Thermographic visualization of cell death in tobacco and Arabidopsis

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ABSTRACT
Pending cell death was visualized by thermographic imaging in bacterio-opsin transgenic tobacco plants. Cell death in these plants was characterized by a complex lesion phenotype. Isolated cell death lesions were preceded by a colocalized thermal effect, as previously observed at sites infected by tobacco mosaic virus (TMV) (Chaerle et al. 1999 Nature Biotechnology 17, 813–816). However, in most cases, a coherent front of higher temperature, trailed by cell death, initiated at the leaf base and expanded over the leaf lamina. In contrast to the homogenous thermal front, cell death was first visible close to the veins, and subsequently appeared as discrete spots on the interveinal tissue, as cell death spread along the veins. Regions with visible cell death had a lower temperature because of water evaporation from damaged cells. In analogy with previous observations on the localized tobacco–TMV interaction (Chaerle et al. 1999), the kinetics of thermographic and continuous gas exchange measurements indicated that stomatal closure preceded tissue collapse. Localized spontaneous cell death could also be presymptomatically visualized in the Arabidopsis lsd2 mutant.

Key-words: Arabidopsis lsd2 mutant; hypersensitive response; infrared thermography; programmed cell death; salicylic acid; stomata; thermal imaging; tobacco bacterio-opsin transgenics; transpiration.

INTRODUCTION
Infection of a resistant plant by an avirulent pathogen leads to an incompatible interaction, resulting in a localized programmed cell death called the hypersensitive response (HR) (Greenberg 1997). Previously, the HR of resistant tobacco (Nicotiana tabacum L. cultivar Xanthi NN) to tobacco mosaic virus (TMV) has been detected thermographically before any visual damage became apparent (Chaerle et al. 1999). At infected loci, rapidly expanding spots of increased temperature were visualized. Both maximum extension and intensity of the spot were reached when the necrotic lesions were visible as pinpoints. Salicylic acid (SA) is known to accumulate in this incompatible interaction prior to necrosis (Enyedi et al. 1992). In several plant species, SA plays a central role as a signalling molecule in plant disease resistance (Malamy et al. 1990; Métraux et al. 1990; Uknes et al. 1993). In addition, SA can close stomata (Larqué-Saavedra 1979; Manthe, Schulz & Schnabl 1992), and in tobacco, this action was specific when compared to SA analogues (Chaerle et al. 1999). In the incompatible interaction between tobacco and TMV, the onset of SA accumulation was shown to coincide with an increase in stomatal resistance, which led to the thermographically recorded increase in the temperature of the leaf surface (Chaerle et al. 1999).

Pathogen-independent cell death was first described in disease lesion mimic mutants of maize (Zea mays L.) (Johal, Hulbert & Briggs 1995). More recently, lsd (lesions simulating disease) and acd (accelerated cell death) mutants in Arabidopsis thaliana (L.) Heynh. (Dietrich et al. 1994; Greenberg et al. 1994) and cdr (cell death and resistance) mutants from rice (Oryza sativa L.) (Takahashi et al. 1999) were described, all of which displayed discrete disease-like symptoms. The formation of the lesions mimicking pathogen-induced cell death was accompanied by the activation of defence responses and the induction of systemic acquired resistance. Moreover, several plants expressing transgenes of plant and non-plant origin formed spontaneous necrotic lesions resembling HR and accumulated SA. The transgenes were as diverse as a structural variant of ubiquitin (Bachmair et al. 1990), cholera toxin (Beffa et al. 1995), bacterio-opsin (bo) (Mittler, Shulaev & Lam 1995), glucose oxidase (Kazan et al. 1998), and invertase (Herbers et al. 1996). In addition, several mutants and transgenic plants that accumulated photoactive intermediates as a result of an alteration in the porphyrin synthesis pathway also formed spontaneous lesions and accumulated SA (Hu et al. 1998; Mock et al. 1998; Molina et al. 1999). In all these cases, cell death is probably due to an unbalanced biochemical state linked with metabolic stress (Mittler et al. 1995; Greenberg 1997). Programmed cell death consists of an initiation and a propagation phase. The formation of delimited, isolated lesions on leaves and stems of bo transgenics was considered to be reminiscent of a mutant affected in cell death initiation (Mittler et al. 1995).

