Infrared detection of early biotic and wound stress in plants

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Introduction

Based on previous results, demonstrating that spraying of tobacco leaves with salicylic acid (SA) induced an increase in leaf surface temperature (1), we hypothesised that the endogenous production of SA during the hypersensitive response of resistant tobacco to tobacco mosaic virus (TMV) (2) would also lead to an increase in leaf temperature, associated with the infection sites. Moreover, since mutant and transgenic plants in which cell death spontaneously occurs were known to accumulate high levels of SA, we expected to visualise a comparable phenomenon in these plants. The possibility to identify plant tissue committed to cell death (either pathogen-induced or spontaneous) by thermography would enable specific sampling. Early events could then be characterised biochemically and molecular-genetically, before the appearance of the resulting cell death.

The model system resistant tobacco - tobacco mosaic virus (2) was used for thermographic visualisation of a plant - pathogen hypersensitive response. In initial experiments, using a

Summary

When plants are able to recognise an attacking pathogen, they mount a hypersensitive response, characterised by cell death at the site of infection. In certain mutant and transgenic plants, such a process occurs spontaneously. In the model system of resistant tobacco challenged with tobacco mosaic virus, an increase in surface temperature was thermographically observed, localised within the infection sites, before any visual cell death symptoms became apparent. In addition, an increase of leaf surface temperature was visualised immediately after infection. This response was independent of the presence of tobacco mosaic virus, and is caused by abrasion damage during the infection procedure. In conclusion, thermography has a clear potential to visualise early plant leaf responses to wounding and to infections resulting in cell death.

Key words: thermography, robotisation, plant transpiration, pathogen infection, wounding, cell death
random infection method and low-resolution thermal pictures, indications for a localised increase in temperature were observed. By optimising the infection method and the measuring conditions, the colocalisation between the thermal effect and the visual cell death symptoms was proved (3). The thermal response was characterised by a presymptomatic appearance of ‘hot-spots’ at the sites of infection (on average 8h before pinpoint cell-death lesions were visible by eye). A maximal temperature increase of 0.4 °C was measured at the centre of the thermal spots. The expansion of the thermal effect was very rapid, when compared with the visual symptoms. The maximum size of the thermal effect was reached after 2 days, whereas the visual symptoms needed on average 7days to expand to the same final size. When visual symptoms appeared, co-localised regions of lower temperature became visible at the centres of the ‘hot-spots’ in the thermal images.

Cell death is known to be associated with bursting of cells and hence evaporation of their contents, explaining the lower local temperature. When growing resistant tobacco plants infected with TMV at temperatures above 28°C, virus multiplication is not inhibited (4). When ‘shifting’ such plants back to 21 °C, a massive resistance response is mounted by the plant, resulting in both more rapid and more extended cell death, when compared with plants that were kept at 21 °C. Temperature-shifted plants displayed a more rapid expansion of the thermal effect and a shorter time span between thermal effect and visual cell death (3).

Two processes were studied that were thought to be responsible for the increase in leaf-surface temperature: respiration and transpiration. SA is known to induce the metabolic upsurge in flowers of thermogenic plants by increasing respiration, mediated by the non-energy conserving alternative respiration pathway (5). At the moment of visual lesion appearance, infected tobacco leaf tissue displayed higher levels of total and alternative respiration than control tissue. However, taking in account the small amount of heat produced and the physical characteristics of a leaf blade (6), metabolism was estimated to account for maximum 1% of the measured temperature increase. In general, metabolism is negligible in the energy balance of a plant leaf; on the other hand, transpiration has a major share in it. Plant leaves have tiny valves called stomata, which optimise CO₂-uptake for photosynthesis and minimise water loss by transpiration. SA is known to induce the closure of these leaf ‘pores’ in certain species (7). A decrease in transpiration causes a rapid increase in leaf-surface temperature. By using infrared gas analysis (IRGA) equipment, it was proven that the onset of the increase in leaf temperature after TMV-infection of resistant tobacco, as observed by thermography, coincided with a decrease in transpiration (3). The robotised thermography system permitted to visualise the response of several plants, allowing for the specific sampling of plant tissue during the early hypersensitive response. A time-course of accumulation of SA was obtained with a time-resolution of 1h. The start of accumulation of SA correlated with the emergence of the local increase in surface temperature. We therefore concluded that the observed local increase in leaf temperature was mainly due to a decrease in transpiration, presumably caused by the accumulation of SA or other induced compounds.

Tobacco plants transformed with a bacterial proton pump (bacterio-opsin, bO) were described to spontaneously form islands of cell death on their older leaves (8). When thermographically visualising these plants, several other cell-death ‘phenotypes’ were observed. In the case of isolated flecks, reminiscent of the TMV-infection loci, a thermal effect was visualised before appearance of cell-death. In most cases however, cell death was visualised as a front of higher temperature that moved from the leaf base to the leaf tip, ahead of the expanding cell death (9). When the propagating thermal front reached the measuring chamber of the IRGA-equipment, transpiration started to decrease. When cell death became apparent on the measured leaf area, transpiration increased again. Also in mutant Arabidopsis plants that spontaneously display lesions simulating disease (lsd) (10), a presymptomatic increase in leaf temperature was observed before the cell death lesions became clearly visible.

