Thermal and Chlorophyll-Fluorescence Imaging Distinguish Plant-Pathogen Interactions at an Early Stage

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Different biotic stresses yield specific symptoms, owing to their distinct influence on a plant’s physiological status. To monitor early changes in a plant’s physiological status upon pathogen attack, chlorophyll fluorescence imaging (Chl-FI) and thermography, which respectively visualize photosynthetic efficiency and transpiration, were carried out in parallel for two fundamentally different plant–pathogen interactions. These non-destructive imaging techniques were able to visualize infections at an early stage, before damage appeared. Under growth-room conditions, a robotized set-up captured time series of visual, thermal and chlorophyll fluorescence images from infected regions on attached leaves. As a first symptom of the plant–virus interaction between resistant tobacco and tobacco mosaic virus (TMV), thermal imaging detected a local rise in temperature while Chl-FI monitored a co-localized increase in fluorescence intensity. Chl-FI also revealed pre-symptomatic high-intensity spots for the plant–fungus system sugar beet–Cercospora beticola. Concomitantly, spots of lower temperature were monitored with thermography, in marked contrast with our observations on TMV-infection in tobacco. Knowledge of disease signatures for different plant–pathogen interactions could allow early identification of emerging biotic stresses in crops, facilitating the containment of disease outbreaks. Presymptomatic monitoring clearly opens perspectives for quantitative screening for disease resistance, either on excised leaf pieces or attached leaves.

Keywords: Cercospora beticola Sacc. — Chlorophyll fluorescence imaging — Hypersensitive response — Plant–pathogen interaction — Thermography — Tobacco mosaic virus.

Abbreviations: Chl-FI, chlorophyll fluorescence imaging; DPI, days post infection; F_L, chlorophyll fluorescence image captured after low intensity excitation; F_H, chlorophyll fluorescence image captured after high intensity excitation; FIS, fluorescence imaging system; HR, hypersensitive response; PEA, plant efficiency analyzer; SA, salicylic acid; TMV, tobacco mosaic virus.

Introduction

The use of non-destructive imaging methods holds great promise for early, efficient and objective detection of plant responses to various stresses (Buschmann et al. 2000, Chaerle and Van Der Straeten 2000, Govindjee and Nedbal 2000, Chaerle and Van Der Straeten 2001). Imaging is well suited to visualize heterogeneity in plant response to stress; localized responses can be clearly revealed against a background of unaffected plant tissue. The combination of information from images captured in different spectral regions has the potential to generate specific signatures for particular (categories of) stresses. By using chlorophyll fluorescence imaging (Chl-FI) and thermography to compare the localized symptoms of a viral and a fungal infection we aimed to validate this concept.

Light-energy absorbed by plants is distributed over three competing processes: photosynthesis, thermal dissipation and chlorophyll fluorescence emission (Buschmann 1999). An increase in Chl-FI thus implies a decrease in photosynthesis and/or thermal dissipation, and vice versa. Chl-FI of plant infections was carried out previously for a number of different viral and fungal plant–pathogen systems (Rolfe and Scholes 1995, Balachandran et al. 1997, Chou et al. 2000, Lohaus et al. 2000, Chaerle and Van Der Straeten 2001). Quantitative response analysis based on fluorescence imaging could lead to more efficient cultivar characterization, as opposed to visual ‘scoring’ (Chaerle and Van Der Straeten 2000, Meyer et al. 2001, Soukupova et al. 2003). The process of the hypersensitive response (HR)-linked necrotic cell death during incompatible interactions involves the destruction of chlorophyll, as witnessed by paling of the infected tissue as symptoms first become visible. However, multiple other processes that occur early during this cellular deconstruction (Jones 2001, Lam et al. 2001) can possibly affect photosynthetic efficiency. We thus expected to reveal a change of fluorescence emission upon the HR to tobacco mosaic virus (TMV).