Although the severity of the lesion mimic phenotype was influenced by environmental conditions, these plants devel-
oped normally (Mittler et al. 1995). Cell death in bO plants is preceded by a rise in SA (Mittler et al. 1995), comparable to what happens in TMV-infected plants (Malamy et al. 1990). A method was established to synchronize TMV infection of resistant tobacco (Malamy, Hennig & Klessig 1992). When plants were grown above 28 °C, the HR was inhibited, allowing TMV multiplication and spread. When shifted back below 28 °C, a massive and rapid reaction was mounted by the plant. Thermostatic measurements indeed proved a faster expansion of the thermal spots of higher temperature at the sites of infection (Chaerle et al. 1995). In addition, a shorter time lag between thermal effect and cell death was observed, compared with the interaction at 21 °C. In bO transgenic tobacco plants, cell death can be synchronized by the same procedure (Mittler et al. 1995). Given the above-mentioned analogies, we postulated that thermographic analysis would allow us to presymptomatically visualize cell death in bO transgenic tobacco plants. Using conductance measurements on samples from temperature-shifted plants, cell death was detected earlier in bO transgenic than in TMV-infected tobacco plants (Mittler et al. 1995). This difference in timing was suggested to result from a bypass of the recognition step in the plant–pathogen interaction. Based on these observations it was inferred that the thermal spots would appear earlier after temperature shift than upon TMV infection.

In the Arabidopsis lsd6 and lsd7 lesion mimic mutants, SA was proved to be necessary for cell death (Weymann et al. 1995). SA promotes necrosis in tobacco (Van Der Straaten et al. 1995), although endogenous SA accumulation does not necessarily lead to cell death (Yu, Parker & Bent 1998). Moreover, lesion formation in the dominant initiation mutants lsd2 and lsd4 was shown to be SA independent, because a cross with NahG plants, which degrade SA through the action of an SA hydroxylase (Delaney et al. 1994), does not alter their lesion phenotype (Hunt et al. 1997). By thermographically monitoring the initiation of SA-independent cell death in lsd2 mutants of Arabidopsis, we wanted to find out whether a thermal effect is a more general forerunner of cell death.

MATERIALS AND METHODS

Plant material and growth conditions

Bacterio-opsin (bO) transgenic plants of tobacco (Nicotiana tabacum L. cv. Samsun NN) (line EL-301.A) and the isogenic wild type were a generous gift from E. Lam (AgBiotech Centre, Rutgers – The State University of New Jersey, USA). Line EL-301.A was proved to have a high level of bO expression (Mittler et al. 1995). The Arabidopsis thaliana (L.) Heynh. lsd2 mutant (in Col-0 background) was kindly provided by L. Friedich (Novartis Crop Protection Inc., Research Triangle Park, NC, USA).

Tobacco plants were grown in sterilized potting soil at 21 ± 1 °C and at 60 ± 10% relative humidity, under fluorescent lamps with an intensity of 50 ± 10 µmol m⁻² s⁻¹ and a photoperiod of 16 h light : 8 h dark. The previously described growth conditions for bO transgenics (Mittler et al. 1995) could not be met because of technical limitations on light intensity in the growth chamber and in the measuring cabinet. Arabidopsis lsd2 seeds were sown in sterilized soil. After 2–3 weeks, seedlings were potted in flats containing sterile soil. Due to infrared reflection on plastic surfaces, it was necessary to use flats (23 cm × 36.5 cm; six plants per flat) without internal divisions. Thus, the background on the thermal images consisted solely of moist soil. The plants were grown under the same conditions as tobacco.

Parallel infrared and visual image capture and visualization

The measuring cabinet had a stable temperature of 21 °C and the light intensity was comparable to the above-mentioned conditions. Tobacco plants were placed in continuous light, whereas Arabidopsis plants were kept under a 16 h light : 8 h dark photoperiod. At the start of the experiment, the relative humidity varied between 50 and 60%. Seven- to 9-week-old tobacco plants were decapitated prior to measurements, allowing unrestricted views of four to six fully expanded leaves. Temperature shifts were performed by raising the temperature of the measuring cabinet to 32 °C, keeping it above 28 °C for at least 24 h, and then cooling down to 21 °C. Temperature measurements on thermal images, captured with a Ther movision 900 LW/ST long-wave Stirling-cooled thermal imaging system, were obtained with the Erika software running on the system computer (FLIR Systems, Portland, OR, USA; http://www.flir.com).

