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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.

2.2. Conductivity measurements

Membrane damage was detected by measuring ion leakage from inoculated and control whole pepper leaves with a conductivity meter (Radelkis OK-102/10, Budapest, Hungary) as described by Barna et al. (1993). Leaves were cut at their petioles from ObPV-, PMMoV-, mock-inoculated and untreated plants and floated on 10 ml of distilled water in Petri dishes. Ion leakage was measured at different time points after inoculations.

2.3. Contents of chlorophyll a, b and total carotenoids

At various time periods after inoculation leaf discs (diameter 9 mm) were cut randomly from ObPV-, PMMoV- and mock-inoculated and from untreated leaves in order to represent the whole leaf area. After measuring their weights, leaf tissues were homogenized in ice-cold 80% (v/v) acetone and the chlorophyll *a*, *b* and total carotenoid contents were determined spectrophotometrically as described by Lichtenthaler (1987). Pigment contents of leaves were calculated on both fresh weight and leaf surface basis.

2.4. Measurements of chlorophyll a fast fluorescence kinetics

The energy flow in PSII was characterized by measuring the parameters of chlorophyll *a* fast fluorescence with a Plant Efficiency Analyzer (PEA; Hansatech Ltd. King's Lynn, Norfolk, England) as described earlier (Skoczowski et al., 2011). Shortly, fluorescence parameters of pepper leaves were measured after 30 min of adaptation to darkness (clips with a 4 mm diameter hole) at 20 °C. Changes in fast fluorescence were registered during illumination within a period of 10 μs to 1 s. During the initial 2 ms data were collected every 10 μs. After this period, the frequency of measurements was reduced automatically. Based on the obtained data the following parameters of energy flow efficiency were calculated: energy absorbed by photosynthetic antennae (ABS/CSm), energy trapped in reaction centers (TRo/CSm), energy used for electron transport (ETo/CSm), the energy dissipated as heat (DIo/CSm) and the maximal quantum yield of PSII (Fv/Fm). CS means the sample cross section. Measurements on virus-inoculated, mock-inoculated and untreated control leaves were performed in 10 replicates.

2.5. Chlorophyll a fluorescence imaging

Chlorophyll *a* fluorescence images were captured with a FluorCAM imaging system (PSI, Brno, Czech Republic). By this technique the changes in four different photosynthetic parameters could be detected: maximal PSII quantum yield (Fv/Fm), efficiency of PSII at light adapted state (ΦPSII), photochemical quenching (qP), and nonphotochemical quenching (NPQ). Before each measurement plants were dark adapted for 20 min inside the measuring chamber. Dark-level fluorescence yield (F_o) was recorded at a very low light intensity (less than 1 μmol m^{-2} s^{-1}) and then the maximum fluorescence yield (F_m) was detected at a saturating light-flash of about 2500 μmol m^{-2} s^{-1}. The saturation pulls lasted 1 s. Afterward plants were light-adapted under red-orange LED actinic light (180 μmol m^{-2} s^{-1}). Actinic light intensity was chosen on the basis of previous measurements as the highest light level that was almost completely used in photochemical reactions (qP close to 1). After 10 min of light adaptation the current fluorescent yield (Ft) and the maximum light-adapted fluorescence ($F^{m\prime}$) were measured (with a saturating flash as before). In these measurements Ft = Fs. Photochemical quenching coefficient (qP) and non-photochemical quenching (NPQ) were calculated according to Schreiber et al. (1994) and Bilger and Björkman (1991), respectively. All parameters were analyzed 6, 48 and 96 hpi in at least three replicates. The effects of viral inoculations were compared to mock-inoculated plants at each time point.

2.6. Heat emission measurements

Measurements of specific thermal energy were performed by using virus-inoculated, mock-inoculated and untreated control pepper leaves, which were detached at different time periods following inoculations. All measurements were performed on the fourth leaf. The thermal energy emitted by leaves were measured 48 and 96 hpi, by using an isothermal calorimeter TAM III (Thermometric 3101 TA Instruments) in 5 repetitions at 20 °C, as described previously (Stoklosa et al., 2006). Briefly, detached leaves were placed into measuring ampoules containing 2 ml of distilled water. The same amount of water without plant material was used in a reference ampoule. Tightly sealed ampoules were allowed to equilibrate and then the metabolic energy emitted by the leaf was continuously measured for 10 min. After completion of the measurement leaves were lyophilized and weighed in order to calculate the specific thermal energy on dry weight basis.

2.7. Fourier transformation Raman spectroscopy

Near-infrared Fourier transformation Raman measurements were performed directly on detached leaves of virus- or mock-inoculated as well as of untreated control pepper plants. FT-Raman spectra were recorded using a Nicolet NXR 9650 FT-Raman spectrometer equipped with an Nd:YAG laser emitting at 1064 nm and a germanium detector cooled with liquid nitrogen. The spectrometer was provided with an *xy* stage, a mirror objective and a prism slide for the redirection of the laser beam. The measurements were performed with a spectral resolution of 8 cm^{-1} in the range of 150–3700 cm^{-1}. All spectra were accumulated from 256 scans, measured with a laser power of 300 mW using an unfocused laser beam of a diameter of approx. 100 μm. Raman spectra were recorded by the Omnic/Thermo Scientific software program.

2.8. RNA extraction and gene expression analysis by RT-PCR

Total RNA was extracted from 0.1 g virus-inoculated, mock-inoculated or untreated pepper leaf material ground under liquid nitrogen with a Total RNA Miniprep kit (Viogene, Sunnyvale, CA, USA). Reverse transcription (RT) of 2.5 μg total RNA was carried out with a RevertAid H Minus First Strand cDNA Synthesis kit (MBI Fermentas, Vilnius, Lithuania) using an oligo(dT) primer. Semi-quantitative PCR for assaying a pepper carotenoid dioxygenase gene (GenBank accession Y14164) expression was conducted with a PTC 200 DNA Engine extended with an ALS-1296 sample holder (Bio-Rad, Hercules, CA, USA). The PCR reaction mixtures contained 10 pmol of each primer, 0.5 U of Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania), 0.8 mM of each dNTP, 2 mM $MgCl_2$ and 2 μl of template cDNA in a total volume of 25 μl. Expression of a pepper actin gene (GenBank accession AY572427) served as a constitutive control. Gene-specific oligonucleotide primer pairs were designed as follows: carotenoid dioxygenase forward primer: 5′-GCCGCCTTATGAATCCAGACCTA-3′, reverse primer: 5′-GGATATGGCATATCGGGGTGTATAG-3′ (product length: 611 bp); actin forward primer: 5′-AGCAACTGGGACGATATGGAGAAGA-3′, reverse primer: 5′- AAGAGACAACACCGCCTGAATAGCA-3′ (product length: 198 bp). The amplification started with 2 min denaturation at 94 °C, followed by 25 cycles of 30 s at 94 °C, 45 s at 55 °C (annealing), 45 s at 72 °C and terminated by 10 min extension at 72 °C. The amplified PCR products were separated by gel electrophoresis in 1% agarose gels and visualized by GelRed nucleic acid gel stain (Biotium Inc., Hayward, CA, USA).

