Multicolor Fluorescence Imaging of Leaves—A Useful Tool for Visualizing Systemic Viral Infections in Plants†

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ABSTRACT

Multicolor fluorescence induced by UV light is a sensitive and specific tool that may be used to provide information about the primary and secondary metabolism of plants by monitoring signals of the chlorophyll fluorescence (Chl-F) and blue-green fluorescence (BGF), respectively. We have followed the systemic infection of Nicotiana benthamiana plants with the Pepper mild mottle virus (PMMoV) by means of a multicolor fluorescence-imaging system, to detect differences between two strains of PMMoV during the infection process and to establish a correlation between the virulence and changes induced in the host plant. Changes in both BGF and Chl-F were monitored. BGF increased mainly in the abaxial side of the leaf during pathogenesis and the corresponding images showed a clear vein-associated pattern in leaves of infected plants. HPLC analysis of leaf extracts was carried out to identify compounds emitting BGF, and determined that chlorogenic acid was one of the main contributors. BGF imaging was able to detect virus-induced changes in asymptomatic (AS) leaves before detection of the virus itself. Chl-F images confirmed our previous results of alterations in the photosynthetic apparatus of AS leaves from infected plants that were detected with other imaging techniques. Fluorescence ratios F440/F690 and F440/F740, which increase during pathogenesis, were excellent indicators of biotic stress.

INTRODUCTION

In the last decade, UV-excited fluorescence signals have emerged as a sensitive and specific tool for evaluating the physiologic state of plants before the symptoms induced by different environmental stress factors become evident (1–3).

The excitation of leaves with long-wavelength UV radiation (320–400 nm) results in a total of four characteristic fluorescence bands with peaks near to 440 nm (blue; F440), 520 nm (green; F520), 690 nm (red; F690) and 740 nm (far-red; F740) (1,2,4). Taken together, F690 and F740 are also called chlorophyll fluorescence (Chl-F), as these fluorescence signals are emitted by chlorophyll a (Chl). Often, F440 and F520 are treated as blue-green fluorescence (BGF), as F520 is rarely present as a distinct peak (3).

During the monitoring of multicolor fluorescence (MCF) signals by point measurements on randomly selected leaf areas, the information about fluorescence gradients over the whole leaf surface is missed unless one makes time-consuming, repeated point measurements. In contrast, the UV-excited fluorescence imaging systems offer rapid analysis and high spatial resolution from the leaf to the remote sensing scale (1,2,5–7).

Blue-green fluorescence is primarily emitted from the epidermis and from both the mesophyll cell walls and the leaf veins by several phenolic compounds that are covalently bound to cell wall carbohydrates. In contrast, Chl-F nearly exclusively emanates from mesophyll cells (except for the guard cells of the epidermis which contain some chloroplasts). Cell wall-bound ferulic acid has been reported to be the main emitter of BGF in several plant species (8–10), showing that soluble plant phenolic compounds present in the vacuole of plant cells contribute little to the overall BGF emission in most of the species that have been investigated so far. More recently, chlorogenic acid (CGA) has been revealed to be one of the main BGF fluorophores in artichoke leaves (11). This hydroxycinnamic acid (HCA) is also predominant in tobacco plants, and its levels increase with leaf age (12,13).

The use of the fluorescence ratios F440/F520, F440/F690, F440/F740 and F690/F740 is widespread as BGF and Chl-F have distinct origins and can change independently in response to stress factors. F440/F690 and F440/F740 are very early stress indicators, and F440/F520 can change after a long exposure to stress (2,4,10). The F690/F740 ratio has an inverse relationship with the Chl content in the leaf (3,4,6,14,15).

Several limiting agricultural factors have been monitored through MCF, in most cases using imaging systems—mineral deficiencies (2,16–20), water stress (2,6,21,22) and high-temperature stress (23). Imaging is especially valuable for the visualization of plant–pathogen interactions where, according to the symptomatology, no uniform alteration in the foliar stress indicators, and F440/F520 can change after a long exposure to stress (2,4,10). The F690/F740 ratio has an inverse relationship with the Chl content in the leaf (3,4,6,14,15).

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pathogen-infected plants. Fungal infection has been reported to increase F440 either by the fungus autofluorescence (39) or by the production of plant phytoalexins (40). Chinese cabbage infected with *Turnip yellow mosaic virus* showed changes in the fluorescence ratios (41). Using MCF imaging, early visualization of small punctures made by tobacco flies on leaves as well as a mite attack on beans was achieved (2). More recently, the hypersensitive response (HR) to *Tobacco mosaic virus* (TMV) infection was revealed by a BGF increase linked to scopoletin accumulation (42).

