Life at the edge – imaging the hypersensitive response induced by TMV

K.M. Wright & S. Santa Cruz

The hypersensitive response (HR) is a mechanism by which resistant plants can defend themselves against infection by viral, bacterial, fungal and nematode pathogens. It is frequently characterised by the rapid death of a limited number of cells near the invading pathogen. This sacrifice of cells blocks further progression of infection and results in the formation of necrotic lesions (Fig. 1).

Figure 1 Necrotic lesions on *Nicotiana edwardsonii* resulting from the hypersensitive response to TMV.

Gene-for-gene resistance Triggering of the HR is usually a highly specific event, which depends on a matching specificity between a disease resistance gene in the plant and an avirulence gene in the pathogen. We have investigated the HR induced in a species of tobacco, *Nicotiana edwardsonii*, that carries the *N* gene for resistance to *Tobacco mosaic virus* (TMV). This *N* gene-mediated response is temperature dependent, the plant only responding defensively at temperatures below 27°C. This characteristic allows plants to be inoculated and maintained at temperatures at which the resistance response is inoperative, prior to lowering the temperature to initiate and synchronise the induction of the HR.

Visualising the virus Until recently, the viral infection sites could not be localised non-invasively before the appearance of visible symptoms. However, by using TMV expressing the green fluorescent protein (GFP), we are now able to localise precisely virus-infected cells in relation to the host response. Under UV illumination, the infected cells fluoresce green and the infection sites can be seen as green dots against the purple colour of the leaves (Fig. 2).

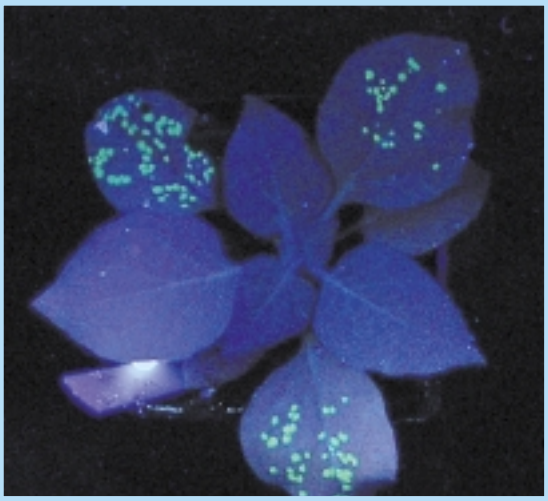

Figure 2 TMV infection sites fluorescing green under UV illumination.

We investigated the course of development of the HR in *Nicotiana edwardsonii* plants using four approaches.

1. By eye or under bright-field illumination using a stereomicroscope.
2. Under blue light using a Confocal Laser Scanning Microscope (CLSM) at low magnification.
3. Using the CLSM at higher magnification.
4. Following treatment of the leaf with a red dye, Texas Red, to trace the supply of water to the leaf. Dye uptake was achieved by dipping the leaf petiole in a solution of Texas Red, which travels with the water into and around the leaf via the xylem.

Xylem Restriction In all experiments, plants were inoculated with TMV-GFP and maintained at 32°C for 48 hours to allow virus infection and growth. The plants were then transferred to 20°C to initiate the hypersensitive response. Using these methods, the first effect of the HR was seen in the supply of water to the infection site. Under normal conditions, the labelling of water with Texas Red shows the water in all areas

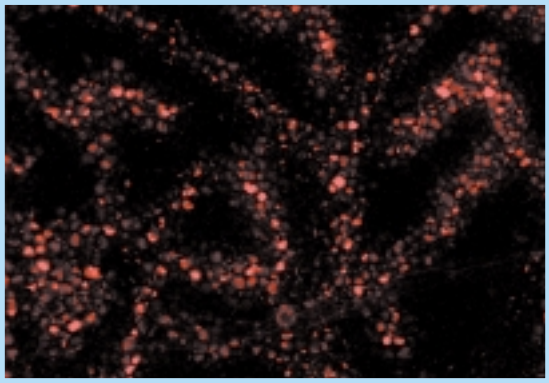

Figure 3 Accumulation of Texas Red in the vacuoles of cells surrounding the xylem.

of the leaf, with the dye accumulating in the vacuoles of individual cells surrounding the xylem, hence the red dots in Figure 3. However, at about 11 hours after the temperature shift, the HR led to a restriction in the supply of water to the infection site, resulting in no red marking of the infected area (Fig. 4a-c).