2.9. Data analysis

Data presented are means of three or more independent parallel experiments. Analysis of the results was performed on the basis of Statistica 9.0 for Windows (StatSoft, Inc., Tulsa, OK, USA). Data were statistically analyzed using an analysis of variance test (ANOVA) and significant differences ($p \leq 0.05$) were calculated by using the Tukey Test.

3. Results

3.1. Disease symptoms and membrane damage

There were no visible symptoms on pepper leaves until 3 dpi following inoculation with ObPV or PMMoV. Infection of pepper leaves with ObPV resulted in the appearance of visible necrotic lesions only 72 hpi in accordance with earlier studies (Csilléry et al., 1983; Tóbiás et al., 1989). The necrotic spots slightly increased until 96 hpi (Fig. 4 ii). However, the membrane permeability of inoculated leaves increased much earlier (18 hpi) indicating the breakdown of cell membranes during this hypersensitive response. The rate of ion leakage caused by ObPV markedly increased until 96 h post-inoculation (Fig. 1), which correlates with the strength of gradual membrane damage in the detached leaves. PMMoV inoculation caused no visible symptoms on infected leaves until 96 hpi (Fig. 4 iii) as found earlier (Tóbiás et al., 1989). Although PMMoV inoculation caused no symptoms, interestingly it led to a gradual and significant increase of ion leakage. However, this effect was substantially weaker than that caused by ObPV. Mock-inoculations did not cause any significant membrane damage as compared to untreated control leaves (Fig. 1).

3.2. Changes of chlorophyll a, b and total carotenoids contents after virus inoculations

ObPV inoculation led to a marked decrease of chlorophyll *a* content in inoculated pepper leaves as compared to mock-inoculated leaves. This decline of chlorophyll *a* content became significant from 48 hpi (Fig. 2A and D). When chlorophyll *b* and total carotenoid contents were calculated on fresh weight basis in ObPV-inoculated leaves 72 hpi, their levels significantly increased (Fig. 2B and C, respectively). However, when these pigment contents were calculated on leaf surface basis, no significant differences were found between mock- and ObPV-inoculated leaves (Fig. 2E and F, respectively). Therefore we calculated the leaf fresh weight/leaf disc surface ratios for each sample. Significant changes were found in the case of leaf discs cut from the ObPV-inoculated leaves, which lost 28% and 17% of their weight 72 and 96 hpi, respectively, as compared to mock-inoculated control leaf discs. PMMoV inoculations did not influence significantly the above ratio at any time points (data not shown). These results clearly showed that the apparent increases in chlorophyll *b* and total carotenoid contents were caused by the marked weight loss of leaves as a consequence of ObPV inoculation. The hypersensitive reaction elicited by ObPV resulted in elevated leaf transpiration and decreased water content of leaf tissues, which finally led to decreased leaf fresh weights. On the other hand, PMMoV inoculation did not change significantly neither the leaf water content nor the pigment content of pepper leaves (Fig. 2A–F).

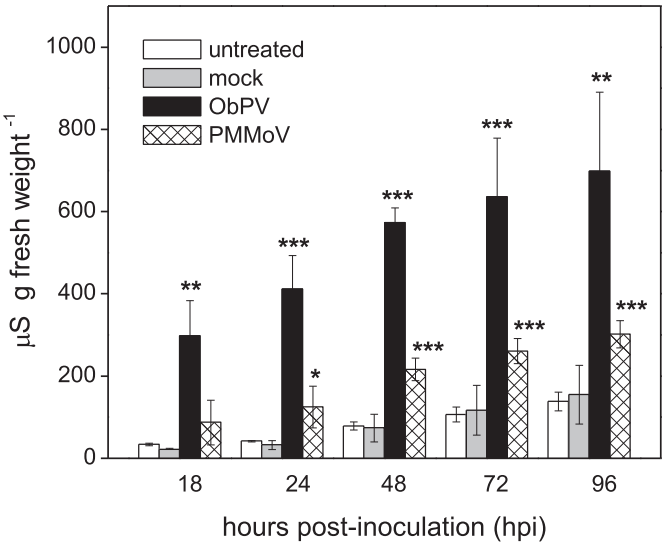

Fig. 1. Ion leakage from pepper leaves following inoculation with *Obuda pepper virus* (ObPV) or *Pepper mild mottle virus* (PMMoV) as measured with a conductometer. Mock-inoculated and untreated leaves were used as controls. Mean values of 4 independent parallel experiments ± SD are shown. The symbols *, ** and *** show significant differences between untreated and inoculated plants at $P = 5\%$, 1% and 0.1%, respectively.

3.3. Alterations in the effectiveness of energy flow in PS II after virus inoculations

Both virus inoculations significantly reduced the efficiency of PSII in comparison to mock-inoculated leaves, but ObPV caused substantially stronger disturbances of energy flow in pepper leaves than PMMoV as detected by PEA measurements (Fig. 3). In ObPV-inoculated leaves a significant reduction of energy flow was observed already 72 hpi: the energy absorption by antennae (ABS/CSm), the energy trapped in reaction centers (TRo/CSm) and the energy transfer on electron transport chain (ETo/CSm) decreased by 22, 30 and 42% in comparison to mock-inoculated leaves, respectively (Fig. 3). Four days after ObPV inoculations the parameters TRo/CSm and ETo/CSm dramatically dropped to near-zero values, while the ratio of maximum quantum yield (Fv/Fm) decreased to 33% of control. The amount of energy was dissipated as heat (DIo/CSm) elevated only 96 hpi in ObPV-inoculated leaves. Interestingly, PMMoV-inoculated plants showed also significantly diminished values of ABS/CSm, TRo/CSm, ETo/CSm and Fv/Fm as compared to mock-inoculated leaves 96 hpi, while the parameter DIo/CSm did not change. A spider-graph summarizing the values of all five photosynthetic parameters determined in mock-, ObPV- and PMMoV-inoculated leaves 96 hpi clearly shows the markedly stronger damaging effect of ObPV in comparison to PMMoV (Suppl. Fig. 1).