In the present work, using an MCF imaging system, we monitor a systemic viral infection in *Nicotiana benthamiana* plants infected with either the Spanish or the Italian strain of *Pepper mild mottle virus* (PMMoV-S, PMMoV-I). The origin of these strains has been previously reported (43,44), respectively. The PMMoV-S strain is more virulent than PMMoV-I, and the magnitude of the symptoms in the infected plants correlates with the degree of virulence. In earlier studies, we have shown that the oxygen-evolving complex of photosystem II is the main target of this tobamovirus in the chloroplast (45-49). Kinetic Chl-FI studies reveal a correlation between nonphotochemical quenching and the spread of PMMoV in asymptomatic (AS) leaves (38). Imaging thermography can detect this infection earlier than the former technique through the increase in leaf temperature caused by stomatal closure (37). In order to obtain a more complete picture of the host plant response to PMMoV infection, information about secondary metabolites involved in signaling processes and the response of *N. benthamiana* to pathogens is needed. Hence, we have monitored changes in both BGF and Chl-F in *N. benthamiana* leaves infected with either PMMoV-S or PMMoV-I using a compact flash-lamp MCF system. Fluorescence values (F440, F520, F690 and F740) and fluorescence ratios (F440/F520, F440/F690, F440/F740 and F690/F740), as well as their corresponding images, of whole leaves from control and infected plants were tracked over the course of the infection. In an attempt to identify the compounds involved in BGF emission, imaging was complemented by HPLC analysis of leaf extracts.

**MATERIALS AND METHODS**

*Plant growth and PMMoV infection. Nicotiana benthamiana* cv. Gray plants were cultivated in a growth chamber with 120 μmol m⁻² s⁻¹ photosynthetically active radiation, generated by white fluorescent lamps (HPI-T 250 W; Phillips, Eindhoven, The Netherlands), with a photosynthetically active radiation, generated by white fluorescent lamps (HPI-T 250 W; Phillips, Eindhoven, The Netherlands), with a 15/9 h (26/20°C) light/dark photoperiod and relative humidity of 60%. The three lowest leaves of plants with six to seven leaves fully expanded were carburundum-dusted and mechanically inoculated with the Spanish or the Italian strains of PMMoV (PMMoV-S and PMMoV-I, respectively) using 50 μl of inoculum per leaf (50 μg of virus per mL of 20 mm sodium phosphate/biphosphate buffer pH 7.0). Mock-inoculated plants were treated with buffer only.

At 5 (PMMoV-S-infected plants) and 7 (PMMoV-I-infected plants) days postinoculation (dpi), visual symptoms appeared—new leaves that developed after the inoculation showed severe curling and wrinkling (symptomatic leaves, S). In contrast, no symptoms were detected in leaves that were already fully expanded when the plants were inoculated (AS leaves). Inoculated leaves showed no symptoms of disease. In summary, we can distinguish three kinds of leaves in the infected plants—inoculated, AS and S. Inoculated leaves were not used in this study because they had mechanical damage. Stunting of the plants was clearly evident at 14 dpi. PMMoV-I-infected plants were able to recover from 21 dpi onward—plant height again increased and plants developed new, noncurly leaves. In contrast, no new leaves appeared in PMMoV-S-infected plants after 14 dpi, and plants died at 21 dpi, except for the batch of plants in which the fluorophore content was determined which died at 23 dpi.

Starting with the lowest leaf and numbering leaves in ascending order, we analyzed the fifth leaf as the AS one. The second youngest leaf at each dpi was tested as the S one.

**Fluorescence imaging.** Fluorescence imaging measurements were made in control and virus-infected plants at different postinfection times: 3, 5, 7, 10, 12, 14, 17 (for plants infected with both viral strains), 21, 24 and 28 dpi (for controls and plants infected with PMMoV-I). The imaging system used is briefly described below, since fluorescence excitation and detection were essentially identical to that described in detail earlier (50).

Fluorescence-emission images of the abaxial (AB) and adaxial (AD) surfaces of leaves were made with a compact flash-lamp fluorescence imaging system (FL-FIS; 2, 51). Leaves were fixed in front of the flash lamp by an air stream generated by a ventilator. Excitation light was provided by a pulsed xenon lamp with a UV-transmission filter (transmission maximum at 355 nm). Fluorescence images were acquired with a CCD camera that was synchronously gated with the flash lamp. Leaves were placed at 390 mm from the camera, to produce images 80 mm in width. The multispectral fluorescence imaging for the blue (F440), green (F520), red (F690) and far-red (F740) fluorescence was acquired sequentially from an identical field of view, each with the appropriate filter selected from a filter wheel in front of the CCD camera. The acquisition of the fluorescence-emission images required the accumulation of 800 images.

Images were processed by specific software (Camille 1.05 software; Bruker, Karlsruhe, Germany). The raw image was corrected for nonuniform excitation with the pixels of an image showing uniform fluorescence (blue fluorescence image of a white paper). Black and white images of both measured fluorescence intensity and fluorescence ratios (F440/F520, F440/F690, F440/F740 and F690/F740), acquired by a division of fluorescence intensity at the corresponding wavelength of each pixel were set at the appropriate intensity and either false color or a black and white scale were applied by the ImageJ software package (http://rsb.info.nih.gov/ji).