An XYZ positioning system with thermographic (Agema T HV900LW; FSI, Portland, OR, USA) and video camera mounted side by side was built into the measuring cabinet (Chaerle et al. 1999). This set-up was used to consecutively image the upper leaves of up to five tobacco plants per experiment. High-resolution thermographic images were obtained by capturing several slightly overlapping pictures of each leaf and joining them subsequently. To ensure maximum temperature contrast, the subpictures were visualized within a temperature window of 1 °C. Slightly different median temperatures were chosen to reveal the full temperature range of the leaf blade. This introduced discontinuities within the panels in some of the figures presented. Moreover, sometimes it was necessary to highlight local temperature differences on slightly distorted leaves affected by cell death. The robot was used once in manual mode at the start of the experiment in order to enter all positions for the thermographic camera by a teach-in procedure. The corresponding video positions were calculated automatically by applying a constant offset for the three axes. Subsequently, cycles of image capture at these programmed positions were performed automatically, at fixed intervals, for approximately 2 weeks. Collected parallel video and infrared pictures were converted after each cycle and combined with ImageMagick software (http://www.imagemagick.org), allowing imme-
diate visualization of plant responses. Image sequences were generated every day, and analysed during and after the experiments. The distance of the infra-red scanner to the leaf was such that the width of the image roughly corresponded to that of a tobacco leaf. The resolution was approximately 0·5 mm pixel\(^{-1}\) (15 cm for 272 pixels), as previously (Chaerle et al. 1999). In the 15 experiments carried out, 85 leaves of 25 plants were visualized. Younger leaves of Arabidopsis \textit{bad}2 rosettes were screened with the robot by automatic cyclic capture of thermographic and video images on programmed positions, every 2 h for several weeks. A stack of pictures was taken at incremental steps along the \textit{Z} axis (each 2 mm, corresponding to the depth of field of the thermographic system), in order to anticipate growth and movement of the leaves. The low contrast of the video images, taken at short-object distance, was due to a suboptimal illumination level. The thermal camera was used without extra lens. The resolution of the infrared images was 0·1 mm pixel\(^{-1}\). Three experiments, with 18 plants each, were performed.

Infrared gas analysis measurements

Continuous transpiration was measured with a dual channel ADC 2250 (ADC, Hoddesdon, UK) gas exchange equipment and two measuring cuvettes with an internal diameter of 14 mm (PP Systems, Hitchin, UK). The infrared gas analysis (IRGA) of water vapour was carried out in parallel with thermography in the measuring cabinet. The cuvettes had to be placed on interveinal tissue to ensure tight fitting. The thermal response of the enclosed tissue could not be assessed thermographically, because glass is not transparent for long-wave infra-red radiation. Likewise, visualization of the development of cell death inside the cuvettes by video imaging was problematic, due to reflections on the glass windows of the cuvettes. A cuvette placed on an equivalent leaf of a wild-type tobacco plant was used as a control. The constant flow of air supplied to the cuvettes was set at a minimum of 1 mL s\(^{-1}\) to mimic the still-air conditions in the cabinet at best. With these settings, it took approximately 1 min to flush the air inside the system. Each cuvette was sampled automatically every 10 min. Measurements switched between the cuvettes every 5 min. Data were recorded approximately 4 min after switching, when the equilibrium was reached. Of the 15 experiments carried out with bO transgenics, 11 were combined with IRGA measurements.