3.4. Effects of virus infections on photosynthetic processes in pepper leaves

To get a deeper insight into the temporal and spatial patterns of alterations in the efficiency of the photosynthetic apparatus as a consequence of viral inoculation the photosynthetic performance was evaluated also by chlorophyll *a* fluorescence imaging. This technique enabled us to analyze the entire surface of virus- and mock-inoculated leaves. The changes in three parameters, the maximal PSII quantum yield (Fv/Fm), the efficiency of PSII at light adapted state (ΦPSII), and the nonphotochemical quenching (NPQ) were detected 6, 48 and 96 hpi (Figs. 4–6). The values of photochemical quenching (qP) did not change significantly in any virus- or mock-inoculated leaves (data not shown).

Chlorophyll *a* fluorescence imaging revealed dynamic disease progress already at early time points (6-96 hpi) following both viral inoculations. The presence of visible lesions on the ObPV-

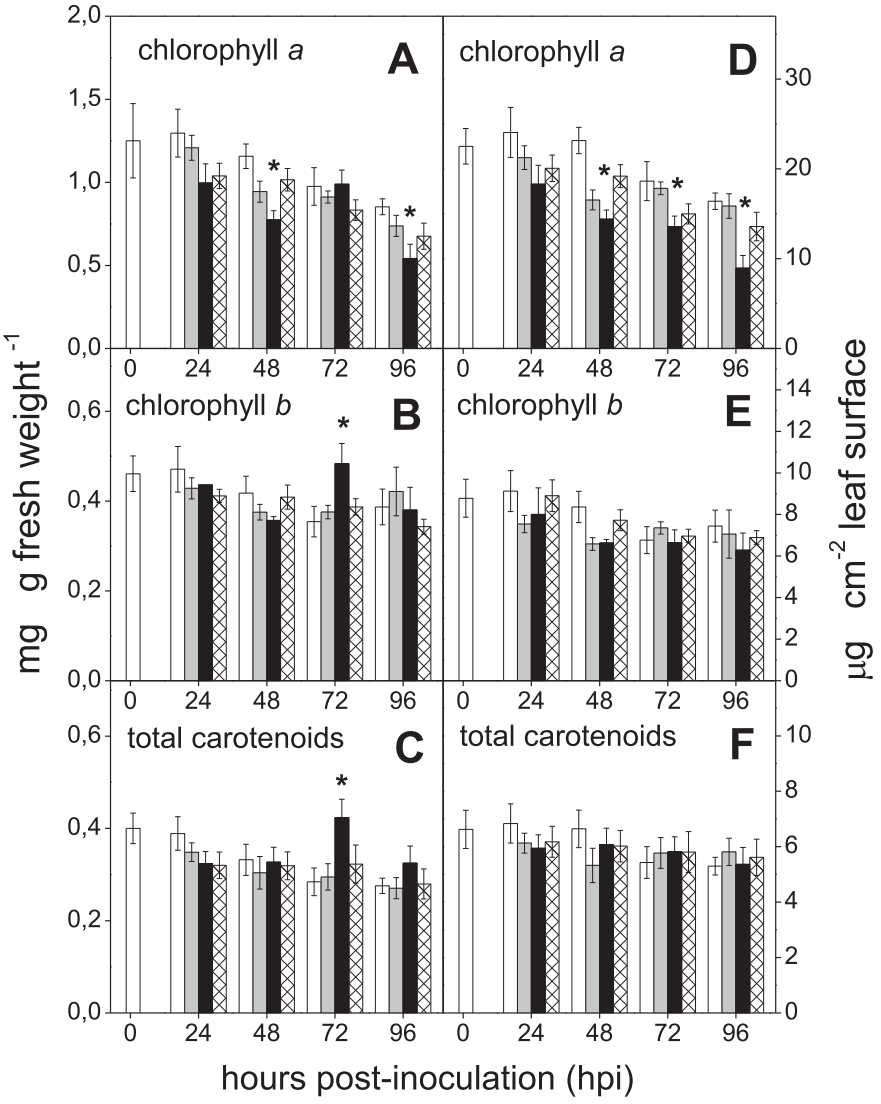

Fig. 2. Changes in the total contents of chlorophyll *a*, *b* and carotenoids in pepper leaves following inoculation with *Obuda pepper virus* (ObPV) or *Pepper mild mottle virus* (PMMoV). Mock-inoculated leaves were used as controls. Mean values of 5 independent parallel experiments ± SD are shown. Empty, gray, black and hatch-crossed patterned columns represent untreated, mock-, ObPV- and PMMoV-inoculated leaves, respectively. The symbol * shows significant differences between mock- and ObPV-inoculated plants at $P = 5\%$. Pigment contents were calculated on fresh weight basis (left panels A, B, C) as well as on leaf surface basis (right panels D, E, F).

inoculated leaves at 96 hpi correlated with the marked changes of Fv/Fm, ΦPSII and NPQ values. ObPV inoculation of pepper leaves led to elevated Fv/Fm and ΦPSII levels already before the appearance of visible symptoms (48 hpi) in comparison to mock controls (Figs. 4E and 5E, respectively). The veinal areas of leaves showed higher efficiency than interveinal mesophyll parts. Following the development of visible necrotic lesions in ObPV-inoculated leaves (96 hpi), the Fv/Fm, ΦPSII and NPQ values drastically decreased on extended leaf areas, mainly in the distal (older) part of leaves. However, adjacent to these inactive spots, the infected leaves showed markedly elevated Fv/Fm, ΦPSII and NPQ levels, particularly at the proximal (younger) part of leaves (Figs. 4F, 5F and 6F, respectively).

PMMoV inoculations resulted in decreased Fv/Fm and ΦPSII levels 96 hpi as compared to mock control (Figs. 4I and 5I, respectively). The ΦPSII level slightly increased in PMMoV-inoculated leaves already 6 hpi as compared to mock control, but later gradually declined to control levels (Fig. 5G–I). PMMoV inoculation resulted in the substantial decrease of NPQ level already 6 hpi (Fig. 6G). With the progress of the disease, the NPQ levels remained lower in PMMoV-inoculated leaves than in mock control leaves until 96 hpi (Fig. 6G–I).

3.5. Heat emission of inoculated leaves following virus inoculations

The specific heat energy emitted by detached pepper leaves was measured after two different incubation periods (48 and 96 hpi). ObPV inoculation led to a substantial, approx. 2-fold increase of heat emission in infected pepper leaves 48 hpi as related to mock-inoculated leaves. In contrast, PMMoV inoculation did not alter the amount of emitted heat 48 hpi (Fig. 7). The heat emission remained elevated also at 96 hpi in ObPV-inoculated leaves, although at this time point it was only 42% higher than in mock-inoculated leaves. However, the specific heat energy production was significantly elevated also by PMMoV inoculation 96 hpi (Fig. 7).