Thermal imaging is a straightforward choice for visualizing stress-induced changes in leaf transpiration, given controlled environmental conditions (Jones 1999). Otherwise, the influence of environmental changes would be superimposed on the thermal signature of the stress under study. Under controlled conditions, thermal imaging has enabled the isolation of...
stomatal mutants through screening assays (Chaerle and Van Der Straeten 2000, Mustilli et al. 2002) as well as the presymptomatic visualization of a number of biotic plant stresses (Chaerle et al. 1999, Boccara et al. 2001). The HR of resistant tobacco to TMV infection is a well-characterized model system. Using thermography, we previously measured a pre-symptomatic temperature increase at sites of TMV infection, coinciding with localized salicylic acid (SA) accumulation (Chaerle et al. 1999). We then proposed that the local temperature is caused by stomatal closure induced by the pre-necrotic accumulation of resistance-associated compounds. The HR of plants to invading pathogens occurs spontaneously in a series of mutants from different plant species. Previously, we showed high-contrast thermal detection of pre-necrotic symptoms in tobacco and Arabidopsis (Chaerle et al. 2001). We then hypothesized that any local plant cell death phenomenon would be detectable by thermal imaging at an early stage. Cercospora leaf spot is a necrotrophic fungal infection of sugar beet, characterized by the formation of dark circular necrotic spots (Skaracis and Biancardi 2000). This symptomatology thus provided a testing opportunity for the wider applicability of early thermal (and possibly fluorescence) disease detection. Plant resistance in general, with or without the manifestation of HR, inevitably has repercussions on general metabolism and physiology (Somssich and Hahlbrock 1998). A recent report indicates that inhibition of metabolic reactions with no clear link to photosynthesis can have an influence on chlorophyll fluorescence emission (Barbagallo et al. 2003). Depending on the metabolic reactions affected by plant–pathogen interactions, different effects on photosynthesis and transpiration can likely be revealed by chlorophyll fluorescence imaging (Chl-FI) and thermography, respectively.

In this study we used a thermal and a fluorescence imaging system, mounted side by side on a gantry robot, for parallel time-lapse imaging of plants under constant growth conditions, during the development of two different plant–pathogen interactions. For the viral and fungal infections tested, the formation of clearly delimited necrotic symptoms allowed correlation of presymptomatic responses with the delayed co-localized visual damage. Interestingly, the two studied interactions displayed a completely different early thermal presymptomatic response, while the differences in early chlorophyll fluorescence were subtler. These observations demonstrate the discriminating power of multispectral imaging in identification of stress agents on plants. In addition we have tested these imaging techniques on leaf pieces, in order to establish their use as a rapid assay for disease resistance screening.

Results

Imaging of TMV local lesion development in attached tobacco leaves

A single spot between the side veins of a tobacco leaf was TMV-inoculated. The infection locus corresponds to the centre of the images in Fig. 1, panels a–f. Fig. 1g depicts the evolution of the size of this single lesion, as imaged by the respective cameras. One hour before infection, no local effects could be seen at the infection site in the (from upper left clockwise) colour reflectance, thermal, F\textsubscript{H} and F\textsubscript{L} chlorophyll fluorescence images. The first pre-symptomatic indication of the HR to TMV-infection was revealed by Chl-FI as a single spot of high intensity in the image taken under low intensity excitation (F\textsubscript{L}) (Fig. 1b, lower left). The whitish spot appeared a few hours before the first thermal symptoms. In the thermal images, a local developing lesion first appeared as a spot of higher temperature and had already considerably expanded 36 h after infection (Fig. 1c, upper right). The temperature increase due to the HR is represented by a yellow colour. The co-localized F\textsubscript{L} fluorescence image shows a white spot (high fluorescence intensity) with centred grey (lower intensity) patches (Fig. 1c, lower left panel). At this time point, a pinpoint necrotic lesion is not yet visible in the colour reflectance or thermal image (Fig. 1c, upper panels). The high temperature spot showed a fast expansion during this pre-necrotic period, reaching their maximal expansion about 2 d after infection. The spots of high fluorescence emission expanded much slower, and rapidly displayed a central grey (lower intensity) core (Fig. 1b–d, lower left panels). In the fluorescence image captured under high intensity excitation (F\textsubscript{H}) no white zone of high intensity fluorescence emission was visible (Fig. 1c–f, lower right). The lower intensity ‘core’ seen in the F\textsubscript{L}-image was visible on average 2 h earlier in the F\textsubscript{H} images (compare Fig. 1b lower right to Fig. 1c, lower left). The low intensity patches in the fluorescence images showed up on average 20 h before pinpoint visual symptoms became apparent (compare Fig. 1c–e). Measurements on the reflectance images at locations of emerging pinpoint TMV-lesions show a constant intensity until 58 h after infection (that is between the timepoints of Fig. 1d, e). Thereafter intensity increases sharply, associated with a relative decrease of the share of the green part of the spectrum. Local measurements on pre-symptomatic TMV-infected loci were also made with a portable plant efficiency analyser (PEA) non-imaging fluorometer. A pre-necrotic decrease in maximal fluorescence under saturating illumination (Fm) was measured, when compared to mock-inoculated sites (see supplementary Fig. 1). After 29 h the Fm starts to drop for infected plants, this correlates well with the drop in F\textsubscript{H} at the infection site depicted in Fig. 1b.