Numerical data from a region of interest corresponding to the leaf outline were processed by Camille 1.05 software. Fluorescence profiles across the leaves (created using ImageJ) were made by drawing a line perpendicular to the main vein, covering 266 pixels of AS leaves. The main vein was located in the middle of the line (133 pixels to the edge of the leaf).

**Fluorophore determination.** Phenolic compounds were warm (70°C) extracted from 20 mm² leaf disks with methanol for 30 min. The volume of the extract solutions was diluted to 5 or 10 mL (depending on the type of sample) with methanol, and then frozen at ~80°C until they were analyzed.

Leaf extracts were analyzed by the HPLC method described previously (11). This method revealed the presence of one (most abundant) phenolic acid, which was identified as CGA by comparing its retention time and UV absorption spectrum with that of CGA (pure standard [purity >97%], Fluka Chemie GmbH, Buchs, Switzerland) in methanol. Prior to analysis, the sample extracts were thawed and filtered through a 0.45 μm polycylinylene fluoride filter. High-performance liquid chromatography was performed using an HPLC system (Waters Alliance 2795 HPLC system; Waters Corp., Milford, MA) equipped with an on-line degasser, autosampler module (4°C), and column oven (40°C). A diode-array detector (Waters 2996 UV–visible) was used for analyte detection. The column used was an analytical HPLC column (Waters Symmetry C18, 250 mm x 4.6 mm i.d., dp = 5 μm) with a 20-mm guard column made of the same material. The mobile phase consisted of a gradient system of solution A, MilliQ water, and solution B, HPLC gradient methanol-acetic acid (97.5:2.5; vol/vol) and solution B, HPLC gradient methanol-acetic acid (97.5:2.5; vol/vol) at a flow rate of 1.0 mL min⁻¹. The gradient program was as follows: linear gradient from solution A-B (70:30) to solution A-B (51:49) in 15 min, isocratic at solution A-B (51:49) for 10 min, followed by a linear gradient to solution A-B (70:30) in 5 min, finally isocratic at solution A-B (70:30) for 5 min. The injection volume was 15 μL. Automated instrumentation was controlled by an HPLC data system (Hystar PostProcessing; Bruker Daltonik GmbH). Quantification was based on peak-area measurements.
at 325 nm. Standard solutions were prepared with CGA, and were examined by HPLC in order to determine the retention time (4.1 min). A multipoint calibration was performed from 1.25 to 40 µm. The linear relationship was as follows: \( Y = 9.28X + 1.14 \) (\( R^2 = 0.9999 \)). Naphthol (299% GC; Riedel-de Haën, Seelze, Germany) was used as internal standard (retention time, \( t_R = 0.0 \)).

**Statistics.** The statistical analysis was carried out with specific software (SigmaPlot 8.0 software; Systat Software, Inc., San Jose, CA). The Student's t-test was used to compare control with infected plants, and PMMoV-I-infected plants with PMMoV-S-infected plants. Graphs show mean values (obtained from at least four different plants per leaf type) and their corresponding standard errors. Tables 1 and 2 indicate significant differences between treatments, according to the Student's t-test and the three significance levels tested: \( P < 0.05 (*) \), \( P < 0.01 (**) \) and \( P < 0.001 (***) \). Statistical analysis presented 6 degrees of freedom.

### RESULTS

We have obtained fluorescence images (F440, F520, F690, F740 and their corresponding ratios) from both the AB and AD leaf surfaces of AS and S leaves in control and PMMoV-infected plants at different postinfection times. In addition, fluorescence values averaged in the whole leaf were obtained and compared in control and infected plants during the infection. The statistical significance of the differences between both kinds of plants is summarized in Tables 1 and 2 (tables show the dpi when significant differences occur compared with the control).

#### Blue-green fluorescence emission of leaves

The most obvious changes in the BGF emission of both AS and S leaves from infected plants occurred on the AB surface (Fig. 1). BGF emitted by AS leaves from infected plants and their equivalent controls increased with leaf age. The AB surface of AS leaves from PMMoV-infected plants showed higher BGF emission compared to control values from 10 and 12 dpi onward (PMMoV-S and -I, respectively; Fig. 1 left panel, Table 1). In addition, BGF emission from plants infected with PMMoV-S was higher compared to those infected with PMMoV-I. BGF emission decreased to values similar to that of the control in the last PMMoV-I-infection steps (21–28 dpi), coinciding with the recovery phase in these plants.

The increase in BGF fluorescence from leaf surfaces during pathogenesis was even higher in S leaves from infected plants.

### Table 1. Summary of the statistical analysis of the intensity of the fluorescence emitted in the blue, green, red and far-red region by intact leaves of control and PMMoV-infected *N. benthamiana* plants.