RESULTS

Early detection of cell death using thermography

Here, the visualization of a cell death-associated increase of leaf temperature in bO transgenic tobacco plants (Mittler et al. 1995), prior to the appearance of colocalized visual leaf damage, is reported. The bO transgenics displayed various patterns of thermal effects and subsequent cell death. In Table 1, as a summary of all experiments conducted, the frequency of the different types of response is given. A thermal front propagating over the leaf was seen in most cases. Different leaves of an individual plant and, occasionally, regions on the same leaf could display several types of response. The major observed categories will be described in detail below. To synchronize the induction of cell death in bO transgenic plants, temperature shifting (TS) from 32 to 21 °C was applied in most experiments.

Isolated lesions

Formation of isolated lesions was only apparent in a few bO transgenic plants studied. When not submitted to a TS, lesion formation was preceded by a colocalized thermal effect visible 4 h prior to lesion formation and remained limited to a few spots per leaf (data not shown). In the case of the TMV–tobacco interaction, the local temperature increase became visible 8 h before cell death was initiated (Chaerle et al. 1999). After TS, the lag phase between thermal and visual effect upon TMV infection was reduced from 8 to 4 h (Chaerle et al. 1999). Temperature shifting induced a thermal effect in bO transgenics (Fig. 1a), although it was not synchronized as expected, because new thermal spots appeared over a period of approximately 10 h. Moreover, the time lag between thermal effect and subsequent cell death was increased to at least 12 h. Importantly, cell death lesions remained very small (Fig. 1b, video image) compared to the extent of the thermal effect (Fig. 1a, thermal image), hampering early detection of the cell death-associated decrease in temperature. Formation of homogenous, isolated thermal spots after TS (Fig. 1a) could either be followed by the development of colocalized patches of isolated pinpoint lesions or individual necrotic lesions (Fig. 1b). After cell death had appeared as single isolated lesions, halos of higher temperature remained visible around the lesion (data not shown), comparable with previous observations on TMV-infected tobacco (Chaerle et al. 1999). This phenomenon was observed in experiments at constant temperature (21 °C) as well as at some locations after temperature-shift. However, the local thermal response observed after TS was more intense than that

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Leaves (85)</th>
<th>Plants (25)</th>
<th>Exp. (15)</th>
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<tbody>
<tr>
<td>Small lesions</td>
<td>5</td>
<td>3</td>
<td>3</td>
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<tr>
<td>Patches of small lesions</td>
<td>3</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Starting from leaf base</td>
<td>28</td>
<td>13</td>
<td>10</td>
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<td>Starting from mid-vein</td>
<td>13</td>
<td>10</td>
<td>9</td>
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<tr>
<td>Starting at leaf tip</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Isolating big lesions</td>
<td>13</td>
<td>11</td>
<td>10</td>
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<td>Reticulate pattern</td>
<td>8</td>
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*Out of 85 observed leaves, 54 displayed cell death; on some leaves, several phenotypes occurred.
occurring at 21 °C. The temperature difference between the intense thermal spot (indicated in Fig. 2a) and the unaffected adjacent leaf surface was 0·4 °C at maximum extension, before appearance of colocalized cell death. In Fig. 1b, the thermal effect at the indicated location had almost disappeared. A thermal effect associated with the mid-vein was observed emerging from the leaf base (Fig. 1a & b).

**Expanding thermal front**

The most striking and most frequently observed effect was the establishment of a moving thermal front of higher temperature, starting near or at the base of the leaf (Fig. 2a), and expanding sideways and towards the leaf tip, while following the mid-vein (Fig. 2b & c). This thermal front was succeeded by cell death, thermographically visualized as a pattern of colder spots expanding along main and side veins (Fig. 2b & c). In the video images, darkening of the veins (in some cases extended to the petiole and stem) and the formation of associated, irregularly shaped necrotic flecks in the interveinal tissue was apparent (Fig. 2b & c). Newly initiated cell death, following the thermal front and associated with the veins, was thermographically most distinct (Fig. 2b & c). In general, necrotic cell death is accompanied with loss of integrity of cell membranes (Pennazio & Sapetti 1982). The ensuing water loss implicates evaporation and thus cooling of the affected region. At a later stage, the dead tissue dried, and its temperature increased concomitantly (Fig. 2c & d). The damage to the veins was extensive, because leaves and petioles bent (Fig. 2c & d) and, eventually, snapped. The difference in average temperature between an area of interveinal tissue, close to the mid-vein, and an area not attained by the thermal front (indicated by circles in Fig. 2b) evolved from 0·01 °C before arrival of the thermal front (Fig. 2a) to a maximum of 0·61 °C (Fig. 2b). Thereafter the difference decreased to 0·1 °C (Fig. 2c) and finally reached −0·1 °C (Fig. 2d). The minimal temperature due to cell death on the veins was 0·88 °C below that of the control region.