Only chlorophyll fluorescence imaging allowed the detection of multiple necrotic infection foci within a single TMV-infection spot (Fig. 1b, c). Later on these foci coalesced to form a single HR lesion (see colour reflectance image Fig. 1f). Coinciding with the appearance of necrotic spots in the visual reflectance images (first apparent 58 h post infection), co-localized black spots of zero fluorescence intensity appeared in the grey zones of the F\textsubscript{L} fluorescence images (Fig. 1d, e). At about the same time a distinctive colder, darker central spot emerged in the high-temperature spot on the thermal image.
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A halo of increased fluorescence remained visible around the expanding central dark zone for the duration of the experiment. The thermal halo, however, faded and disappeared, while the central region of the lesion dried and displayed an increased temperature (Fig. 1f). At this stage, the formerly grey zone in the fluorescence images had turned completely black. In terms of symptom expansion the thermal effect reached its maximum area very rapidly, in contrast with the fluorescence effect in the FL images, which lagged behind (Fig. 1g). Animations of the TMV-infection process are available as supplementary material. Local increases in temperature were consistently observed for 25 local infections in the independent experiments carried out. The temperature increase was specific for the HR of resistant Xanthi NN tobacco to TMV, since no response was observed in susceptible Xanthi nn tobacco (Fig. 1h). This indicates that the thermal response can be used as a marker to screen for disease resistance. Chlorophyll fluorescence imaging detected pre-necrotic symptoms in all experiments; however, the intensity of these early effects was variable among different plants. In susceptible Xanthi nn tobacco no change was observed in chlorophyll fluorescence emission (data not shown).

Imaging of TMV local lesion development in leaf discs

Using a leaf-disc assay, the presymptomatic increase in surface temperature at infection sites of TMV could not be detected. Leaf discs adapt to the temperature of the water they are floating on; consequently thermography revealed an equal
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surface temperature until the necrotic lesions started to dry 4 d post infection (DPI) (see supplementary Fig. 2). The colour reflectance image of Fig. 2a (upper panel) does not yet show any signs of necrosis 30 h after infection. The small dark spot at the site of infection in the FL fluorescence image of Fig. 2a was present from the time-point of infection on, and is due to superficial wounding during inoculation. In the FL images captured later on no local presymptomatic increase in fluorescence emission was detected (lower panels of Fig. 2b, c). Two days after infection necrotic lesions are apparent in the colour reflectance image, and are also discernable in the FL image as black spots (Fig. 2b). Around the black spots in the fluorescence image a grey area can be distinguished (Fig. 2c). This correlates with a lighter colour around the necrotic lesions in the visual images. In another experiment, however, a decrease in fluorescence emission was observed before such visual symptoms (and associated black spots in the FL images) became apparent (see supplementary Fig. 2). The visible pale necrotic lesions in Fig. 2b and c (upper panels) appear as sharply delimited black spots in the corresponding chlorophyll fluorescence images (lower panels). Lesions became visible in visual reflectance and chlorophyll fluorescence images at about the same time-point: (40 h after infection).

**Does exogenously applied SA have an effect on chlorophyll fluorescence?**

As shown previously, exogenously applied SA caused an increase in leaf surface temperature in tobacco (Van Der Straeten et al. 1995). In addition, localized ‘hot spots’ were visualized by thermography after droplet-application (Chaerle et al. 2002). Here, droplet application was monitored with the combination of thermal and chlorophyll fluorescence imaging. Fig. 3a shows the leaf surface before droplet application. First, the sites of application of both SA- and buffer-solution cooled down (darker colour in Fig. 3b, lower panel) compared to the rest of the leaf surface, due to evaporation of the applied droplets. The thermal image of Fig. 3b has a temperature span of 4 degrees; the other panels display a temperature range of 1 degree between black and bright yellow. After 1 h, when all the fluid has evaporated only the sites of SA-application started to increase in temperature. A maximal effect was attained 3 h after SA-application, indicated by lighter, yellow coloured spots (Fig. 3c, lower panel). The yellow spots at the SA-droplet application area were visible for 7 h, albeit gradually decreasing in intensity. At the corresponding sites in the fluorescence images, however, no intensity changes were observed (Fig. 3c, d). In Fig. 3b, the tissue around the liquid droplets appeared to have a slightly higher intensity. There was however no distinction between SA and control treatments. Likely, this effect is due to diffraction of the excitation light by the droplets.

