman mapping, which characterizes the distribution of these compounds (Skoczowski and Troc, 2013). FT-Raman spectroscopy was successfully applied to detect changes of the carotenoid level in plant leaves at ambient temperature and pressure without any need for pre-processing (Baranski et al., 2005).

In the present study non-invasive detection techniques including chlorophyll *a* fluorescence, isothermal calorimetry and infrared Raman spectroscopy as well as biochemical and molecular methods were used to detect early changes in the metabolism of virus-infected pepper plants between 6 and 96 h post-inoculation (hpi). Leaves of a pepper cultivar harboring the L^3 resistance gene were infected with two different tobamoviruses (ObPV and PMMoV) in order to compare an incompatible and a compatible plant/virus interactions. ObPV inoculation of pepper leaves containing the L^3 resistance gene results in the appearance of necrotic lesions (incompatible interaction, hypersensitive reaction), while PMMoV causes only very slight chlorotic symptoms in inoculated leaves (compatible interaction) (Tóbiás et al., 1989). Non-invasive techniques proved to be appropriate tools to reveal significant differences in the early defense responses of pepper plants to ObPV and PMMoV inoculations.

2. Materials and methods

2.1. Pepper variety and virus inoculations

Seeds of the pepper (*Capsicum annuum* L.) cultivar TL 1791 harboring the L^3 resistance gene were planted into soil and grown under greenhouse conditions (18–23 °C; about 16 h daylight with 160 μmol m^{-2} s^{-1} supplemental light for 8 h per day; relative humidity: 75–80%). Pepper seeds were kindly provided by Dr. Lajos Zatykó (Research Institute of Vegetable Crops, Budatétény, Hungary). For each experiment 55–60 day old plants were used.

The whole surface of the third and fourth true leaves (3rd and 4th leaf position above hypocotyl) of plants were uniformly inoculated with a suspension of ObPV or PMMoV. The ObPV strain was isolated in Hungary (formerly used synonym: Ob strain of *Tomato mosaic virus*) (Csilléry et al., 1983; Tóbiás et al., 1989) whereas the L^3-resistance-breaking strain of PMMoV was isolated in Louisiana, USA (formerly used synonym: Samsun latent strain of *Tobacco mosaic virus*) (Greenleaf et al., 1964; Tóbiás et al., 1989). Viral inoculations were carried out with carborundum as an abrasive according to Tóbiás et al. (1989). In all experiments mock-inoculated leaves (inoculation with carborundum and buffer but without any virus to test the effect of the slight mechanical injury caused during virus inoculation) were used as controls. Virus-inoculated, and mock-inoculated control plants were kept at 25 °C in a growth chamber with 16/8 h light/dark cycles.

For all analyses samples were taken from the virus-infected third and fourth true leaves of plants together with samples taken from corresponding mock-inoculated leaves. At each measurement, except photometric chlorophyll and carotenoid determinations, entire leaves were sampled with or without symptoms depending on the virus and the time of inoculation. For comparison, leaves of untreated plants were also analyzed.