Methods

Plant material and infection

Tobacco plants (Nicotiana tabacum L.) cv. Xanthi NN (resistant to TMV infection) and cv. SR1 (cultivar susceptible to TMV) were grown in a growth room at 21 ± 1°C with 60 ± 10% RH and under a 16-h/8-h light/dark cycle. TMV inoculum was prepared from SR1 plants infected with TMV strain U1. Leaf edges and
main veins of the selected leaf from Xanthi NN plants were copied on a parafilm sheet. The desired infection pattern was created by punching holes in the parafilm leaf outline. The parafilm sheet with infection grid was then placed on the leaf and the infection was carried out at each defined site by applying 0.5 µg of fine sand and 0.5µl of inoculum followed by gentle abrasion with a fine glass rod. As a control, mock-inoculation was similarly carried out with ground healthy SR1 tissue. Alternatively, regions between side-veins where infected as a whole by powdering with sand and applying several 0.5µl droplets before abrasion with a bent glass rod (the bent-over part matching approximately the width of the infected area).

After infection, the plants were placed in the custom-built measuring chamber set at 32 °C to allow virus multiplication. After 1 day temperature was decreased to 21 °C to induce the resistance response (temperature shift experiment). Experiments without temperature shift were carried out at 21 °C.

Robotised thermal imaging of plant leaves

At the start of this research project, it proved impossible to carry out reproducible measurements over long periods of time either due to diurnal or climatisation-induced temperature fluctuations. In addition, the resolution of the used FLIR/Agema THV900LW Stirling cooled thermography system proved insufficient to image several attached leaves from different tobacco plants simultaneously. To correlate the thermal signature of the leaf with visual symptoms of the infection, a customer-grade video camera was used. However, a fixed viewpoint combined with a short object distance introduced important parallax-deviations between thermal and video images.

To circumvent these 3 problems, a water-cooled cabinet with built-in Cartesian positioning system for both cameras was designed and installed. A manual teach-in procedure is used to enter the image-capture positions for the thermal camera. The system then captures thermal and video images at fixed intervals of 1 hour for the duration of the plant response to the infection (typically one week).

After each capture cycle, thermal and video images are transferred over a local network, and processed on a Solaris8 Intel workstation. Thermal images are converted into PC bitmap images, with a user-defined region of interest on the leaf surface as a reference. For each studied plant, thermal and video images are combined into a concatenated image, to allow for rapid detection of changes. This visualisation method proved ideal to guide sampling of tobacco tissue for SA-determination, before visual symptoms became apparent (thermography-aided tissue sampling). Each day, concatenated ‘overview’ images are converted into MPEG image sequences, allowing visualisation of the evolution of the thermal effect.

Results

TMV infection depends on small wounds at the surface of the tobacco leaf. To guarantee successful infection an abrasive as sand or boronundum is commonly used.

In experiments conducted to optimise the localised (spot wise) TMV infection method, the effect of superficial wounding through abrasion was visible as cold, dark regions in the thermal images. While no visual damage was apparent, the low temperature at the infection spots could remain visible up to the time of emergence of the thermal effect due to TMV infection. An example of successful infection with minimal abrasion damage is shown in figure 1.

The upper left panel of Fig. 1 shows a fast increase in temperature 30 minutes after infection, attaining 0.6 – 1 °C above the temperature of the surrounding leaf tissue. This effect reaches a maximum difference of 1.1 – 1.6 °C at 1 hour post infection (right panel on the top row). This effect passed undetected in previous experiments, due to later start-up of the imaging procedure. In both thermal images, the uninfected tissue has a heterogeneous ‘patchy’ appearance with surface temperature varying from 27.1 to 28.6 °C. Leaf margins and tip show a higher temperature of 28.5 to 30°C. The main vein also has a higher temperature ranging from 28 to 28.8 °C. Leaf margins and tip show a higher temperature of 28.5 to 30°C. The main vein also has a higher temperature ranging from 28 to 28.8 °C. The spots of higher temperature loose intensity very quickly thereafter. In the lower left panel taken 8 hours after infection, the commonly observed cold spots at the sites of infection are clearly discernable and are 0.3 – 0.4 °C colder than the unaffected tissue. Thereafter the pattern of lower temperature spots disappeared gradually. One day after infection (lower right panel) the leaf tissue between the major veins has again a uniform appearance. Comparing the ‘TMV’ pattern in the 3 first panels from Fig. 1 with the pattern in
the thermal images from Fig. 2 proves that the early temperature increase, the ensuing temperature decrease and the thermal effect preceding visual damage are precisely in the same place. An early higher-temperature effect due to tissue damage was visualised after both infection and mock infection of leaf areas (see Fig. 3), proving that this immediate thermal signature was due to local wounding. A higher level of abrasive wounding (lower temperature in upper right panel Fig. 3) correlates with a higher level of infection (higher temperature in lower right panel Fig. 3). Additional animations of this experiment are available at the PlantIR website: www.plantgenetics.rug.ac.be/PlantIR/Thermology.