**Monitoring of Cercospora symptoms on attached beet leaves**

Spraying beet leaves with a *Cercospora beticola* spore solution typically resulted in the unsynchronized appearance of lesions. Fig. 4a shows a beet leaf fixed between two layers of gauze; at 2 DPI the site where the first isolated lesion will later develop (centre of red circle in video image panel b) had a homogeneous appearance. First whitish fluorescence symptoms were apparent at 6 DPI (see supplementary Fig. 3). In the centre of the high intensity regions a black (low intensity) central spot was discernable at 6.5 DPI. This coincided with the appearance of a co-localized pinpoint visual lesion. At 7 DPI, thermal and fluorescence symptoms for the indicated first lesion were at their maximum intensity (Fig. 4b). The thermal response was characterized by a low temperature (black) spot at the site of infection. The fluorescence effect had a higher intensity in the images captured at the highest illumination level (compare F_H to F_L image). In the colour reflectance image...
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taken at 7 DPI, a single spot of lighter colour is present (Fig. 4b, centre of circle), which precisely co-localises with the dark spot in the fluorescence images. A thermal image with broader temperature span (2 degrees) makes it possible to identify sub-spots within the single cold (black) spot from the 1-degree span thermal image shown in Fig. 4b. This correlates with the shape of the high-intensity area in the fluorescence images (see supplementary Fig. 4).

Cercospora infection sites first became apparent as regions of high intensity chlorophyll fluorescence emission in the $F_H$ and $F_L$ fluorescence images. A few hours later, dark spots of lower temperature appeared in the thermal image. See supplementary Fig. 5 for the development of a second isolated lesion (starting at 7.6 DPI) within the same imaged region as depicted in Fig. 4. For the first spot described above only fluorescence and reflectance data are available at the earliest time points (see supplementary Fig. 3). An experiment was conducted with thermal imaging visualizing the underside of a spray-infected, attached but unsupported leaf. Confirming the above observations, local cooling was already visible by 7DPI, and expanded rapidly thereafter (see supplementary Fig. 6).

In Fig. 4c, new infection spots emerged in fluorescence and thermal images captured at 8 DPI. The centre of the first isolated lesion has dried and displays a warmer core; the extension of the cooling effect has dramatically decreased. One day later (9 DPI), the effects associated with infection spots covered the whole surface of the leaf in the fluorescence and thermal images. In the colour reflectance image, these additional lesions are hardly visible due to their small size and low colour contrast with the surrounding tissue.

Beet leaf strip assay for Cercospora infection

At 7.3 DPI, leaf strips infected with C. beticola displayed a pattern of high-intensity fluorescence spots. In three of these spots a central dark core was present (red circles in Fig. 5). Both $F_H$ (depicted in Fig. 5) and $F_L$ images (data not shown) displayed the white fluorescence spots. The chlorophyll fluorescence intensity at the infection sites increased from $F_L$ to $F_H$.

In the visual reflectance images, the appearance of red-brown pin-point lesions coincided with the visibility of the dark spots in the chlorophyll fluorescence images (Fig. 5b, c). The control leaf strips did not display any change in chlorophyll fluorescence emission pattern. In Fig. 5c (8 DPI), the area of the
high intensity spots has expanded and new white high intensity spots emerged. The three black central spots present in the Fig. 5b increased in size, while new dark spots appeared in the centre of several white spots. A white halo remained visible around the dark cores. One day later (Fig. 5d, 9 DPI), expansion of white fluorescence symptoms had almost ceased. However, up to 12 DPI halos remained visible around the dark spots. Comparable to the results for TMV infection in leaf discs, the thermal images of the leaf-strips did not reveal local temperature differences associated with infection (data not shown). The contact of the leaf strips with the water-agar most likely leads to rapid temperature equilibration (to the temperature of the water agar).

Discussion

Here we show for the first time presymptomatic detection of the agriculturally important C. beticola infection of sugar beet, with both thermography and chlorophyll fluorescence imaging. Both techniques also enabled visualization of pre-necrotic symptoms in the HR of tobacco to TMV. Interestingly, these plant–pathogen interactions could be distinguished on the basis of the imaging measurements, before visual damage appeared. This indicates the potential of combined thermal and chlorophyll fluorescence imaging to generate specific signatures for plant–pathogen interactions, usable as a fingerprint for early disease identification. Early detection of diseases, as well as other stresses, will be beneficial for optimal crop management. We will first discuss the observations made on the two plant–pathogen interactions, and then expand on the wider applicability of multispectral presymptomatic imaging.