**Uniform thermal effect**

In contrast with the previous descriptions of lesion formation, some leaves displayed a uniform thermal response. A thermal effect appeared immediately after TS at the edges of the leaf (Fig. 3b). Subsequently, the thermal effect expanded inward between the veins (Fig. 3c) until most of the leaf blade showed a thermal response. Only apparent at 37 h post TS pinpont lesions formed all over the leaf surface, while the thermal effect diminished concomitantly (Fig. 3d & e). The difference between the region with the

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**Figure 1.** Formation of isolated thermal spots of higher temperature in bO transgenics upon shifting from 32 to 22 °C. (a), 24 h after shifting plants back to 22 °C the yellowish spots of higher temperature have attained their maximal expansion although cell death is not yet apparent (thermal images, left). The white arrow indicates the thermal spot on which temperature measurements were carried out. On the video image, no cell death is visible; (b), 94 h after temperature shift, the thermal effect on isolated spots has disappeared; a thermal effect associated with the mid-vein is still visible. On the video image, patches of small lesions colocalize with single thermal spots on the thermal image in panel (a). Due to movement of the leaf, partly caused by starting necrosis of the petiole, the venation pattern should be taken as a reference to compare panels (a) and (b). Movies of the evolution of thermal and visual symptoms can be seen at http://www.plantgenetics.rug.ac.be/~lacha/PE.html.
Figure 2. Propagating cell death in bO transgenic plants. (a). A thermal effect is visible at the right side of the mid-vein at the leaf base (yellowish patch). Initial cell death is seen as a dark red spot in the yellow area; (b), +32 h. The thermal effect has expanded to the right of the mid-vein. Visual cell death is visible on the right side of the mid-vein and the attached side veins. Black circles indicate the regions of the thermal front chosen for temperature measurements. A thermal front is also visible on the lower leaf. On the video image, cell death is apparent as isolated flecks loosely associated with the veins; (c), +72 h. The thermal effect has expanded further along the mid-vein, towards the leaf tip. The yellow thermal front is now also visible on the left side of the leaf lamina. Recently initiated cell death, associated with the veins, appears as cooler regions (dark blue), due to evaporation. The visual image shows newly formed necrotic flecks. The mid-vein and the petiole have started to lose colour, and due to veinal damage, parts of the leaf start to bend; (d), +153 h. The thermal front has disappeared. Cell death zones are still colder than the surrounding tissue. The visual images show the maximal extension of cell death.

Figure 3. Uniform pinpoint lesion formation after temperature shift (TS) of bO transgenics. (a), 4 h after TS: apparently asymptomatic leaf, although at higher resolution an initial thermal effect is already visible; (b), 13 h after TS: thermal effect at leaf margins appear as yellow patches, no visual damage detectable in video images. (c), 39 h after TS: spread of patchy pattern towards the mid-vein; a few pinpoint necrotic lesions start to appear on the video image; black circles correspond to the areas used for temperature measurements; (d) and (e), 73 h and 112 h post TS: thermal effect diminishes and eventually disappears. A uniform pattern of pinpoint lesions is established. The temperature range was set to 1 °C for maximum contrast.
highest temperature and an unaffected area (Fig. 3c, black circles) was 0.47 °C compared with 0.07 °C (Fig. 3a) and 0.1 °C (Fig. 3b). This difference declined, associated with cell death, from 0.2 °C (Fig. 3d) to –0.03 °C (Fig. 3e).

