Visualization of early stress responses in plant leaves

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ABSTRACT

Plant leaves possess 'microscopic valves', called stomata, that enable control of transpirational water loss. In case of water shortage, stomata close, resulting in decreased transpirational cooling. The ensuing temperature increase is readily visualized by thermography. Salicylic acid, a central compound in the defense of plants against pathogens, also closes stomata in several species. In previous work, thermography permitted to monitor an increase in temperature after infection of resistant tobacco by tobacco mosaic virus, before visual symptoms appeared. Furthermore, cell death was visualized with high contrast in both tobacco and Arabidopsis. In addition to transpiration, photosynthetic assimilation is a key physiological parameter. If the amount of light absorbed by chlorophyll exceeds the capacity of the photosynthetic chain, the surplus is dissipated as light of longer wavelength. This phenomenon is known as chlorophyll fluorescence. If a plant leaf is affected by stress, photosynthesis is impaired resulting in a bigger share of non-utilized light energy emitted as fluorescence. The potential of an automated imaging setup combining thermal and fluorescence imaging was shown by monitoring spontaneous cell death in tobacco. This represents a first step to multispectral characterization of a wide range of emerging stresses, which likely affect one or both key physiological parameters.

Key-words: thermography, visible-light-induced chlorophyll fluorescence imaging, transpiration, photosynthesis, incompatible plant pathogen interaction, salicylic acid, spontaneous plant cell death

1. INTRODUCTION

Plants control the transpiration stream that enables uptake of water and nutrients from the soil by regulating stomatal aperture. Upon drought, levels of the hormone abscisic acid (ABA) increase and cause stomatal closure\textsuperscript{6}. Thermal imaging was used to screen for barley mutants unable to respond to ABA\textsuperscript{5}. Recently, the interest for such screenings increased again\textsuperscript{4}. Historically, thermography was first used for plant research in a remote-sensing context\textsuperscript{7}. In the mid seventies, a thermal imaging system was used at lab scale to study the necrotising damage caused by tobacco mosaic virus\textsuperscript{8}.

Plants of some so-called thermogenic species increase the temperature of their flowers by increasing respiration. The kinetics of these processes have been recorded by thermal imaging\textsuperscript{9}. Salicylic acid (SA) was discovered to trigger the metabolic upregulation resulting in the observed heat-production. Thermal imaging indicated that SA increased the temperature of leaves when applied by spraying on tobacco leaves\textsuperscript{10}. In addition, SA was shown to close stomata in other species, including common bean\textsuperscript{11}. These data were obtained by microscopical observation and measurement of the increase in humidity of air circulated through cuvettes clamped on leaves (direct transpiration measurements). Importantly, salicylic acid is a signaling compound in the defense reaction of plants to pathogens\textsuperscript{12}. After infection of resistant tobacco by tobacco mosaic virus (TMV), the model system for incompatible plant-pathogen interaction, SA rises to high levels before any visual necrotic symptoms become apparent\textsuperscript{13}. During this plant-pathogen interaction, presymptomatic increases in temperature were visualized which coincided with the later occurring necrotic cell death\textsuperscript{14}. Thermal imaging permitted to discern the increase in temperature 8 hours before the emergence of pinpoint necrotic lesions. More important, 2 days after infection, the area with increased temperature reached its maximal extension, corresponding to the final necrotic lesion formed 4 days later. Direct transpiration measurements proved the major share of the temperature increase was due to stomatal closure. In support of this finding, plant leaves have a high surface to volume ratio, making it impossible to accumulate metabolic heat\textsuperscript{15}. The process of cell death in response to invading pathogens is mimicked in spontaneous cell death mutant or transgenic plants\textsuperscript{16,17}. Thermal imaging monitored a
temperature increase before the appearance of the spontaneous cell death\textsuperscript{16}. In addition, the cell death process is visualized with high contrast, since the dying tissue evaporates water from disrupted cells, and appears in dark shades in thermograms. Thermography thus clearly visualizes pending damage to plant leaves. This would enable early monitoring of disease outbreaks or screening for increased disease-resistance.