Thermal imaging

The HR to TMV infection in tobacco can be detected thermographically as rapidly expanding localized hot-spots, about 8 h before the appearance of visible co-localized necrosis (Chaerle et al. 1999; Fig. 1). However, in contrast to the reaction to TMV infection, the early phase of C. beticola attack in beet is not characterized by a raise in temperature, but rather by an immediate cooling (Fig. 4b; Suppl. Fig. 5). Cercospora infection sites display a lower temperature for approximately 1.5 d. The development of visual necrotic symptoms at TMV-infected sites is accompanied by a decrease in temperature in the centre of the hot-spot. During on average 1 d of further lesion development, the hot spot stays detectable as a warm halo.

Infection of a tobacco leaf with TMV proceeds by virus ingress through damaged cells associated with surface wounds. The HR to TMV first affects the infected epidermal cells, which finally burst. This was microscopically observed as a loss of turgor and collapse of the epidermal layer, before cells of the mesophyll succumb to the infection (Wright et al. 2000). The evaporating epidermal cellular liquid contents accounts for the low surface temperature appearance of infected spots displaying visible necrosis. Strikingly, stomata do not collapse during the HR. However, the measured decrease in temperature cannot be solely related to reopening of the stomata due to
loss of turgor of the epidermal cells (Blatt et al. 1999), since the epidermal layer has already disintegrated by that time.

*C. beticola* infection of beet occurs by penetration of open stomata by germination tubes growing from spores. According to histological information on *Cercospora* infection (Lieber 1982), the mycelium then grows into the mesophyll leaving the epidermis intact. The local decrease in temperature (and thus increase in transpiration; Fig. 4b) could be due to toxin-induced collapse of mesophyll cells and evaporation of cellular fluid within the substomatal cavity. The *C. beticola* fungus produces toxins that influence cell membrane permeability by different mechanisms, including membrane peroxidation for cercosporin, pore formation and inhibition of plasma membrane proton pumps in the case of beticolin. These effects finally cause cell membrane rupture, leading to cellular collapse (Goudet et al. 2000). Alternatively, direct effects of the infection on stomatal aperture could be involved. Infection of plants by the pathogens producing fusicoccin and necrosis-inducing peptides cause stomata to open by stimulating plasma membrane proton pumps (Hammond-Kosack et al. 1996). No such compounds are known from *C. beticola*. Stomatal closure occurs if membranes of stomata get depolarized by the presence of certain pathogen toxins (Digioigio et al. 1996). After *C. beticola* infection no initial increase in local leaf surface temperature could be recorded at *Cercospora* infection sites. On the other hand, an increase in stomatal opening can also be explained on a purely mechanical basis. Stomata normally open by an increase in turgor and swelling of the two guard cells. If the surrounding supposedly intact epidermal cells lose turgor (possibly through toxin-induced depolarization), it is possible that the stomata further open by absence of an equilibrating pressure (Blatt et al. 1999).

At a late stage of the HR to TMV, when the infection is contained, and the visible lesion has reached its maximal size, the affected leaf area starts to dry. This causes the local surface temperature to rise above that of the surrounding healthy, transpiring surface (Fig. 1f). The same drying phenomenon was visualized during *C. beticola* lesion development (Fig. 4c, d).

**Chlorophyll fluorescence imaging**

The first signs of an increase in local chlorophyll fluorescence intensity at TMV-infection sites were visible as whitish spots, co-localized with and appearing slightly before the thermal hot-spots (Fig. 1b, c). The pre-necrotic spots of higher intensity were only visible in the $F_4$ images (low intensity excitation). The photosynthetic system in these regions could already be light saturated at the low intensity excitation light level, probably due to (partial) inhibition of Photosystem II (PSII) photosynthetic electron transport. The affected tissue thus stands out from the surrounding not light-saturated tissue by its higher chlorophyll fluorescence. After high intensity excitation ($F_6$ images) both regions would be saturated, and thus emit a comparable amount of chlorophyll fluorescence. In contrast with the HR to TMV, the whitish fluorescence symptoms at *Cercospora* infection sites increase in intensity with higher excitation intensity ($F_6$ versus $F_4$) (see Fig. 4b). This likely indicates that the highest excitation light level ($F_6$ image) does not saturate the surrounding unaffected sugar beet tissue; consequently fluorescence emission is not maximal. The observed local increases in chlorophyll fluorescence might also be caused by a local minor chlorophyll level decrease at infection sites, by diminishing the likelihood of chlorophyll fluorescence reabsorption within this leaf region (Gitelson et al. 1998). However, measurements on the reflectance images do not indicate a change in intensity or color until 58 h after infection, when a decrease in chlorophyll fluorescence intensity is already clearly observable (see Fig. 1d, e). The subsequent increase in reflection is consistent with the paling of the tissue (Fig. 1e, f). The percent increase in reflection is higher for red and blue light when compared to green light, resulting in the fading of the green color at the infected spot, as expected during chlorophyll breakdown.