In this case, the thermal and visual symptoms were very similar to what could be observed after uniform TMV infection in resistant tobacco (unpublished results). In contrast, TMV-induced cell death appeared already 4 h after the initial thermal effect (10 h post TS). The final size of the lesions was smaller than TMV lesions, indicating a slower expansion and consequently, a later detection. As generally observed, the plant shown in Fig. 3 displayed several phenotypes of cell death, which appeared to be controlled in a developmentally dependent way. Spreading fronts of cell death appeared preferentially on upper leaves, whereas isolated lesions were mainly observed on older leaves. Intermediate leaves could show combinations of both phenomena. Senescence was apparent at a late stage, in leaves that were characterized by the uniform thermal effect. The plant shown in Fig. 4 started to display a uniform pattern of lesions early in development, at approximately 5 weeks, on three of its older leaves. These leaves subsequently showed a rapid and uniform progression of senescence (Fig. 4, video image). The senescing tissue did not show a decrease in temperature. Presumably, no increase in water loss occurred. The younger leaves of this plant did not senesce, and displayed emerging discrete lesions. Due to a colocalized thermal effect, these lesions appeared 0.2 °C warmer than the surrounding tissue (Fig. 4, small leaf at the bottom of the image). Overall, leaves of intermediate age appeared 0.2 °C colder than the senescing leaves. In comparison, the youngest leaves were still 1.4 °C colder (black on Fig. 4).

IRGA of cell death in bO transgenics

In a previous study on tobacco challenged with TMV, small round cuvettes were centred on predetermined infected locations (Chaerle et al. 1999). Continuous measurements were made during the course of these experiments. In bO transgenics, it was assumed that changes in transpiration would be recorded when the front of increased temperature passed through the cuvette. Transpiration was measured as close as possible to the mid-vein (Fig. 5), where the probability was the highest for the front to pass. However, there was considerable variation in the response of the selected younger leaves between plants and also between younger leaves on the same plant. Thus it proved difficult to anticipate the evolution of a front. Moreover, the lateral extent of the thermal effect was not constant among side veins. In several experiments, either the chosen leaf did not react, or the expanding thermal front did not reach the measuring zone of the cuvette. As visualized in Fig. 5a & b, the thermal front expanded and finally reached the measuring cuvette. In Fig. 5c, the thermal front presumably had attained the measuring zone of the cuvette, given its expansion along the side veins. In Fig. 5d, extensive cell death and its thermal effect is visible on the left side of the leaf, in the region beneath the cuvette. In addition, the thermal front associated with the mid-vein has progressed beyond the cuvette towards the tip of the leaf. The decrease in transpiration presented in Fig. 5e (starting before the indicated time point of Fig. 5c) also supports the notion that the thermal front reached the measuring zone. The subsequent increase (before the time point of Fig. 5d) reflects ongoing cell death reaching the measuring zone. At the end of the experiment, the enclosed tissue was found to be almost completely dead. This proved that the whole measuring zone indeed witnessed a thermal effect prior to cell death.

The temperature difference between an area halfway the mid-vein and the nearest border of the measuring cuvette, and an area not reached by the thermal front (indicated by black circles on Fig. 5a), increased from 0.1 °C (Fig. 5a) to 0.2 °C (Fig. 5b) with the arrival of the thermal front. The difference diminished to ~0.09 °C concurrently with the expansion of cell death (Fig. 5c) and finally increased again.
Thermographic visualization of cell death in plants

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to –0.06 °C due to drying (Fig. 5d). The effect on leaf temperature and transpiration in bO transgenic plants reacting by thermal front formation was less pronounced than in the TMV–tobacco interaction (Chaerle et al. 1999). Cell death moving in the trail of the thermal front, and associated with the venation pattern, might hamper the water supply to the healthy tissue heading the thermal effect (Fig. 5b & c). In addition, the thermal effect and decrease in transpiration could be less consistently correlated in time, due to the inability to follow the expansion of the thermal front in the cuvette (Fig. 5c & d). An increase in transpiration due to loss of integrity, at the time when cell death was visible in the measuring zone, could not always be observed. These observations can be explained as follows. First, cell death did not always cover the whole measuring zone, but was often limited to patches, thus ‘diluting’ the effect. Second, at the time points considered, the petiole of the leaf was also necrotizing, possibly further diminishing water transport to the leaf.

Cell death in the *lsd2 Arabidopsis* lesion mimic mutant

The *lsd2* mutant of *Arabidopsis* displays a spontaneous, albeit conditional, lesion phenotype (Dietrich et al. 1994).