Chlorophyll fluorescence imaging was shown to be a second straightforward way for detection of stress\textsuperscript{17}. Different fluorescence imaging systems (FIS) have been developed, and applied to study biotic or abiotic stress\textsuperscript{18-20}. Light energy absorbed by the chlorophyll pigment in plant leaves is used for photosynthetic carbon assimilation. Energy captured in excess of the capacity of the photosynthetic system is dissipated as emission of light or heat\textsuperscript{21}. When stress - either biotic or abiotic - causes the photosynthetic capacity to decrease, the emission of light (and heat) increases for equal illumination conditions. Chlorophyll re-emits light at specific wavelengths. When excited with visible light, fluorescence is emitted at red (690nm) and far-red (740nm) wavelengths. By equipping the visible light source with a cut-off low-pass blue filter and placing a cut-off high-pass red B+W 092 filter in front of the CCD camera chlorophyll fluorescence can be recorded without interference from excitation light. When using UV-excitation, fluorescence from other leaf constituents can be recorded in addition to chlorophyll fluorescence\textsuperscript{22}.

By combining two imaging techniques each monitoring a major plant physiological parameter, it is expected that many stress situations will be detected at early stages, enabling timely control measures. In addition, monitoring of differences in the kinetics of stress responses among populations of plants could be useful in breeding programs for selection of plants more resistant to stress\textsuperscript{23,24}.

\section{METHODOLOGY}

\subsection{Imaging systems}

A custom-designed measuring chamber was built, in which a constant temperature within 0.1ºC limits is obtained. Inside this chamber a Cartesian positioning system was installed (See fig.1). The thermal imaging system (FLIR-Agema THV-900 LW - Stirling cooled) and a color CCD-camera were fitted one next to the other on a supporting structure at the basis of the Z-axis. Recently a chlorophyll fluorescence imaging system (FIS), consisting of a black and white CCD-camera and a circular high-intensity illuminating system consisting of small halogen bulbs was mounted on the system. Imaging positions are entered into the system by a teach-in procedure, using the thermal or video camera for visualization. The PLC controlling the robot can be set to automatically generate imaging positions for the color-CCD camera and FIS system (see Fig. 2). The resulting images captured from the 3 cameras are thus co-localized. If necessary, these images can be further aligned by using image registration procedures. After teach-in programming, the robot is made to run the programmed image capture cyclus at a fixed time interval (typically 15 minutes, 30 minutes or 1 hour). The images from the three cameras are captured following a continuous numbering scheme. After each capture cycle, images are transferred to a central storage disk. A workstation PC subsequently renumbers the files to reflect
capture position and capture cycle. Next the infrared FLIR Agema files are converted to bitmap. Subsequently, thermal, chlorophyll fluorescence and video images from capture positions belonging to a single leaf or plant are combined in a concatenated file. These files make it possible to visualize on-line changes in thermal patterns of the monitored plants and ultimately to guide sampling of presymptomatic leaf tissue for molecular and biochemical analysis. The overview images are automatically combined into an MPEG animation file, either incrementally after each capture cycle, or a separate file is created each 24h. After completion of the experiment, the MPEG-animation files permit an efficient visual selection of the gathered data. Selected leaves or plants are subsequently analyzed for local leaf temperature, area of temperature increase and area of visual symptom extension.