The accumulation of pathogen or resistance specific compounds could also alter fluorescence emission. The toxin cercosporin produced by *Cercospora* species has a maximum fluorescence emission around 600 nm, followed by an exponential decrease to zero at 700 nm (Daub et al. 1992). The long-pass filter on the FIS transmits 5% light at 680 nm, with 50% transmittance at 695 nm. It is thus unlikely that cercosporin fluorescence contributes significantly to the increase in fluorescence observed in sugar beet.

During the TMV–tobacco interaction, whitish fluorescence spots expanded more slowly when compared to the observations of the ‘hot-spots’ with thermography (see Fig. 1g). It was previously shown that the observed local temperature increase was caused by stomatal closure, presumably through local accumulation of SA (Chaerle et al. 1999). Chl-FI imaging, however, did not detect any local change in fluorescence intensity after topical SA treatment of tobacco leaves, although parallel thermal imaging revealed a local temperature increase (see Fig. 3). Stomatal closure did thus not lead to an increase in chlorophyll fluorescence emission through photosynthetic inhibition by limiting the diffusion of CO$_2$ (Meyer et al. 2001). Taken together with the different expansion kinetics of the thermal and chlorophyll fluorescence effects (Fig. 1g), this likely indicates that distinct processes are responsible for the respective changes in transpiration and photosynthetic electron transfer.

The appearance of visual symptoms is linked to the degradation of chlorophyll, resulting in discolouring of the local infection spots. As a consequence, chlorophyll fluorescence emission is expected to decrease sharply and to drop to zero when lesions become visible. Fluorescence indeed decreased to near-zero levels coinciding with visual lesion development both in the HR to TMV (Fig. 1e–f: black spots) and during *Cercospora* infection (Fig. 4b: single black spot). However, at TMV infection sites a striking decline in chlorophyll fluorescence emission already became apparent at least 20 h before
local discolouration was visible, as witnessed by the appearance of grey spots corresponding with the infection sites. Infections might alter the distribution of light energy absorbed by chlorophyll among the three competing processes: photosynthetic assimilation, chlorophyll fluorescence and thermal dissipation. In the PSII antenna and the PSII reaction centre, chlorophyll is associated with protein complexes. Conformational changes in these light-harvesting complexes can lead to a shift in the share of each process (Elrad et al. 2002). Changes in fluorescence emission could result from alterations in the PSII reaction centre, as for example mentioned in a study on pre-necrotic TMV-infected tobacco leaves. In this research, a decrease in photosynthetic electron flow was measured in a thylakoid membrane assay (Seo et al. 2000). Concomitantly the expression level of a chloroplast-located protease, likely involved in the removal of damaged D1 protein from the PSII reaction centre, decreased. During the HR of Asparagus mesophyll cells to the G-protein activator mastoparan, large decreases in $F_{m}'$ (fluorescence emission after 500 ms of saturating illumination superposed on continuous illumination with 60 $\mu$mol m$^{-2}$ s$^{-1}$ PAR) and in $F_{m}$ (same measurement as $F_{m}'$, but in darkness) were measured using a non-imaging pulse-amplitude modulated fluorometer (Allen et al. 1999). A reduction in chlorophyll fluorescence emission, through increased thermal dissipation, was attributed to a decrease in the antenna size of PSII. In the study presented in this paper on TMV-infected loci during early HR, pre-necrotic decreases in $F_m$ (fluorescence emission after 1 s of saturating illumination of a dark-adapted leaf) were measured using a non-imaging PEA fluorometer (see supplementary Fig. 1). This result is similar to the described decrease in $F_m$ for mastoparan-induced cell death. In addition, the large decrease in $F_{m}'$ observed during mastoparan-induced cell death can be compared to the presymptomatic local fluorescence decrease imaged at the TMV-infection sites, clearly apparent in the $F_{m}$ images captured after 1 s of high-intensity illumination after pre-illumination (Fig 1c, d). Given these similarities with the mastoparan-induced HR, the strong decrease in fluorescence emission upon the HR to TMV could thus be explained by changes in the conformation of the PSII antennae complexes that increase the probability of thermal energy dissipation over chlorophyll fluorescence emission. Reactive oxygen species, which are produced during both the HR to TMV in tobacco (Lam et al. 2001) and the necrotic reaction to C. beticola in beet (Skaracić and Biancardi 2000), could be the underlying trigger. These reactive molecules can cause extensive damage to various components of the photosynthetic system, and could thus induce a presymptomatic increase in fluorescence in both plant–pathogen interactions.