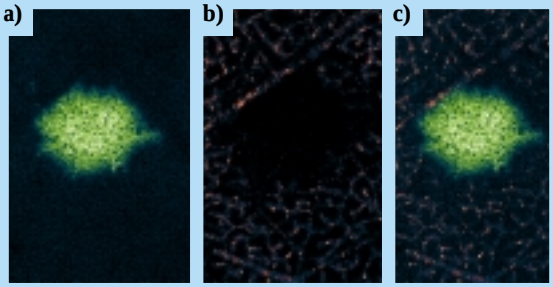

Figure 4 Restriction in water supply 11 hours after HR induction indicated by failure of Texas Red to label the TMV infection site.

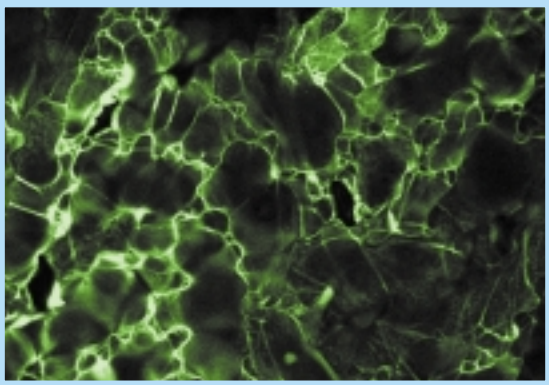

Figure 5 Healthy epidermal cells 11 hours after HR induction.

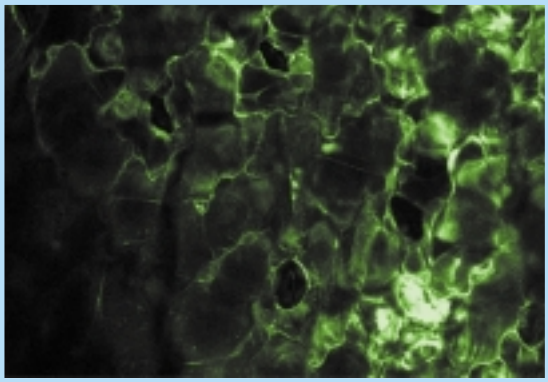

Figure 6 Epidermal cells collapsing onto the underlying mesophyll layer 13 hours after induction.

Cell collapse High magnification of the upper layer of the leaf, the epidermis, revealed that the cells have a rounded, jig-saw-like appearance (Fig. 5). However, after 13 hours, these cells started to collapse onto the underlying mesophyll cells (Fig. 6). This coincided with the first visible signs of collapse seen by eye or under bright-field illumination. The collapsing cells become more obvious over the following 2 hours (Fig. 7). In addition, by 15 hours, the mesophyll cells at the

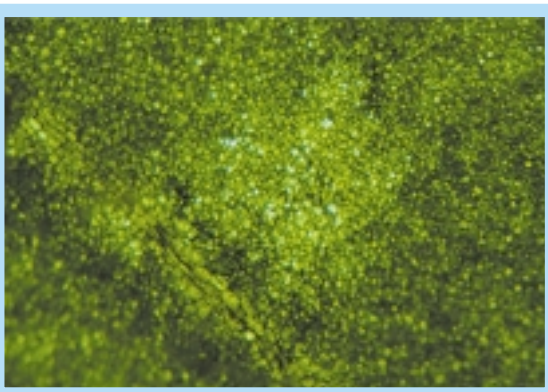

Figure 7 Bright field image of a collapsing infection site 15 hours after HR induction.

centre of the infection site were also seen to be collapsing (Fig. 8), resulting in a dark patch at its centre (Fig. 9).