<table>
<thead>
<tr>
<th>PMMoV</th>
<th>Strain</th>
<th>Abaxial surface</th>
<th>Adaxial surface</th>
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<tr>
<td></td>
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<td>Asymptomatic leaf</td>
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<tr>
<td>F520</td>
<td>-I</td>
<td>▲10–14**, ▼13**, ▲7**, ▼10–17***, ▲12–14**, ▲17**, ▲5**, ▲7*, ▼14–17*</td>
<td>▼10–17**, ▼21–28**, ▼17***</td>
<td>▼10–12**, ▼14–17**, ▼7*, &lt;0.001 (***), Degrees of freedom: 6. Arrows indicate increase (▲) or decrease (▼) in fluorescence emission of PMMoV-infected plants compared with controls. Asymptomatic leaves = old leaves which remain symptomless during the whole infection process; F440 = blue fluorescence; F520 = green fluorescence; F690 = red fluorescence; F740 = far-red fluorescence; PMMoV-I and -S = Italian and Spanish strains of the <em>Pepper mild mottle virus</em>; NS = no significant differences were found at any dpi assayed; symptomatic leaves = young leaves displaying curling from the first week of infection.</td>
<td>▲7*, ▲10–12**, ▼14–17**, ▼7*, &lt;0.001 (***), Degrees of freedom: 6. Arrows indicate increase (▲) or decrease (▼) in fluorescence emission of PMMoV-infected plants compared with controls. Asymptomatic leaves = old leaves which remain symptomless during the whole infection process; F440 = blue fluorescence; F520 = green fluorescence; F690 = red fluorescence; F740 = far-red fluorescence; PMMoV-I and -S = Italian and Spanish strains of the <em>Pepper mild mottle virus</em>; NS = no significant differences were found at any dpi assayed; symptomatic leaves = young leaves displaying curling from the first week of infection.</td>
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<tr>
<td>F740</td>
<td>-I</td>
<td>▲7*, ▼10–14**, ▼17*, ▼7*, ▼10–17***, ▼21–24**, ▼17**, ▼28**</td>
<td>▼10–12**, ▼14–17**, ▼7*, &lt;0.001 (***), Degrees of freedom: 6. Arrows indicate increase (▲) or decrease (▼) in fluorescence emission of PMMoV-infected plants compared with controls. Asymptomatic leaves = old leaves which remain symptomless during the whole infection process; F440 = blue fluorescence; F520 = green fluorescence; F690 = red fluorescence; F740 = far-red fluorescence; PMMoV-I and -S = Italian and Spanish strains of the <em>Pepper mild mottle virus</em>; NS = no significant differences were found at any dpi assayed; symptomatic leaves = young leaves displaying curling from the first week of infection.</td>
<td>▼7*, ▼10–14**, ▼17**, ▼28**</td>
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Table displays days postinoculation where significant differences compared with controls were found at \( P < 0.05 (*) \), \( P < 0.01 (**) \) and \( P < 0.001 (***) \). Degrees of freedom: 6. Arrows indicate increase (▲) or decrease (▼) in fluorescence emission of PMMoV-infected plants compared with controls. Asymptomatic leaves = old leaves which remain symptomless during the whole infection process; F440 = blue fluorescence; F520 = green fluorescence; F690 = red fluorescence; F740 = far-red fluorescence; PMMoV-I and -S = Italian and Spanish strains of the *Pepper mild mottle virus*; NS = no significant differences were found at any dpi assayed; symptomatic leaves = young leaves displaying curling from the first week of infection.
PMMoV-S caused the outstanding signals from 5 to 7 dpi onward (F520 and F440, respectively). In the case of PMMoV-I infection, the first significant BGF increase was recorded at 10 dpi. During the recovery phase of these plants, BGF values of the S leaves decreased to values similar to that of the control plants (Fig. 1 right panel, Table 1).

**Chlorophyll fluorescence emission of leaves**

Values of Chl-F integrated over the entire leaf that were emitted both by AS and by S leaves were higher on the AB leaf side in all cases (Fig. 1). Chl-F decreased with the leaf age of AS leaves in infected plants as well as in their corresponding controls from the middle stage of the infection onward (Fig. 1 left panel). In general, no significant differences in Chl-F emission from these kinds of leaves could be established between PMMoV-S-infected and control plants (except the F740 signal from the AB surface at 17 dpi; Table 1). However, PMMoV-I-infected plants showed a significant decrease in Chl-F from the AB surface at 5–17 dpi (F690 and F740). These differences disappeared during the recovery phase in both sides of the leaf. 

![Figure 1. Time-course of blue (F440), green (F520), red (F690), and far-red (F740) fluorescence emission of asymptomatic (left) and symptomatic (right) leaves of *Nicotiana benthamiana* control and PMMoV-infected plants. Fluorescence signals integrated over the entire leaf at every dpi assayed are displayed; error bars indicate standard error. Abaxial and adaxial surfaces of the leaves are shown. Data are mean ± standard error of *n* = 4.](Photochemistry and Photobiology, 2008, 84 1051)
The S leaves from infected plants also showed a decrease in Chl-F compared to the control (Fig. 1 right panel). The AB side of S leaves from PMMoV-S-infected plants exhibited the first significant changes as early as 5 dpi (F690, Table 1), while the AD surface showed significant differences only from 7 dpi (F690 and F740). PMMoV-I-infected plants had lower Chl-F values from 7 to 10 dpi onward (AD and AB surfaces, respectively). During the late recovery phase (from 24 dpi on) in PMMoV-I-infected plants, the Chl-F emission decreased to values similar to that of the control (see Table 1 for statistical significance).