TMV-infected tobacco plants show a faster expanding and more intense thermal effect when first grown at 32°C and then shifted to 21°C. The temperature shift was initiated 1 day after infection. Eight hours later, the already described thermal effect has reached its maximum extension and the temperature difference with the surrounding tissue amounts 0.3 – 0.4 °C. (Fig. 2, left panel). On the video image in the top right panel no effect is detectable. Five days after infection (lower panel) the extent of visual damage (necrosis) corresponds with the thermal signature of infection of the upper panel. In the accompanying thermal image, some ongoing necrosis is still visible as dark spots of 20 - 20.3 °C, which was 0.2 – 0.3 °C below the temperature of the unaffected surroundings. The dried regions at the infection sites range from 20.6 – 20.9 °C. At this stage of the resistance response, parts of the leaf blade are bent due to side-vein damage. These changes in orientation are reflected in the temperature distribution over the leaf blade. The upper central part of the leaf has temperatures of 20.9 - 21.1 °C, whereas the lower central part only reaches 20.3 – 20.4 °C.

Discussion
The presymptomatic visualisation of TMV infection was proved by co-localisation of the early thermal effect and later necrotic cell death (3). A thermal effect was expected to occur at
Figure 2.
Thermal (left) and visual spectrum (right) images of an attached tobacco leaf, infected with TMV using a localised infection method. After infection, the plant was grown at 32 °C, and then submitted to a temperature shift to 21 °C. In the upper images captured 8h post temperature shift (pTS), the thermal effect has reached its maximum extension and temperature increase (0.3 - 0.4 °C), while no visual effects are apparent. 5 days after the temperature shift (lower images), the visible pattern of cell death has reached its final extent. Each panel was composed from three adjacent images as shown in figure1. Animations of the whole infection process are available at the plantIR website.

Figure 3.
Thermal images of an attached tobacco leaf, infected with TMV using a region infection method. Infection and imaging were carried out at 21 °C. The upper left image shows the leaf surface temperature distribution 30 minutes before infection (average temperature 21.2 °C). One hour after infection (upper right panel) the untreated basal part of the leaf has an average temperature of 20.8 °C. Some areas have a high temperature (0.5 °C higher), while others are considerably colder (0.4 °C lower). The leaf surface has again a pre-infection appearance 18.5 h post infection (pi), with the same average temperature of 21.2 °C. From the lower right panel (40.5h pi) infected regions can be discerned from mock-infected regions by their elevated surface temperature (0.3 – 0.5 °C difference). Animations of this experiment are available at the plantIR website.
the time of salicylic acid accumulation – as shown previously. In addition to this infection-specific local temperature increase, a local increase in temperature was apparent immediately after infection or mock-infection. The effect was only transient and gradually reverted, resulting in cold spots at the infection sites (Fig. 1). Tissue damage is expected to cause a drop in leaf surface temperature, since the contents of bruised cells will evaporate and locally cool the surface until the wound has dried. An analogous, yet more pronounced phenomenon occurs during pathogen-induced and spontaneous cell death (9). The thermal effect specifically associated with TMV-infection results from stomatal closure and associated decrease in transpirational cooling. Transpiration of regions of attached tobacco leaves was measured using infrared gas analysis (IRGA) equipment. Small measuring chambers, through which air is circulated, are clamped on the leaf. The measured increase in water vapour content indicates the amount of transpiration of the enclosed leaf region. By using this technique the process leading to the temperature changes was clearly identified.

Likely the local increase in leaf surface temperature immediately after infection also results from a decrease in transpiration. The infection method using abrasion probably disturbs stomata at the infection site causing a temporary closure. After a few hours the stomata could start function normally again, allowing the damage at the surface to be detected as smaller lower temperature spots. Since the early temperature increase was thermographically visualised in both infected and mock-infected regions (Fig. 3), a wounding-only explanation is prevalent. Direct transpiration measurements were not attempted immediately after infection since the leaf is still partly moist after rinsing. The plants were infected outside the measuring room at 21 °C, and then placed at 32 °C. As can be seen from the upper 2 thermal images in Fig.1, the uninfected leaf tissue has a heterogeneous appearance due to this temperature change and to the evaporation of residual rinsing water. The infected leaf regains its stable temperature distribution before 8h after infection (compare the upper 2 images in Fig1 with the 2 lower and the thermal images from Fig. 2). As can be seen from Fig. 3, a high level of infection is correlated with the amount of abrasive damage. At levels of abrasion that result in homogeneous infection of leaf areas, no temperature increase immediately after infection can be detected. This suggests that the wounding effect masks the stomatal closure response. The level of surface damage visible at the left in the upper right panel of Fig. 3 does not lead to later visual damage. This can be deduced from the ‘recovery’ visualised as a gradual disappearance of the temperature difference with the adjacent leaf regions.

Thermography clearly permits early and high-contrast visualisation of wounding, localised infection and cell death. In addition, the robotisation of combined thermal and video imaging allows to visualise the evolution of these responses over time. Given the importance of the cell-death response in the resistance to pathogens, robotised thermography could become a screening tool to search for mutants in the cell death response pathway.

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References


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