**Imaging of excised leaf pieces**

Local increases in chlorophyll fluorescence were measured in excised beet leaf pieces spray-infected with C. beticola (Fig. 5). The intensity of the fluorescence symptoms was lower in Cercospora-infected excised leaf pieces as compared to attached leaves (data not shown). Likely, the direct contact of the abaxial side of the excised beet leaf parts with the gel surface causes the leaf piece to react differently compared to intact attached leaves. This different environment (probably with limited gas exchange) might cause a general change in the photosynthetic system of the excised leaf tissue, limiting the detectable amplitude of the reaction to Cercospora infection. Moreover, presymptomatic chlorophyll fluorescence increase was not visible for infected leaf discs floating on water, in accordance with our observation with TMV-infected leaf discs (Fig. 2). Flotation on water likely has a more profound effect when compared to the gel-assay, rendering the detection of higher intensity fluorescence emission impossible in the conditions used. The high-contrast visualization of the low intensity spots associated with necrosis is clearly feasible in both setups (Fig. 2b, 5d).

**Perspectives**

The ability of Chl-FI and thermal imaging to monitor a larger affected area at early time-points, compared to visual assessment, could be exploited for early detection of localized infections. Applying Chl-FI and/or thermal imaging, in combination with (semi-)automatic quantification of presymptomatic symptoms, can thus be applied to accelerate the screening of plant populations for resistance. At later time points, the first manifestations of necrosis were monitored with higher contrast by Chl-FI compared to thermography and reflectance imaging. This high-contrast visualization of cell death by Chl-FI can be applied for quantitative assessment of necrosis expansion on leaf pieces or attached leaves.

In the presymptomatic stage, both the here described TMV and Cercospora infection first cause a local increase in chlorophyll fluorescence, but only TMV causes a subsequent decrease in chlorophyll fluorescence before visual symptoms appear. TMV and Cercospora infection can be easily distinguished respectively by their “hot” and “cool” thermal effects at the infection site. These contrasting signatures obtained for the TMV and Cercospora infections illustrate the possibilities of multispectral imaging to narrow down on the causal agent of early-detected stress symptoms on different plant species. This concept can be extended to distinguish the different pathogens of a given crop and could, after lab-scale validation, be applied for surveillance in nursery, greenhouse or field cultivation. Addition of hyperspectral imaging, covering the visual and near-infrared spectral regions, would further expand the discriminating power of such a set-up, and thus allow rapid non-destructive identification of the stress agent.

**Material and Methods**

**Plant material**

Tobacco plants (*Nicotiana tabacum* L. cv. Xanthi NN, resistant to TMV and Xanthi nn, susceptible to TMV) were grown in a walk-in chamber at 21°C, 60–70% relative humidity and under a 16/8h light/
dark cycle. Fluorescent tubes (Philips TLD 33; Koninklijke Philips Electronics N.V., Eindhoven, The Netherlands) provided 50±10 μmol m⁻² s⁻¹ photosynthetically active radiation (PAR). When at the 8th leaf stage, plants were transferred to the measuring room for imaging under continuous light.

Sugar beet plants (Beta vulgaris L.) susceptible to C. beticola Sacc. infection (susceptible tetraploid pollinator line) were grown under the same conditions as described above for tobacco.

Infection and treatments

TMV inoculum was obtained by grinding tissue from N. tabacum L. cv. SR1 plants (susceptible to TMV) infected with TMV strain U1 (Yalpani et al. 1991); 0.1 g tissue ml⁻¹ phosphate buffer (20 mM, pH 7). For imaging of local lesion development, single lesions were obtained as previously described (Enyedi et al. 1992). One leaf per plant (Nicotiana tabacum L. cv. Xanthi NN), was inoculated using this method, with fine sand as an abrasive. Leaf discs with a diameter of 30 mm, centred on localized infection sites, were cut with a scalpel, excluding the main vein, and placed floating on water in six well plates (Falcon 353046, Becton Dickinson Labware, NJ, U.S.A.), one disc per well. Leaves from control plants were mock inoculated with extracts from grinded healthy SR1-tobacco tissue.

SA (3 mM in 20 mM phosphate buffer, pH 7) droplets of 50 μl were applied with a pipette (Gilson Pipetman P) to the leaf surface of supported tobacco leaves.