**Imaging of multicolor fluorescence of leaves**

To analyze the viral-induced spatiotemporal changes in fluorescence emission at different wavelengths, we compared the images obtained before (5 dpi) and after (17 dpi, once the fluorescence emission changes were very evident) changes in fluorescence (Fig. 2) in both S and AS leaves from control and PMMoV-infected plants.

The F440 pattern from control leaves, equivalent to the AS in infected plants, did not significantly change over the experimental period (Fig. 2a). Areas showing the highest fluorescence emission corresponded to the vascular tissues of the AB leaf surface. At 5 dpi, AS leaves from infected plants were very similar to that of the controls. However, an increase in F440 could be detected at 17 dpi in both the veinal and interveinal tissues of AS leaves of infected plants, and was more apparent on the AB surface and in the case of PMMoV-S-infected plants. Viral-induced changes on the F520 pattern were very similar to those of F440.

During the first days after infection, the F690 and F740 pattern (Fig. 2a) of AS leaves from infected plants appeared to be similar to that of control plants; however, at 17 dpi, Chl-F emission of both leaf sides decreased in the whole leaf compared to that of control plants. A fluorescence gradient pattern appeared where the highest Chl-F values were found in the leaf areas surrounding the main veins.

Control leaves corresponding to the S leaves in infected plants displayed a very homogeneous BGF pattern on both surfaces during the period analyzed (Fig. 2b). The S leaf of PMMoV-infected plants did not differ from controls at 5 dpi (increased values recorded in the AD surface were not significant). However, a BGF increase in the whole leaf (both surfaces) was evident at 17 dpi. The vein areas which had the highest values were more apparent on the AB surface of the S leaves and in PMMoV-S-infected plants, as in the case of AS leaf patterns of infected plants. In contrast to the defined Chl-F pattern from AS leaves of infected plants, no clear pattern emerged from the F690 and F740 images of S leaves (Fig. 2b).

**Profiles of asymptomatic leaves at 17 dpi**

Fluorescence profiles of the AS leaf surface at 17 dpi were plotted to visualize the distribution of the different fluorescence signals recorded across these leaves (Fig. 3). The AB leaf surface was chosen because its fluorescence signal was more prominent.

Blue-green fluorescence emission from control leaves was the lowest. BGF levels in AS leaves from PMMoV-infected plants had higher values along the whole profile, were more evident in the vein area and were highest in the case of PMMoV-S-infected plants. Three peaks could be distinguished, corresponding to Type I and II veins. On the other hand, control leaves had the highest Chl-F values. The main veins had the lowest Chl-F values in AS leaves in both the control and PMMoV-infected plants (Fig. 3).

**Fluorescence ratios of leaves**

In AS leaves, the values of F440/F520 ratio decreased with leaf age in all cases (Fig. 4 left panel). A significant increase in this ratio in infected plants compared to the controls was reported in the AB side from 12 and 14 dpi onward (PMMoV-S and -I-infected plants, respectively) (Table 2).

F440/F690 and F440/F740 were the fluorescence ratios that reported the PMMoV infection the earliest, and showed significant increases with respect to control values in the AD surface at 5 (F440/F690) and 7 dpi (F440/F740). In the case of PMMoV-I infection, these ratios were similar to that of the control plants during the symptom-recovery phase (Fig. 4 left panel, Table 2). In the case of PMMoV-S infection, an increase in both ratios was detected at 12 dpi (AB surface, F440/F690), and remained high during the whole infection process.

F690/F740 values were generally higher in the AB surface than on the AD surface (Fig. 4 left panel). This ratio slightly decreased on both leaf surfaces until it was about 10–12 dpi in control and infected plants; from this time onward the ratio rose. Notably, AS leaves for both kinds of infected plants did not have a lower Chl content than the controls, except for the AD side of the AS leaves of PMMoV-S-infected plants at the last infection point (Table 2). Moreover, the AD surface of AS leaves of PMMoV-I-infected plants had lower values of F690/F740 than controls at various times (12, 14, 28 dpi; Table 2).

In the case of S leaves (Fig. 4 right panel), every fluorescence ratio assayed was a good indicator of the stress induced by PMMoV infection in *N. benthamiana* plants, except for F440/F520. However, none of the ratios could detect significant differences between S leaves of infected plants and the equivalent controls before visual symptoms appeared. Alterations in both F440/F690 and F440/F740 ratios due to the infection with both viral strains could be detected as early as 7 dpi (Fig. 4 right panel, Table 2). The recovery phase of PMMoV-I-infected plants was registered as a decrease in these ratios.

Increases in F690/F740 were observed in S leaves from PMMoV-infected plants. The AD surface of leaves from control and infected plants displayed lower values of F690/F740 than the AB surface (Fig. 4 right panel).