Cell death and lesion formation It is clear that many changes take place within the cells to generate the HR. We therefore attempted to identify some of the less visible effects that precede cell collapse. To do this, the leaves were infiltrated with Evan's Blue, a dye that is only able to permeate damaged membranes. Up to 8 hours after HR-induction, all the cells within the infection site excluded the dye (Fig. 10). However,

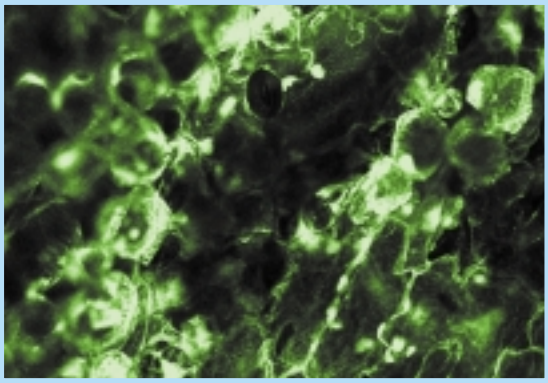

Figure 8 Collapsing mesophyll cells 15 hours after HR induction.

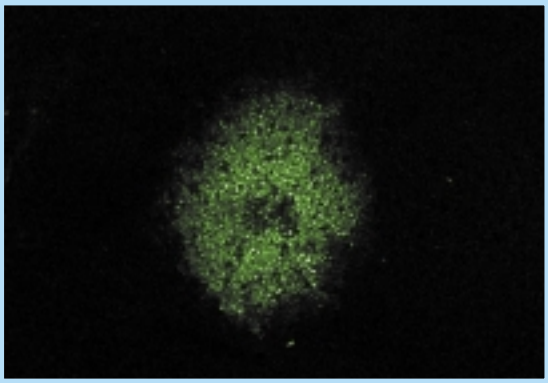

Figure 9 Low magnification CLSM image of a TMV infection site showing collapse of the central cells.

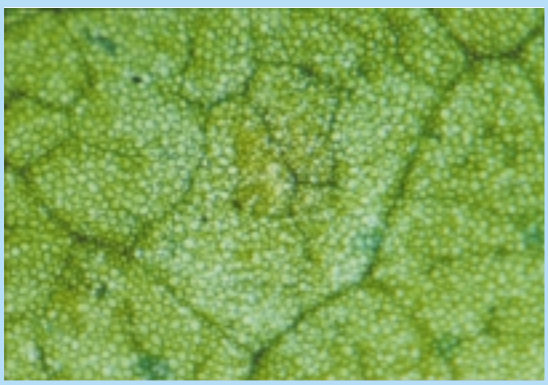

Figure 10 Bright field image of a TMV infection site treated with Evan's blue 8 hours after HR induction showing no staining of cells.

at 9 hours, a subset of infected cells was stained (Fig. 11) indicating damage to their limiting membranes, which leads to cell death.

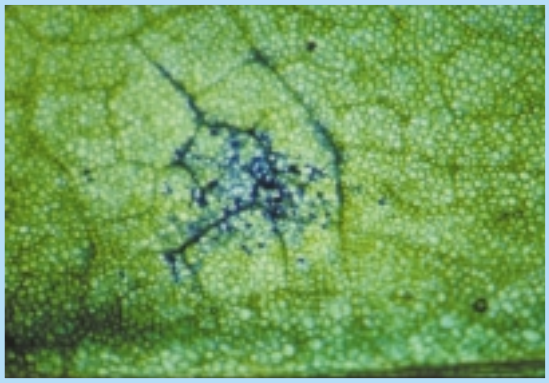

Figure 11 Bright field image of an Evan's blue stained TMV infection site 9 hours after HR induction.