3.6. Leaf metabolites detected by near-infrared Raman spectroscopy

By using Raman spectroscopy spectral information from a leaf surface can be obtained in a non-invasive manner. The Raman

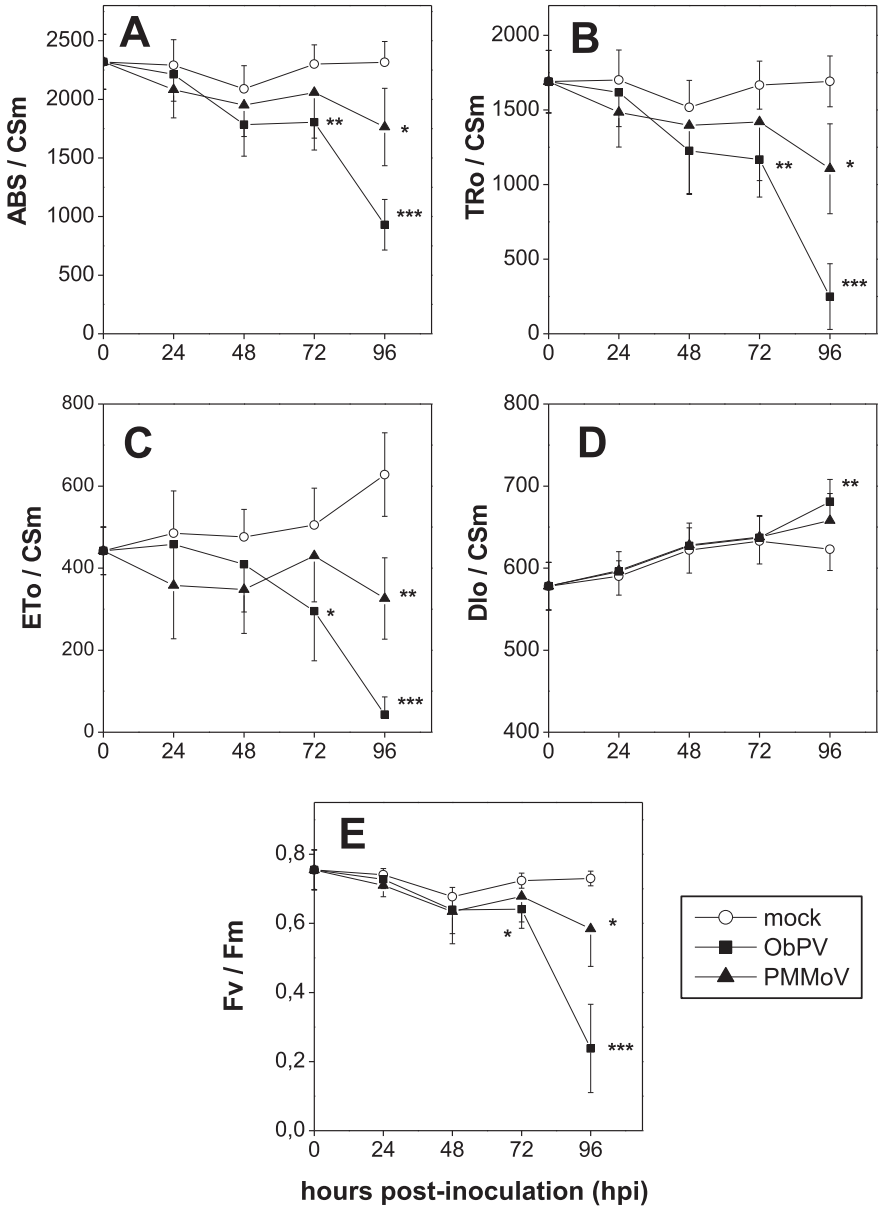

Fig. 3. Changes in the energy flow through the PSII in pepper leaves following inoculation with *Obuda pepper virus* (ObPV) or *Pepper mild mottle virus* (PMMoV) as detected by Plant Efficiency Analyzer (PEA). Abbreviations of PSII parameters: ABS/CSm: absorption of energy by antennae; TRo/CSm: energy transmitted to reaction centers; ETo/CSm: energy transferred to the electron transport chain; DLo/CSm: energy lost as heat; Fv/Fm: maximum quantum yield. Mean values of 10 independent parallel experiments ± SD are shown. The symbols *, ** and *** show significant differences between mock- and virus-inoculated plants at $P = 5\%$, 1% and 0.1%, respectively.

spectra obtained from pepper leaves clearly showed three characteristic bands of carotenoids at 1005, 1156 and 1525 cm^{-1} (Fig. 8). A distinctive characteristic of carotenoid structure is the long central chain with a conjugated double bond system. This system is a light absorbing chromophore responsible for the color of this compounds. The first, most intense C═C stretching vibration of β-carotene is observed at 1525 cm^{-1}. The second, medium intensity band at 1156 cm^{-1} is assigned to C–C stretching vibration. The third, low-intensity band at 1005 cm^{-1} came from the CH_3 groups attached to the polyene chain with C–C bonds (Fig. 8). Some bands of low intensity at 1602, 1325 and 1287 cm^{-1} can be assigned to chlorophyll. Additional bands observed at 1606 and 1662 cm^{-1} arose from the aromatic ring stretch - doublet and connected to the ring breathing at 1189 and 1216 cm^{-1}. Further bands, which appeared at 1463 and 1380 cm^{-1} came from deformation vibrations of CH, CH_2 and CH_3 groups and stretching C–C vibrations of aliphatic carbohydrates, respectively.

Intensities of Raman bands originating from various plant metabolites correlate with the concentration of the individual metabolites. The differences between the spectra recorded in ObPV-, PMMoV- and mock-inoculated leaves indicated a marked increase in the foliar content of carotenoids in ObPV-inoculated leaves 48 and 96 hpi (Fig. 8). In comparison, the carotenoid content was not significantly modified by PMMoV inoculation (Fig. 8).