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**Figure 2.** Images of blue (F440), green (F520), red (F690) and far-red (F740) fluorescence emission of asymptomatic (a) and symptomatic (b) leaves of *Nicotiana benthamiana* control and PMMoV-infected plants. Abaxial and adaxial surfaces of the leaves are shown. Images obtained before (5 dpi) and after (17 dpi) fluorescence changes occurred are displayed. The color scale applied is shown for each panel. Different sizes of asymptomatic (old) and symptomatic (young) leaves are due to leaf age.
Imaging of fluorescence ratios of leaves

Figure 5 displays the images corresponding to the fluorescence ratios of both AS and S leaves before (5 dpi) and after (17 dpi, once changes in ratios values were very evident) the biggest changes in the fluorescence ratios.

A lower $\frac{F_{440}}{F_{520}}$ due to leaf aging was evident in the images of AS leaves from infected plants and their controls (Fig. 5a). No significant differences were found between control and infected plants at 5 dpi; however, images at 17 dpi displayed higher $\frac{F_{440}}{F_{520}}$ values in PMMoV-infected plants compared to the controls, especially in the vein areas of the AB surface.

At 5 dpi, images of the $\frac{F_{440}}{F_{690}}$ ratio did not show differences between control and infected plants (Fig. 5a). Nevertheless, a clear rise in this ratio in AS leaves of infected plants was evident at 17 dpi, and was more prominent on the AD surface. For this parameter, a declining gradient was detectable from the vein (which had the highest values of the ratio) to the rest of the leaf blade. Images of $\frac{F_{440}}{F_{740}}$ displayed a very similar situation, albeit with higher values than $\frac{F_{440}}{F_{690}}$ (Fig. 5a).

During the infection, no significant decreases in Chl content (Table 2) were evident in the $\frac{F_{690}}{F_{740}}$ images from AS leaves of infected plants compared to the control (Fig. 5a), except for the AB surface of the AS leaves from PMMoV-S-infected plants, where Chl loss was evident at 17 dpi. The AD surface of the leaves generally had lower values of $\frac{F_{690}}{F_{740}}$ than the AB surface.

In the case of S leaves, no clear trend emerged from the $\frac{F_{440}}{F_{520}}$ images (Fig. 5b). The only remarkable fact was that the AD surface of the PMMoV-S-infected plants had lower $\frac{F_{440}}{F_{520}}$ values compared to the controls at 17 dpi.

For $\frac{F_{440}}{F_{690}}$ and $\frac{F_{440}}{F_{740}}$, an increase in these ratios was clear in the images of S leaves from infected plants only at 17 dpi, with the vascular tissues emitting the highest fluorescence (Fig. 5b). This vein-associated pattern, very similar to that found in AS leaves of infected plants, was more obvious on the AB surface of the S leaves. Increases in $\frac{F_{690}}{F_{740}}$...
appeared at 17 dpi and only in the S leaves from PMMoV-S-infected plants.

**Fluorophore determination**

Leaf extracts were analyzed by an HPLC method described previously (11), which is able to resolve CGA, caffeic acid and cynarin (1,3-dicaffeoylquinic acid). HPLC analysis of methanolic extracts from S leaves of *Nicotiana benthamiana* control and PMMoV-infected plants revealed the presence of one of the major phenolic compounds, identified as CGA by comparing its retention time and UV absorption spectrum with that of CGA (pure standard) in methanol. The chromatogram also revealed the presence of other very minor unidentified compounds, which were not further analyzed (data not shown). The concentration of CGA increased during the viral infection (Fig. 6). Significant differences with respect to the controls appeared from 7 to 21 dpi for PMMoV-S plants, whereas PMMoV-I-infected plants displayed differences only at 14 dpi. Later (21 dpi) significant differences in the CGA content between control and PMMoV-I-infected plants vanished in accord with the appearance of the recovery phase.

*Figure 4.* Time course of F440/F520, F440/F690, F440/F740 and F690/F740 fluorescence ratios of asymptomatic (left) and symptomatic (right) leaves of *Nicotiana benthamiana* control and PMMoV-infected plants. Averages for the whole leaf at each dpi assayed are displayed; error bars indicate standard error. Abaxial and adaxial surfaces of the leaves are shown. Data are mean ± standard error of *n* = 4.
DISCUSSION

Although in the last few years UV-excited MCF imaging has emerged as a sensitive and specific tool for presymptomatic and nondestructive monitoring of changes in the physiologic state of plants, the application of this technique has not gained ground in the investigation of pathogen attacks, especially viral infections. Nevertheless, Chl-FI has been broadly employed to study such interactions (32,33,35–38,52–54). Information about BGF imaging and the corresponding accumulation of secondary metabolites in virus-infected plants is available only for the HR induced by TMV (42). The present study is the first to elucidate such changes during a systemic infection.