2.2. Plant growth conditions

Tobacco plants (*Nicotiana tabacum* cv. Xanthi NN, Xanthi nc) are grown in a conventional plant growth chamber at 21±1°C and 60±10%RH. At the 6 to 8 leaf stage plants are transferred to the measuring room, conditioned at 21±0.1°C. Since measurements can span several weeks, plants have a constant supply of water to avoid effects of water availability on leaf transpiration and temperature. Fresh tobacco mosaic virus suspension was obtained by grinding infected tissue from *Nicotiana tabacum* cv. SR1. Pumice was used as an abrasive to permit TMV to infect leaf epidermal cells.
3. RESULTS

3.1. Salicylic acid increases leaf surface temperature

Salicylic acid was previously shown to increase leaf surface temperature of tobacco leaves after spraying7. A concentration of 3mM resulted in maximal leaf-surface temperature increase. Higher concentrations were phytotoxic and resulted in necrosis. In order to determine the minimum concentration that results in a measurable temperature increase, single droplets of SA-solutions were applied at the leaf surface. Comparing the reaction of different-leaves from different plants proved difficult due to heterogeneous response at the leaf surface. The result from the application as droplets of a dilution series of salicylic acid on a tobacco leaf surface is consistent with the data obtained by spraying. In both experiments, 3mM resulted in a clearly identifiable temperature increase.

Figure 3. Droplet application of a dilution-series of SA in phosphate buffer pH7 solutions on an attached leaf of a tobacco Xanthi nc plant. The leaf veins delimit application zones. Above the main vein from right to left: control buffer, 10mM, 1mM, 10⁻²mM and 10⁻⁴mM. Under the mid vein from right to left: 5mM, 3mM, 0.1mM, 10⁻³mM and 10⁻⁴mM. The upper panel shows thermal (left) and visual reflectance (right) images, just after the application of the 20 µl droplets. The dark droplets have a minimum temperature of 19ºC, the untreated leaf blade is at 20 to 20.3ºC depending on the position and the main vein reaches 21.5ºC. The pictures of the lower panel were taken 26 hours later. Treatments from 3 to 10mM result in clear symptoms in the thermogram. The maximum temperature at the treated loci was 21.3ºC, whereas the untreated tissue in-between was at 20.9ºC. One and 0.1mM cause very little local increase in temperature.

Figure 4. TMV infection on resistant tobacco leaf sections for thermography-aided presymptomatic sampling. Infection was carried out by dusting the leaf area with pumice, adding TMV-suspension and gentle abrasion with a bent glass-rod. The two pictures from each panel are composed of 2 overlapping images. The attached tobacco leaves are kept horizontal by supporting nylon wires.

The upper panel was taken 28 hours after infection. Neither on the thermal (left) or visual reflectance (right) images symptoms can be seen. The temperature of the leaf blade was on average 20.2ºC. At 41 hours after infection the increase in temperature has reached its maximum (middle panel). The maximum temperature of the warmer, lighter colored regions was 20.6ºC. No visual symptoms were yet detectable at this time point. As a control, the leaf regions in-between the TMV-infected regions were inoculated by the same method, without TMV. These regions have a temperature of 20.2ºC, corresponding with the temperature of the leaf blade in the upper panel. The spread of the infection over the delimiting veins into the control region proved difficult to prevent. The lower panel shows a strong decrease of temperature in the infected areas due to cell death. The dark regions at the infected sites have a minimum temperature of 19.4ºC. The control-infected areas now have a slightly higher temperature of 20.3-20.4ºC, likely due to transport of SA from the infected areas. At this time, visual symptoms are clearly discernable as drying, light-reflecting regions.

3.2. Thermography aided presymptomatic sampling of tobacco-mosaic virus infected leaf tissue

In previous work, a correlation in the kinetics of temperature increase, transpiration decrease and salicylic acid concentration increase was observed8. Sampling for SA-determinations can be done on isolated infection spots (see Fig. 5), or areas bounded by leaf veins can be homogeneously infected (see Fig. 4). Sampling is obviously much faster with the latter method, although some spatial variation is introduced due to uneven infection of the whole region.

Figure 5. TMV infection on resistant tobacco leaf sections for thermography-aided presymptomatic sampling. Infection was carried out by dusting the leaf area with pumice, adding TMV-suspension and gentle abrasion with a bent glass-rod. The two pictures from each panel are composed of 2 overlapping images. The attached tobacco leaves are kept horizontal by supporting nylon wires.