C. beticola inoculum (monosporic strain) was obtained from the Plant Breeding Institute, Christian-Albrechts University, Kiel, Germany. Petri plates containing Cercospora were rinsed with sterilized water. Spore concentration was estimated microscopically with a counting chamber (Bürker type). The solution was diluted to 300,000 spores ml⁻¹. At the 6th leaf pair stage, the upper side of a leaf from the 4th leaf pair was sprayed with the Cercospora spore solution, using a handheld sprayer (Preval, Precision Valve Corp, NY, U.S.A.). Control leaves (second leaf from the selected 4th leaf pair on the same plant) were sprayed with sterilized water. Subsequently the treated plants were placed for 2 d in a high-humidity (100% relative humidity, no condensation) plastic enclosure, with a 16/8 h light/dark cycle. Thereafter the plants were transferred to the measuring room with continuous illumination. Alternatively, leaf strips of 10×70 mm were cut with a scalpel, excluding the main vein and placed on low-density agar (60% containing 20 μm benzyladenine (Sigma, St. Louis, MO, U.S.A.) to prevent early discolouring; in 0.2×0.2 ml Petri dishes (Corning Bioassay plates, VWR International).

Imaging set-up

We previously described the imaging set-up as used in this work (Chaerle et al. 2003) and briefly summarize here it for clarity. Measurements were done in a custom-made walk-in chamber with built-in gantry (Cartesian XYZ) robot with 2×1×0.5 m working area. The temperature in this room is controlled within 0.1°C stable environmental conditions for thermal imaging. Plants were kept at continuous light (60 μmol m⁻² s⁻¹ PAR from fluorescent tubes), 60–70% relative humidity and 21°C. A thermal camera (FLIR-Agema THV900LW, FLIR Systems, Portland, OR, U.S.A.), video camera (Watercolour CCD, LCL-217HS, Las Vegas, NV, U.S.A.) and fluorescence imaging system are mounted together at the end of the vertically moving Z-axis of the robot. These cameras were positioned sequentially above the same plant leaf region to capture the respective co-localized images.

Tobacco leaves were supported horizontally by fine nylon wire to allow plan-parallel imaging. This set-up helped to achieve a more homogeneous illumination with the horizontally fixed FIS. Two opposing beet leaves belonging to the same selected leaf pair were positioned horizontally per plant, supported by plastic coated metal gauze. A second layer of gauze was placed on top, and lightly fixed to prevent beet leaf movements.

The FIS consists of a black and white CCD camera (Pragmatic, U.S.A.) fitted with a cut-off high-pass red filter (B+W 092) to detect light above 650 nm, surrounded by a ring of six halogen lights of alternating 20 and 50 W power, each shielded by a blue cut-off low-pass filter (Schott BG-39) to provide excitation light below 650 nm, with an excitation maximum at 500 nm. The fluorescence image capture was carried out according to the following protocol. First, leaves were illuminated with 250 μmol m⁻² s⁻¹ PAR. After 1 s the F₂ fluorescence image was captured while the lamps stayed on (low intensity excitation F₂ image; three 20 W lamps on). Immediately following this image capture the additional three 50 W lamps were switched on while the 20 W lamps stayed on. After 1 s of illumination with 1,000 μmol m⁻² s⁻¹ PAR the second fluorescence image was captured (high intensity excitation F₂ image; all six lamps on). After completion of the image capture, all lamps were switched off. At the start of the experiment, the measuring unit was programmed to position the FIS at a constant distance of 80 mm from either selected plant leaf areas or dishes with excised leaf pieces. At this distance, the FIS measuring head blocks light from the overhead chamber illumination (remaining light level < 1 μmol m⁻² s⁻¹ PAR).

The FLIR/Agema THV900LW camera (pixel resolution 272×136) was used to capture the images of the TMV infection process; a FLIR SC-300 camera (pixel resolution 160×120) was used for imaging of the SA-treatment; the Cercospora infection process was monitored with a FLIR SC-2000 camera (pixel resolution 320×240). The pixel resolution of the fluorescence and colour images was 300×300 pixels. ImageMagick (www.imagemagick.org) and ImageJ (rsb.info.nih.gov/ij) software packages were used to register (adjust for the difference in field of view and magnification between the three imaging sensors) and process the images from the different optical sensors. The shown fluorescence images were normalized for better visualization. The described measurements were carried out in ImageJ. Intensity was measured on the individual RGB channels of the colour reflectance images. Colour reflectance images were captured under chamber illumination.

Non-imaging chlorophyll fluorescence measurements

Measurements were carried out in the above-described measuring room with a portable PEA (Hansatech, King’s Lynn, U.K.). The measured area was dark-adapted for 20 min before each measurement. The exact same TMV- or mock-infected positions were measured during a period of 1 d.

Supplementary Material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org. In addition to the supplementary material referred to in the text, animations of the infection processes will be made available at http://allserv.ugent.be/~ichaerle/TMV/Cercco/

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