3.7. Up-regulation of a carotenoid dioxygenase gene in virus-inoculated leaves

To gain more information about the metabolism of carotenoids in virus-inoculated pepper leaves we examined the expression

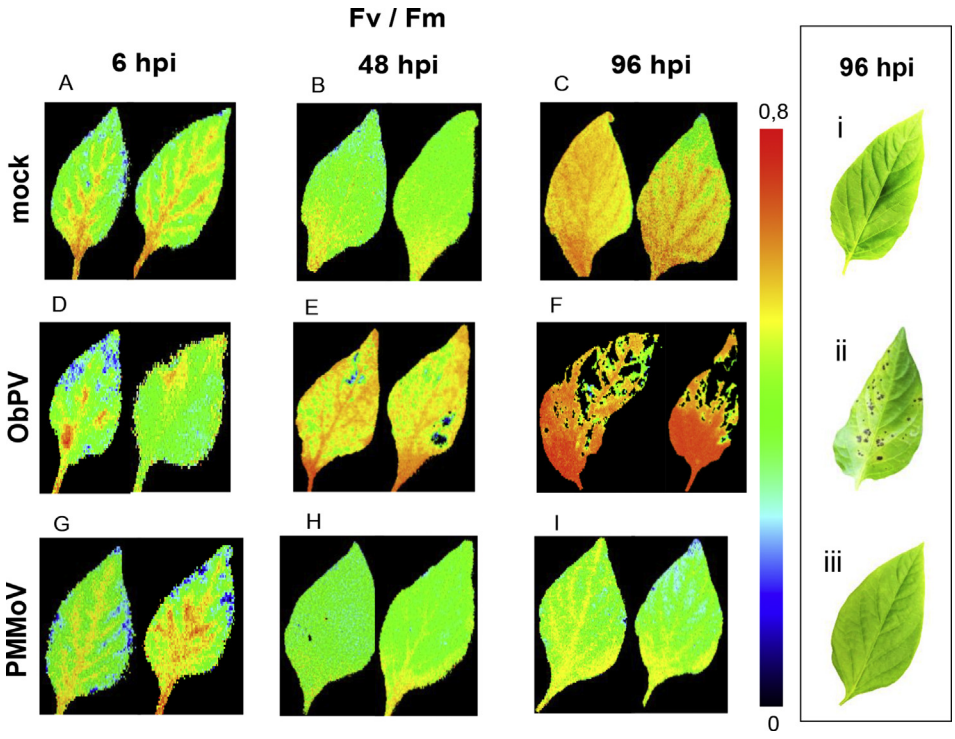

Fig. 4. Imaging of maximal PSII quantum yield (Fv/Fm) in *Obuda pepper virus* (ObPV) and *Pepper mild mottle virus* (PMMoV) inoculated pepper leaves 6, 48 and 96 h post inoculation (hpi). The differences in relative chlorophyll *a* fluorescence yield of different leaf parts are indicated by computer-generated colors with red for high and blue for low fluorescence. Leaves within the right-side frame represent visible symptoms on mock- (i), ObPV- (ii) and PMMoV-inoculated (iii) leaves 96 hpi. Presented images are representative for 3 independent experiments.

level of a carotenoid cleavage dioxygenase (*CCD*) gene (GenBank accession number Y14164; Bouvier et al., 2003), which encodes an enzyme that catalyzes the decomposition of carotenoids. The expression of this *CCD* gene was investigated in ObPV-, PMMoV- and mock-inoculated pepper leaves by semi-quantitative RT-PCR with a gene-specific primer pair. ObPV inoculation led to a marked up-regulation of the pepper *CCD* gene already 24 hpi and the rate of expression gradually increased further until 48 hpi (Fig. 9). In contrast, PMMoV inoculation resulted in a weaker and transient up-regulation, which peaked at 16 hpi, and then the transcript abundance returned to the control level (Fig. 9). Mock-inoculation also resulted in a slight transient induction of gene expression, while no changes were found in untreated control leaves (data not shown). Virus and mock-inoculations did not change significantly the expression of a control, housekeeping actin gene (Fig. 9).

4. Discussion

Few information is available about the defense reactions of pepper plants harboring the L^3 resistance gene following ObPV inoculation. This incompatible interaction leads to the development of local necrotic lesions on inoculated leaves. By largely unknown mechanisms the virus multiplication is restricted to the infected leaves, the virus can not spread systemically into other plant parts. The infected leaves usually fall off the plants approx. 6–7 days following inoculation. To obtain more information about the early biochemical defense reactions of host plants following ObPV inoculations (6–96 hpi), we used biochemical as well as various non-invasive physico-chemical methods. We focused on early metabolic changes because the alterations observed within this short time range determine the outcome of plant/virus interactions (resistance or susceptibility). In order to compare host responses in an incompatible and a compatible interaction, the consequences of PMMoV inoculation in the same pepper cultivar harboring the L^3 resistance gene (compatible interaction) were investigated as well.

ObPV inoculation resulted in the rapid increase of ion leakage as quickly as 18 hpi, well before the appearance of visible symptoms 72 hpi (Fig. 1). This early membrane damage is usually the consequence of enzymatic and non-enzymatic lipid peroxidation. The later process is caused by oxidative stress, *i.e.* the accumulation of ROS, which was observed in both compatible and incompatible virus–plant interactions (Hernández et al., 2006; Hakmaoui et al., 2012). The rate of membrane deterioration progressively rose until 96 hpi in ObPV-inoculated leaves. Interestingly, a significant membrane damage was observed also in symptomless PMMoV-inoculated pepper leaves, although much weaker than that caused by ObPV (Fig. 1).

Subsequently to the appearance of membrane damage, markedly declining chlorophyll *a* contents were observed in the ObPV-inoculated leaves from 48 hpi (Fig. 2D). In contrast, the chlorophyll *b* content did not change significantly following ObPV or PMMoV inoculations (Fig. 2D). Interestingly, chlorophyll *a* was found to be more sensitive than chlorophyll *b* also to damage caused by light-induced ROS production in tobacco leaves (Barna et al., 2012).

Both ObPV and PMMoV inoculations markedly influenced the efficiency of photosynthetic apparatus in the infected pepper leaves. We used two different techniques to detect these changes: PEA and chlorophyll *a* imaging. ObPV inoculation led to a gradual reduction of PSII efficiency from 72 hpi as demonstrated by PEA measurements (Fig. 3). Between 72 and 96 hpi the energy absorption by antennae, the energy trapped in reaction centers, the energy transfer on electron transport chain and maximal quantum yield of PSII strongly decreased (Fig. 3A, B, C and E, respectively). Earlier, several studies reported already the suppression of