Infection in N. benthamiana plants with PMMoV caused an increase in BGF that was higher in the AB side of AS leaves and in the case of PMMoV-S infection; the response of the plant to PMMoV-I is delayed. This is in agreement with the milder symptomatology induced by this tobamovirus, as well as with the lower virulence and accumulation in the host plant that was demonstrated in our previous studies (37,48). Areas surrounding the veins (for N. benthamiana vein classification, see [55]) showed the highest BGF values; viral-induced BGF increases in the interveinal tissue are stronger for F520. This is probably due to the higher accumulation of green fluorophores under long-term stress (2) and the fact that F520 is less efficiently absorbed by the Chl than F440 is (56). This is supported by the decrease in the F440/F740 ratio during the experiment (Fig. 4). The increase detected in F520 and F440 in control old leaves (corresponding to the AS in infected plants, Figs. 1 and 2) over the course of the experiment is probably the result of accumulation of phenolic compounds and changes in the optical properties of leaves that are associated with leaf aging (3,17,57).

Blue-green fluorescence measurements on the AB leaf side seem to be more adequate for following the viral infection. It is well documented that UV-induced fluorescence signals from the upper and lower sides of the leaf are considerably different in dicotyledonous plants. A difference that may account for this observation is that large amounts of Chl from packed palisade cells of the AD side of the leaf reabsorb F440 and F690 more efficiently than the spongy parenchyma of the AB side (2,3).

Imaging of red fluorescence (F690) in PMMoV-infected plants agrees well with our previous data using different Chl-FI systems (37,38). From 14 (PMMoV-S, data not shown) and 17 dpi onward (PMMoV-I), Chl-F showed a heterogeneous leaf pattern on the AD side, with increased values in the tissue surrounding the main veins. It was demonstrated that this pattern is directly related to the spread of the virus in AS leaves (37,38). Fluorescence changes during pathogenesis in these kinds of leaves could not be attributed to alterations in Chl content, because it did not significantly change over the period analyzed (37). The present work also supports this fact, as the F690/F740 ratio (inversely correlated with Chl content) was similar (PMMoV-S) to or even lower (PMMoV-I) than the control values (Figs. 4 and 5). The F690/F740 ratio of the AB surface of AS leaves of PMMoV-S-infected plants displayed higher values than the control very late in the infection process (17 dpi, the last day assayed), but the changes in the Chl-F pattern started earlier (14 dpi).

Blue-green fluorescence changes preceded (10 dpi PMMoV-S; 12 dpi PMMoV-I) the detection of the virus by tissue printing (14 and 17 dpi, respectively, for the two viral strains; [37]) or northern blotting (58) in AS leaves, suggesting an earlier activation of the secondary plant metabolism and the subsequent accumulation of fluorophores.

Increases in the BGF emission of PMMoV-infected plants are also reflected in the higher F440/F690 and F440/F740 ratios, even on the AD leaf side (Fig. 4, Table 2). Similar increases were reported after point measurements in Chinese cabbage infected with Turnip yellow mosaic virus (41). These ratios have been demonstrated to be the best stress indicators (21). It was possible to detect virus-induced stress earlier (5 dpi AD side and 7 dpi AB side) with the F440/F690 ratio than with other fluorescence parameters analyzed in the case of PMMoV-I infection. F440/F520 was not as efficient as BGF/Chl-F in following the PMMoV infection.

The recovery phase of PMMoV-I-infected plants (21–28 dpi) also seems to be evident in the old leaves, as fluorescence values (Fig. 1 left panel, Table 1) and ratios (Fig. 4 left panel, Table 2) of either AD or AB surfaces from AS leaves of these plants are close to the control values.

An analysis of the fluorescence yield along a broader line across the leaf area is possible (22,59). Some authors (20) have used horizontal transects to demonstrate that BGF and Chl-F...
have a negative contrast in variegated leaves. This relationship is also evident in the vein area of AS leaves from *N. benthamiana* infected with PMMoV (Fig. 3). Profiles display the heterogeneous accumulation of blue-green fluorophores, but the Chl-F heterogeneity is poorly represented by these transects, once again establishing that images offer more information about changes triggered by PMMoV infection (37,38).

In order to focus on the S leaves, we first need to summarize some data about viral movement in the host plant. It is well known that tobamoviruses, such as PMMoV, move rapidly from the inoculated basal leaves to the plant apex (60 via phloem (55,61,62). Tissue prints taken from 7 to 28 dpi of the young leaves showed a quick viral accumulation in the whole leaf in the early stages of infection (37). Recently (data not shown) we detected the entrance of PMMoV-S in the leaf at 4 dpi and later (6 dpi) for the less virulent strain PMMoV-I. The rapid viral movement to the S leaves compared with the AS leaves (37), could explain the absence of a dynamic imaging Chl-F pattern in S leaves (Fig. 2b). However, average whole-leaf Chl-F values could distinguish S leaves of infected leaves from their corresponding controls, but not in a presymptomatic manner (Fig. 1 right panel, Table 1). These changes in Chl-F emission preceded the decrease in Chl content (37), as shown by the F690/F740 ratio (Fig. 4 right panel, Table 2).