It was also possible to identify a time when the cellular processes leading to cell death become irreversible. Following an initial temperature shift to 20°C, plants were maintained for progressively longer periods before transfer back to 32°C. After a further 24 hours, the leaves were treated with Evan's Blue. As shown in Fig. 12 irreversible progression to membrane damage began from approximately 5 hours. However, irreversible commitment to HR lesion formation was not established until 10 hours or later. This demonstrates that cell death does not necessarily lead to formation of visible lesions. It is possible that, whilst a threshold number of damaged cells may be necessary for lesion formation, other factors, such as water supply, may also be involved.

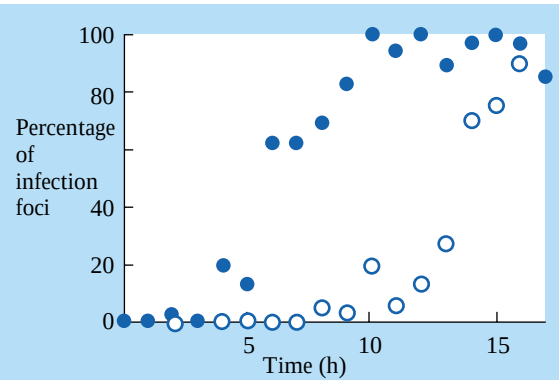

Figure 12 The percentage of infection foci (identified by GFP fluorescence) stained with Evan's blue (closed symbols) and the percentage that developed visible HR lesions (open symbols) after a temperature shift to 20°C for the indicated time followed by maintenance at 32°C for a further 24 hours.

Life at the edge After 17 hours, HR lesion formation progresses with further cell death and collapse, and

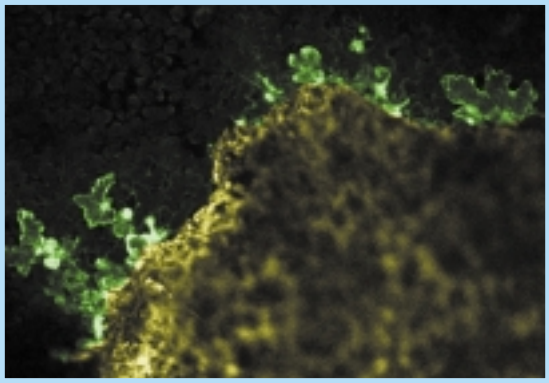

Figure 13 Single TMV-GFP infected cells at the edge of a necrotic lesion 52 hours after induction of HR.

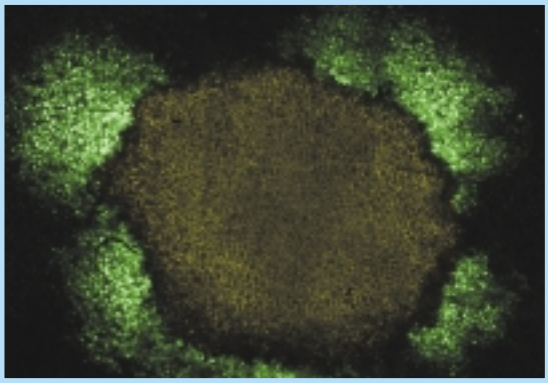

Figure 14 Secondary TMV-GFP infection sites resulting from single cells, following transfer to 32°C for a further 65 hours.

desiccation of the dead area. However, close examination shows that, from about 52 hours onwards, isolated cells become visible at the lesion periphery, persisting for up to 120 hours (Fig. 13). These cells appear to have avoided the initial phase of cell death, presumably because they were only recently infected during the first phase of cell collapse, so that the low level of viral elicitor was insufficient to trigger the HR. It remains to be determined why the virus cannot spread from these cells at low temperature but the inhibition of movement is clearly reversible. If these plants are transferred to high temperature, these cells form the source of virus for development of secondary infection foci (Fig. 14).

Future prospects Using this experimental system, it has been possible to identify the time scale of a number of important changes associated with the HR generated against TMV infection which have previously been obscured due to the inability to identify infected cells. Whilst it is beyond the scope of this report to discuss the biochemical changes and signals which are proposed to be associated with this HR, it should now be possible to link, more accurately, the timing and progression of these changes with the appearance of visible symptoms.