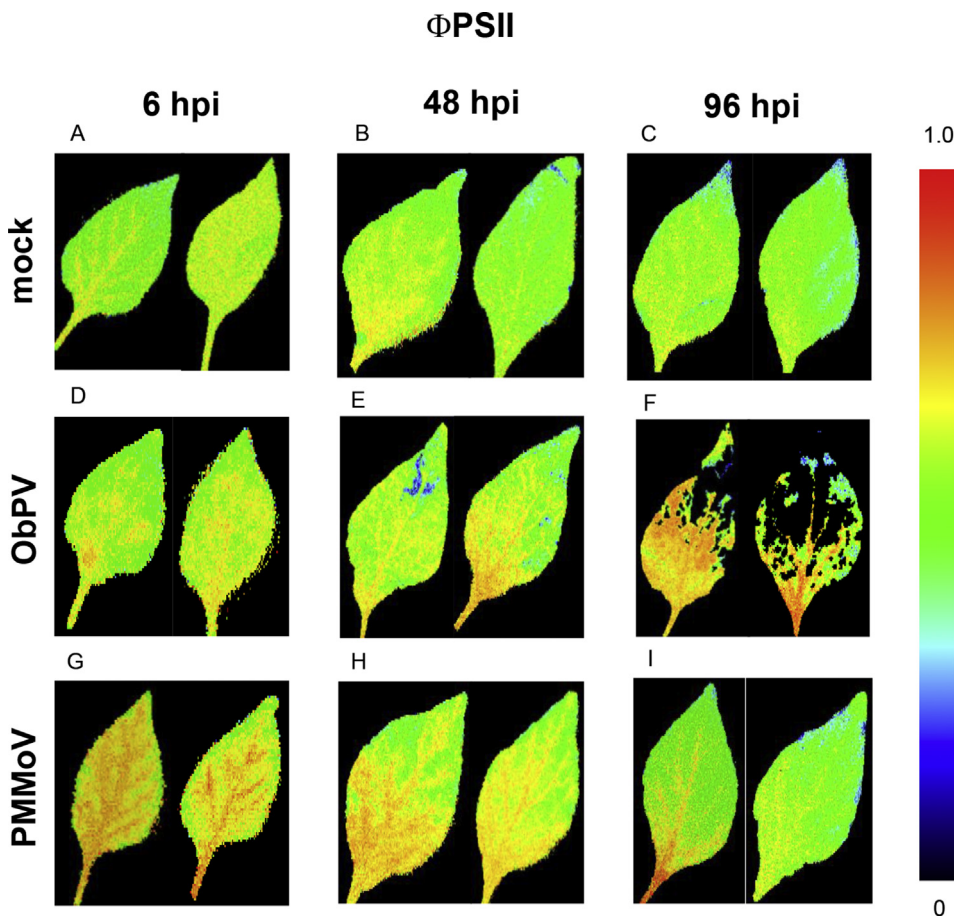

Fig. 5. Imaging of PSII efficiency (ΦPSII) in *Obuda pepper virus* (ObPV) and *Pepper mild mottle virus* (PMMoV) inoculated pepper leaves 6, 48 and 96 h post inoculation (hpi). The differences in relative chlorophyll *a* fluorescence yield of different leaf parts are indicated by computer-generated colors with red for high and blue for low fluorescence. Presented images are representative for 3 independent experiments.

photosynthesis in different plant/virus interactions. Lowered efficiency of energy flow was caused by structural/functional disturbances in membrane or directly by diminished chlorophyll content (Almási et al., 2001; Chaerle et al., 2007). In our experiments, chlorophyll *a* imaging results confirmed the substantial decrease of PSII efficiency in ObPV-inoculated leaves detected by PEA analysis. Four days after ObPV inoculation large photochemically inactive areas were revealed by this imaging technique in the inoculated leaves. These inactive areas appeared mainly on the older, upper part of leaves (Figs. 4F, 5F and 6F). On the other hand, on the younger, basal part of leaves elevated Fv/Fm and ΦPSII values were detected (Figs. 4F and 5F, respectively). The changes of photosynthetic parameters detected by PEA or chlorophyll *a* fluorescence imaging did not closely correlate, because PEA measurements were taken at randomly selected points on the leaf surfaces, while the imaging technique was able the provide spatial pattern information about the entire leaf surface thereby revealing areas with strongly different fluorescence levels. Thus, chlorophyll *a* fluorescence imaging proved to be more suitable for the characterization of changes in photosynthetic parameters in the virus-infected leaves than PEA.

Interestingly, PMMoV inoculation changed almost all parameters of PSII efficiency as well (Fig. 3), but later and to a much lesser extent than ObPV. Earlier, Rahoutei et al. (2000) already observed the attenuated photosynthetic electron transport in PSII in PMMoV-inoculated *Nicotiana benthamiana* leaves. Viral infection affected the polypeptide pattern of the OEC in thylakoid membranes. The levels of a 24 and a 16 kDa proteins were markedly reduced as compared to control. The loss of OEC extrinsic proteins affected the oxygen evolution rates of thylakoid membranes (Rahoutei et al., 2000). The non-photochemical quenching (NPQ) of excess energy in PSII was suggested to be the most adequate chlorophyll fluorescence parameter to assess the effect of PMMoV infection in *N. benthamiana*. A correlation was observed between the increase in the local NPQ values and the areas invaded by the pathogen (Pérez-Bueno et al., 2006). In our studies, very early changes of photosynthetic parameters were detected by chlorophyll *a* imaging. Already 6 hpi increased ΦPSII as well as diminished NPQ levels were detected in PMMoV-inoculated pepper leaves (Figs. 5G and 6G, respectively). The decrease in NPQ values in the front of virus invasion detected by Pérez-Bueno et al. (2006) might correlate with the early decrease of NPQ values in our experiments. The movement of PMMoV through the vascular system and its subsequent spread into the leaves was also detectable by chlorophyll *a* fluorescence imaging (Pineda et al., 2011). The altered photochemical energy conversion by both ObPV and PMMoV would suggest that the xanthophyll cycle is involved in antiviral response of pepper.

In our experiments, the NPQ levels strongly decreased in ObPV-inoculated leaves 96 hpi (Fig. 6F), whereas PMMoV inoculation only slightly diminished this parameter (Fig. 6I). It is noteworthy that NPQ-inactive areas appeared mainly on the older, upper part of