**Figure 5.** Infra-red gas analysis (IRGA) measurements on a moving thermal front in a bO transgenic. (a), 7 h after TS: a yellow thermal front is visible, which was already expanding along the mid-vein before the TS. The tissue at the base of the leaf, on the right hand of the mid-vein, displayed a local intense thermal response earlier on, leading to necrosis of the minor veins and visible shrivelling; black circles indicate the areas for temperature measurement. (b), 19 h post TS: the thermal front has reached the outside of the measuring cuvette, but a distance of 8 mm (the metal housing of the cuvette) still separates it from the measuring zone; damage associated with the veins, visible as darker regions, trails behind the thermal front. (c), 41 h after TS: cell death is visible on the mid-vein next to the cuvette; given the extension of cell death, the thermal front presumably has reached the measuring zone inside the cuvette. (d), 76 h post TS: cell death has further expanded. The thermal front and trailing cell death associated with the mid-vein have passed the cuvette and are by now visible at the leaf tip. Part of the leaf is bent due to loss of strength of the veins; (e), IRGA data: continuous plot of the difference in transpiration rate E between the cuvette shown and a cuvette placed on a control leaf. The time points of the panels (a–d) are indicated. The onset of a decrease in transpiration of the enclosed bO transgenic tissue is apparent between the time points of panels (b) and (c). When comparing panel (a) to panel (b), the thermal front has expanded, during 12 h, over a distance corresponding approximately to the thickness of the metal border of the cuvette. If the same expansion speed is assumed for the next 12 h, the start of a decrease in transpiration is expected around 31 h after the TS. This prediction is thus roughly in agreement with the graph shown. The increase in transpiration starting at 55 h post TS is correlated with cell death reaching the measuring zone between the time points of panels (c) and (d).
The phenotype has been reported to be dependent on the light period, i.e. short-day conditions induced cell death. In our hands, long-day conditions at the given light intensity and quality did not suppress lesion formation and were therefore maintained along the observations. Cell death appeared symmetrically oriented with respect to the mid-vein of the leaf. Typically, two pairs of spots appeared in an unsynchronized way. The affected leaf blade showed a tendency to wrinkle at the position of lesion formation. Together with leaf movement and overlap due to growth, leaf curling made it difficult to follow lesion formation for an entire week.

Comparable to the observations on tobacco TMV infection and bO transgenics, the thermal effect was maximal before the appearance of any visual effect (Fig. 6b, white arrows indicate lesion formation). The temperature of the thermal spots was 0·3–0·4 °C higher than that of surrounding tissue (Fig. 6b; data not shown). Together with the appearance of cell death, first visible as chlorotic flecks of a lighter green colour, this temperature difference dropped to 0·15 °C (Fig. 6c). Importantly, lsd2 lesions immediately appeared as an extended chlorotic spot without obvious subsequent expansion. In some instances, a small warmer halo remained visible around the affected tissue (data not shown). As lesions dried and contrasted more with the healthy tissue, evaporation of water eventually ceased, and the temperature difference with the surrounding tissue increased again to 0·34 °C (Fig. 6d). Thereafter, probably because of a change in orientation and wrinkling of the leaf, the difference diminished to 0·09 °C (Fig. 6e).

DISCUSSION

Cell death phenotypes observed by thermal imaging

The described types of cell death patterns on leaves of bO transgenic plants contrasts with the former report on formation of isolated lesions (Mittler et al. 1995). These marked differences probably indicate that environmental cues can influence developmentally determined patterns of cell death. In contrast to the expansion from leaf base to leaf tip as observed here, isolated spots of cell death were
reported to develop first at the tip of mature leaves (Pontier et al. 1999).

Temperature shifting of tobacco plants challenged with TMV caused a decrease in the lag time between thermal effect and cell death (Chaerle et al. 1999). Conversely, the thermal phenomena associated with pinpoint lesion formation started to be visible much longer before lesion appearance. In this case, the detection of cold spots typically associated with cell death was masked, probably by lateral diffusion of heat in the leaf (Jones 1999).