Either PMMoV detection in the S leaf preceded alterations in BGF emission or the two processes took place concomitantly (F520 of AB surface from S leaves of PMMoV-I-infected plants, Table 1). The changes in fluorescence emission on the AB surface of S leaves were more significant in the case of AS leaves for the same reasons described above. The accumulation of phenolic compounds in S leaves induced by PMMoV, contrary to AS ones, did not precede virus detection. The decrease in BGF values in the S leaves of PMMoV-I-infected plants between 21 and 28 dpi, compared with previous points of measurement, was indicative of the symptom-recovery phase affecting young leaves.

To identify the major compounds responsible for the emission of BGF in *N. benthamiana* plants, HPLC analyses of leaf extracts were performed. Wolfbeis (63) reviewed a series of natural compounds which are known to be present in leaves and which emit BGF upon UV excitation. The two main types of BGF emitters are HCAs and nicotinamide and flavine nucleotides (3,16,64). In the methanolic extracts of control and S leaves from PMMoV-infected *N. benthamiana* plants, CGA was the only HCA detected in high concentrations. A boost in the CGA concentration in S leaves was correlated with the virulence of the viral strain analyzed—PMMoV-S induced the most dramatic changes; and HCA values for the PMMoV-I-infected plants decreased to levels close to that of the controls during the recovery phase. In addition, the CGA concentrations in S leaves concur well with the BGF values (F440, F520) and in both cases increased during the infection with both viral strains. The characteristics of the UV-induced absorption and excitation spectra that we have recorded previously (65) in leaf pieces from PMMoV-infected plants match those of the CGA.

It is well known that biotic and abiotic stress factors induce the phenylpropanoid metabolism (66,67). An increase in phenolic compounds has been observed in plants infected with *Wheat streak mosaic virus* (68), as well as during a combined infection with TMV and the *Potato virus X* (PVX) (69).

Solanaceae, such as the genus *Nicotiana*, accumulate CGA as the major phenolic compound (70). This HCA was found to be most abundant in tobacco plants (13), and was also present in these plants under different environmental stress conditions, as UV light or mineral deficiencies ([12] and references therein).

In the past few years, the antioxidant function of CGA, one of the main products of the phenylpropanoid pathway, has been emphasized. In addition, it has been proposed that the involvement of CGA in an alternative pathway for photochemical energy dissipation under stress has the added benefit of enhancing the antioxidant capacity of the cell (70).

Different authors have suggested that CGA functions as a preformed agent against biotic challenges and operates through its antioxidant activity. Tomato plants with elevated levels of CGA showed a slower progression and reduced levels of infection by *Pseudomonas syringae*. Similar results were reported for tobacco with different levels of CGA, which were infected with either the fungus *Cercospora nicotianae* or *P. syringae* var. tomato ([71] and references therein).

The induction of the synthesis of phenolic compounds such as CGA for biologic control of the phytopathogenic fungus *Phytophthora nicotianae* has been also reported (72). However, little information obtained from BGF imaging is available about the leaf distribution of phenolic compounds that are produced during pathogenesis, as existing data are only concerned with the HR (42).

It is noteworthy that we found similarities between the images of AS leaves of PMMoV-infected plants with a defined spatial pattern of BGF emission at 10–12 dpi (data not shown) and those of the leaf thermal pattern (37) at 7–10 dpi (viral strain dependent) as well as with those of the nonphotochemical quenching pattern at 17 dpi (PMMoV-I) (38) from our previous studies. In addition, the images were in good agreement with later viral immunolocalization on tissue prints at 14–17 dpi. As the thermal changes in these leaves (the temperature increase was due to stomatal closure) were the earliest ones during pathogenesis and prior to the viral detection, some systemic plant factors are presumably involved. In our system, greater BGF emission corresponding to activation of the secondary metabolism occurred 3 days later than thermal changes for every viral strain analyzed. Alterations in the Chl-F pattern were evident when the virus was already present in the leaf. Some of these changes must play a part in the defense against the pathogen, because photoinhibition indicating photosystem II damage was only evident in PMMoV-infected plants in the final infection steps (38,48).

In conclusion, the BGF imaging of the AB side of the leaf was able to distinguish between the two viral strains; and the magnitude of the changes correlated with their virulence. The response was also delayed in the less virulent strain. A symptom-recovery phase in PMMoV-I-infected plants, affecting only the young S leaves, in the last infection steps was also detected by these measurements. BGF emission also differs between S and AS leaves. BGF imaging could detect virally induced metabolic changes in the absence of symptoms in AS leaves preceding even viral detection. The situation in S leaves, where the virus arrives earliest, is more complex, due to the quick invasion by the pathogen; viral spread and changes in BGF emission take place concomitantly. CGA was
demonstrated to be the main contributor to BGF emission in *N. benthamiana* plants. In addition, there was a correlation between its levels in developing leaves and the virulence of the tobamovirus. Taken together, these results indicate that UV-induced fluorescence is a promising tool for visualizing a systemic infection before the appearance of visible symptoms and before the pathogen is present.

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