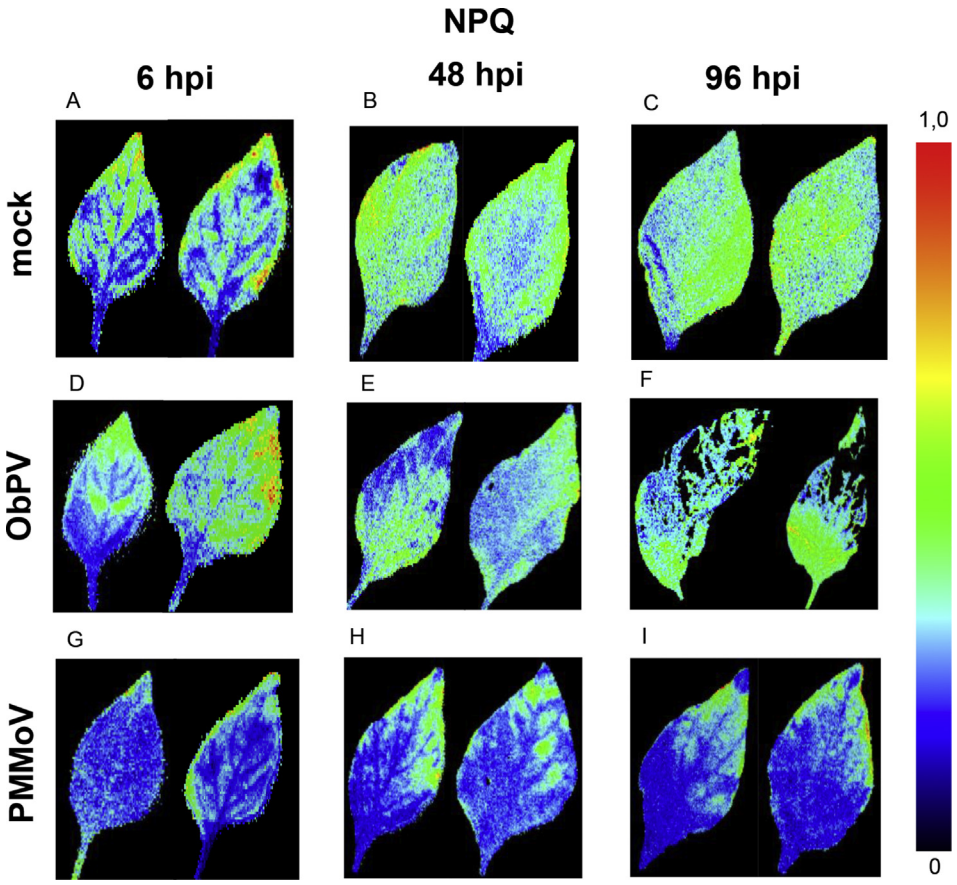

Fig. 6. Imaging of non-photochemical quenching (NPQ) in *Obuda pepper virus* (ObPV) and *Pepper mild mottle virus* (PMMoV) inoculated pepper leaves 6, 48 and 96 h post inoculation (hpi). The differences in relative chlorophyll *a* fluorescence yield of different leaf parts are indicated by computer-generated colors with red for high and blue for low fluorescence. Presented images are representative for 3 independent experiments.

ObPV-inoculated leaves (Fig. 6F). On the other hand, slightly elevated NPQ levels were detected on the younger, basal part of leaves in comparison to those observed at earlier time points (Fig. 6F).

By microcalorimetry, the increased heat emission of ObPV-inoculated leaves was demonstrated already 48 hpi and the effect was sustained until 96 hpi (Fig. 7). The elevated heat production reflects a high level of general metabolic activity of ObPV-inoculated leaves showing HR. Lipid peroxidation and heat production have been already shown to accompany the hypersensitive response in TMV-inoculated resistant tobacco leaves. Probably the increased oxygen uptake and the accumulation of ROS significantly contributed to the increased heat production (Chaerle et al., 2006; Fodor et al., 2007). The leaf surface temperature is known to be controlled by transpiration in infected plants (Chaerle et al., 2004, 2006). An increase in leaf temperature, initiating from the tissue

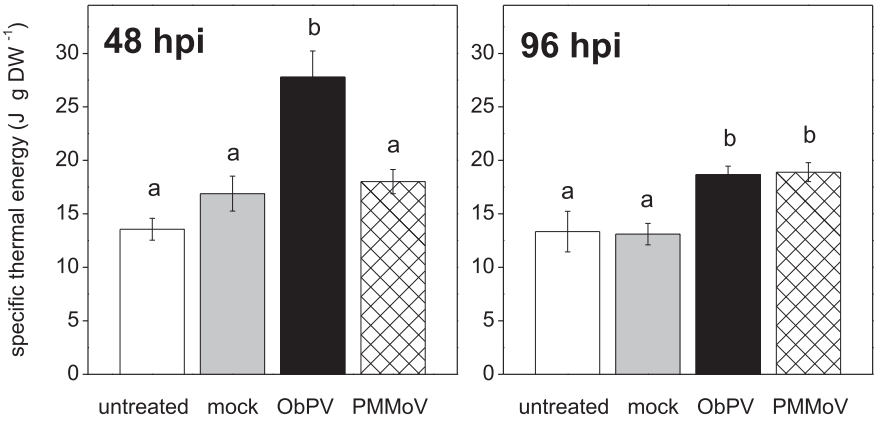

Fig. 7. Amount of the specific heat energy [Jg DW^{-1}] emitted by *Obuda pepper virus* (ObPV), *Pepper mild mottle virus* (PMMoV), and mock-inoculated pepper leaves 48 and 96 h post-inoculation (hpi). The mean values of five independent parallel measurements ± SD are shown. Columns with different letters are significantly different according to Tukey's multiple range test ($p \leq 0.05$).

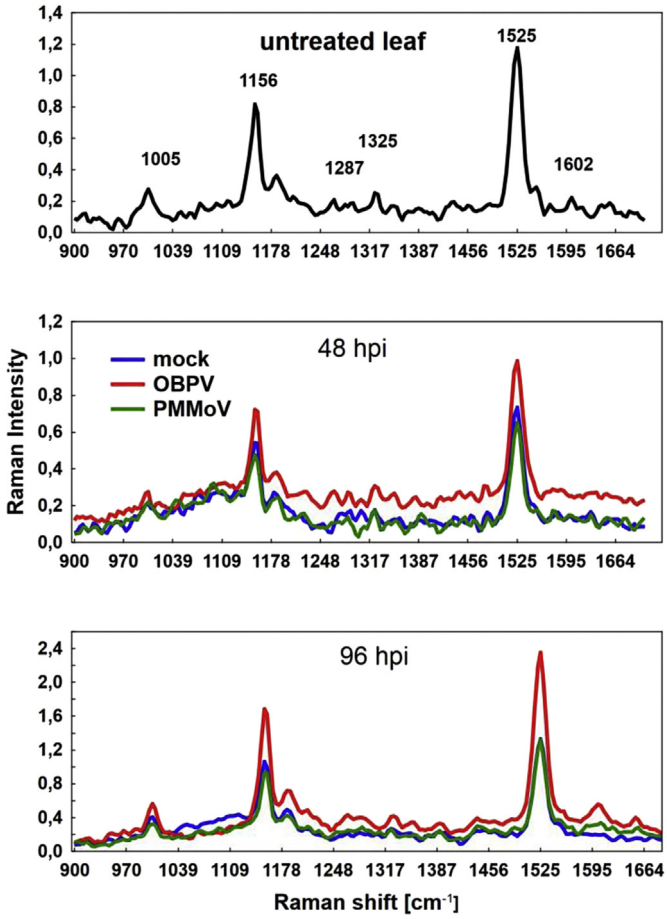

Fig. 8. Carotenoid range of Fourier-transformation Raman spectra of untreated pepper leaves (top panel) as well as *Obuda pepper virus* (ObPV), *Pepper mild mottle virus* (PMMoV), and mock-inoculated pepper leaves 48 and 96 h post inoculation (hpi) (middle and bottom panels, respectively). Representative results of three independent experiments are shown.

adjacent to the main veins, was observed also in *Nicotiana benthamiana* leaves after inoculation with different PMMoV strains. The temperature increase associated with the veins was shown to be related to stomatal closure. This temperature increase indicated a systemic plant response to infection, involving the control of water loss (Chaerle et al., 2006).