The observation, in bO transgenic tobacco, of rapid senescence following a dense pattern of cell death (Fig. 4) illustrates the hypothesis that senescence can be linked to a late phase of the cell-death process (Pontier et al. 1999). Because senescence also becomes apparent in tissue surrounding lesions at a late stage of the HR of tobacco challenged with TMV, it was suggested to function as a supplemental barrier against pathogen ingress (Pontier et al. 1999). Senescence is also regarded as a form of programmed cell death (Pennell & Lamb 1997). In contrast to necrosis, membrane integrity is maintained until the final phase of this breakdown process. Thus, leakage and subsequent evaporation of cellular content resulting from cell burst is probably minimal. This may explain the absence of a decrease in temperature (Fig. 4).

Lesion formation in Arabidopsis lsd2 mutants differed markedly from the observed necrosis associated with TMV infection on tobacco. The HR of tobacco challenged with TMV is characterized by lesions starting as pinpoints, that subsequently expand until their final size is reached. Again, in contrast with TMV-induced necrosis, cooling of the cell death region below the temperature of surrounding tissue was not thermographically apparent. This observation possibly indicates the involvement of a different, probably apoptosis-like form of cell death, as already described in Arabidopsis (Levine et al. 1996).

Stomatal closure: relevant to HR cell death or a side-effect of the defence response?

The described kinetics of transpiration in an incompatible interaction between TMV and tobacco resulted from two opposing effects (Chaerle et al. 1999). First, SA accumulation coincided with local stomatal closure, diminishing transpiration and increasing leaf temperature. Subsequently, cell death coincided with loss of water, evaporating at the leaf surface, and with an ensuing lower leaf temperature. A similar sequence of events (stomatal closure followed by cell death) was thermographically monitored in this study of bO transgenics. It is however, important to keep in mind that, apart from SA accumulation, there are other defence-related phenomena that can possibly induce the initial stomatal closure (Blatt et al. 1999; Lee et al. 1999; McDonald & Cahill 1999).

Specific to the bO transgenics, functional translocation of H+ through the bO proton channel is essential to the cell death phenotype (Mittler et al. 1995). This observation points to an important role of pH, and possibly more generally ionic homeostasis, in controlling commitment to cell death. In this respect, the mutant gene from dnd1 (defense no death) of Arabidopsis, which does not manifest HR cell death in response to avirulent pathogens, was reported to encode a membrane channel, supporting a direct link between ion channel activity and HR cell death (Carrington et al. 1999). Identification of this channel could shed more light on the possible connection between cell death and stomatal closure.

A direct advantage for the plant of narrowing stomatal apertures would be blocking pathogen ingress through the stomata, although most pathogens that gain entry through stomata can penetrate them even closed (Agrios 1997). Thus, the thermal effect most probably results from the influence on stomata of compounds accumulating during defence responses and does not represent a phenomenon that is functionally relevant to cell death.

Monitoring transpiration associated with cell death by thermography

Early monitoring of cell death in hypersensitive reactions to pathogens is usually accomplished by conductance measurements (Mur et al. 1997). The loss of ions from infected leaf parts represents a hallmark of irreversible membrane damage during HR. This degree of damage implies loss of the cellular contents and thus thermographically detectable transpirational cooling. Visual assessment of early damage to infected leaves by video imaging is difficult owing to the low contrast between tissues developing cell death and healthy tissues. In contrast, thermography can continuously provide high resolution and high-contrast images of the phenomenon and has the additional advantage of being non-invasive (Figs 1–6). Thermal imaging is also well suited for long-term monitoring of transpiration, because it gives an immediate overview of the distribution of leaf surface temperature (Chaerle et al. 1999). In addition, the surface temperature obtained by thermography could be quantitatively correlated with stomatal resistance measurements (Jones 1999).

In conclusion, robotized thermal imaging clearly allows in planta visualization of the earliest stages of cell death and its further evolution on a continuous basis. The technique can also be used to visualize localized changes in transpiration associated with cell death, without the drawback of possible entrapment effects during gas exchange measurements. Finally, this technique could be applied to assess the homogeneity of a population of transgenics with respect to changes in transpiration pattern.

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