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3.3. **Tobacco-mosaic virus infection induces local presymptomatic leaf surface temperature increase**

In a previous publication the exact spatial colocalization of the local thermal effect with the subsequently formed patch of dead tissue was proven. Figure 5 illustrates thermal imaging at high resolution of the previously published presymptomatic increase in leaf surface temperature at the local infection site.

3.4. **Combination of thermography and chlorophyll fluorescence imaging**

Spontaneous cell death is preceded by a temperature increase, as was observed previously. We wanted to find out if chlorophyll fluorescence imaging would also permit presymptomatic detection of pending cell death. From figure 6 it can be concluded that, as is the case with thermal imaging, regions with different color can be discerned in asymptomatic tissue. However, these regions are not of higher contrast or extent compared to the thermal effects. From these preliminary images, it can be seen that cell death is visualized with very high contrast compared to the visual color reflection images.

Figure 5. Close-up of a TMV-infection site. The region to be infected was encircled with a black felt marker. Pumice was deposited in the center, followed by a 20µl droplet of TMV-suspension. A fine glass rod was used to infect locally by gentle friction. The images of panel a were captured 1 day after infection. The average temperature inside the marked region of the thermal image (left) was 21.4ºC (min. 21.3ºC and max. 20.5ºC). Half a day later (panel b) a thermal effect is visible, although no visual symptoms can be observed. The temperature has increased by 0.2ºC (avg. 21.6ºC, min. 21.5ºC, max. 21.7ºC). Panel c (2d post infection) shows necrosis at the site of infection. Thermally, these regions have a low temperature (21ºC, dark blue). Visually, the necrotic fleck has a brownish appearance. Two and a half day after infection, the necrotic lesion has dried. Evaporation has completely ceased, causing the region to appear much warmer (max. 22.3ºC) than the surrounding tissue. The width of the images is 2.75 cm.

Figure 6. Parallel thermal and chlorophyll fluorescence imaging of tobacco bO spontaneous cell death mutant showing spreading necrosis. The temperature of the thermal images spans 1ºC, from 21ºC (dark blue spots) to 22ºC (yellow regions). The unaffected portions of the leaf blade have a temperature of 21.5ºC. In the thermal images (left column) emerging cell death is visible as dark blue spots. In panel a cell death is visible at the bottom of the image. One day later (panel b), cell death has expanded along the major and side veins. A faintly warmer front is discernable at the left of the cell death zone. A more detailed pattern of cell death is visible from the chlorophyll fluorescence image (middle column), which provides a much higher contrast than the visual images (right column). A thermal front 0.4ºC higher in temperature has expanded during the next 12h. New initiation of cell death is visible in panel d (2d after first frame). The spread of cell death via the side veins is clearly visible in the chlorophyll fluorescence image. In the last panel (3d post infection), the thermal effect is more diffuse. Th dark regions in the chlorophyll fluorescence image correspond to patches of dead cells where chlorophyll is completely degraded and thus fluorescence emission is absent.
4. CONCLUSIONS

Imaging technology clearly has a lot of potential to assess the physiological status of plants. In addition to the here illustrated thermography and chlorophyll fluorescence imaging, which respectively monitor transpiration and photosynthetic efficiency, hyperspectral reflectance imaging can provide additional information on changes in leaf composition, structure or orientation. Information on accumulation or degradation of a particular compound can be obtained from images taken from specific narrow spectral regions (of the order of 10nm). For each particular stress-situation, multiple images captured in the visual or near-infrared spectrum can be analyzed for deviations. In Table 1 a concluding overview of the three mentioned imaging techniques is given along with their pros and cons. By combining hyperspectral reflectance imaging with thermography and chlorophyll fluorescence imaging, a multispectral imaging setup would become available to realize a so called 'stress-catalogue', enabling timely determination of emerging stresses. This would extend the application of such a multispectral monitoring system beyond the point of simply visualizing an emerging stress condition.

<table>
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Table 1: Overview of imaging techniques that could be combined into a versatile multispectral imaging system.

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REFERENCES