Raman spectroscopy was already used earlier to characterize root exudates from two gladiolus cultivars with different degrees of resistance to *Fusarium oxysporum* f. sp *gladioli*. Resistant plants showed an ability to inhibit conidial germination of this pathogen mainly due to the presence of a high amount of aromatic-phenolic compounds (Taddei et al., 2002). In our experiments, near-infrared FT Raman spectra of pepper leaves showed three characteristic bands of carotenoids at 1005, 1156 and 1525 cm^{-1}. The band with a maximum at 1525 cm^{-1} clearly indicated the presence of carotenoids, which have 9 conjugated double bonds in their polyene chain and this signal can be assigned to the total carotenoids of plant tissues.

Raman spectra showed that ObPV inoculation led to a significant rise in the carotenoid content of inoculated pepper leaves (Fig. 8). In accordance, increased chlorophyll *b* and total carotenoid contents were detected in ObPV-inoculated leaves 72 hpi by spectrophotometric measurements as well, when calculated on fresh weight basis. However, when these pigment contents were calculated on leaf surface basis, no significant differences were found between mock- and ObPV-inoculated leaves. This apparent increase of carotenoid content 48 and 96 hpi was probably due to the significant water loss in the infected leaves as a consequence of hypersensitive reaction elicited by ObPV. This partial desiccation of leaves is linked to plant membrane damage caused by ObPV (Fig. 1). The contradiction between the carotenoid levels measured by Raman spectroscopy and by the traditional spectrophotometric method can be explained by several factors. The water loss or the alteration in surface membrane structures can markedly influence the intensity of Raman bands. In addition, Raman spectrometry may detect a different set of compounds within the large and structurally divergent group of carotenoids in comparison to the spectrophotometric method.

Although no significant change of total carotenoid content was observed in pepper leaves following ObPV inoculation by spectrophotometric analysis (Fig. 2F), the concentration of some minor individual carotenoids might still be substantially altered. Therefore we examined the transcription of a pepper carotenoid cleavage dioxygenase (*CCD*) gene, which catalyzes the oxidative degradation of carotenoids to apocarotenoids (Bouvier et al., 2003; Simkin et al., 2003). In *Arabidopsis thaliana* the CCD enzyme family consists of nine members (Schwartz et al., 2001; Auldridge et al., 2006). In pepper, only one *CCD* gene has been studied in detail (GenBank accession Y14164; Bouvier et al., 2003). When compared to all *Arabidopsis CCD* genes, this pepper *CCD* gene showed the highest homology to *AtCCD1* (GenBank accession NM_116217). The AtCCD1 enzyme catalyzes the symmetrical cleavage of the 9,10 (9′,10′) double bounds of various carotenoids to produce a C_{14} dialdehyde and two C_{13} cyclohexone derivatives (Schwartz et al., 2001). The regulation of *CCD* genes by abiotic or biotic stresses is largely unknown. In our studies, ObPV inoculation led to a gradual and strong up-regulation of the pepper *CCD* gene (Fig. 9), which suggested that the degradation of some individual carotenoids is probably activated in the virus-infected leaves. Indeed the accumulation of a typical CCD1 product, the terpenoid flavor volatile β-ionone was also observed in ObPV-inoculated pepper leaves by gas chromatography-mass spectrometry (D. Frigyes, unpublished result). Since CCDs are involved also in the biosynthesis of abscisic acid (Auldridge et al., 2006), the up-regulation of the pepper *CCD* gene in ObPV-inoculated leaves may be connected to the abscission of infected leaves 6–7 days following inoculation. The detailed analysis of the roles of individual CCD enzymes and carotenoids following ObPV and PMMoV inoculations awaits further studies.

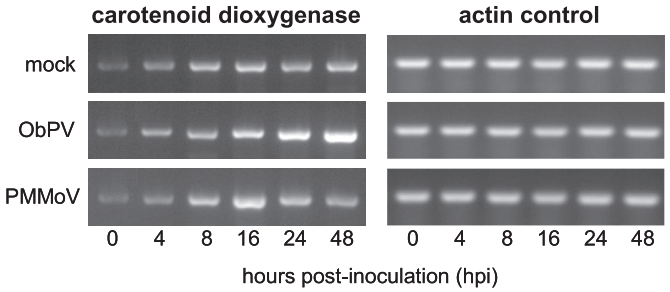

Fig. 9. Up-regulation of the expression of a pepper carotenoid dioxygenase gene (accession number Y14164) following inoculations with *Obuda pepper virus* (ObPV) and *Pepper mild mottle virus* (PMMoV) as well as after mock-inoculation. A housekeeping actin gene was used as constitutive control. Representative results of three independent experiments are shown.

5. Conclusions

ObPV inoculation of pepper leaves resulted in elevated ion leakage, up-regulation of a pepper *CCD* gene, heat emission and

declining chlorophyll *a* content before the appearance of the visible symptoms. Development of hypersensitive lesions was accompanied by partial water loss and a strong inhibition of photochemical energy conversion which led to photochemically inactive leaf areas as revealed by fluorescence imaging technique. However, leaf tissues adjacent to these inactive areas showed elevated ΦPSII and Fv/Fm values proving the advantage of imaging system in comparison to PEA.

PMMoV inoculation also led to a significant rise of ion leakage and heat emission as well as to decreased PSII efficiency and the transient, weak up-regulation of pepper *CCD* gene, but these responses were much weaker than in the case of ObPV. Chlorophyll *b* and total carotenoid contents were not significantly influenced by any virus inoculations as measured by spectrophotometric methods. Pigment contents were calculated on leaf surface basis because of the substantial water loss of ObPV-inoculated leaves as a consequence of hypersensitive lesion formation. 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.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2014.08.013.

Contributions

AS, BB, GG and IT conceived the experiments, BB, GG and IT determined the pigment contents and leakage, AJ measured the photosynthetic parameters, CJ carried out the RT-PCR experiments, DS and AS carried out the isothermal calorimetric determinations, ES made the chlorophyll imaging, GG prepared the manuscript, IT and CJ carried out the virus inoculations, MR and AS carried out the Raman spectrometric studies.

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