A novel *N* gene-associated resistance to the movement of TMV vectors neutralised by a CMV RNA 1 transgene

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The *N* gene is the best characterised resistance gene to a plant virus, viz., *Tobacco mosaic virus* (TMV). This resistance gene was introgressed into tobacco from *Nicotiana glutinosa* in the 1930's and is still in widespread use in field tobacco plants. In the early 1990's, the *N* gene was the first plant virus resistance gene cloned and its resistance mechanism has been analysed extensively[1-3]. Infection of tobacco containing the *N* gene by TMV results in a hypersensitive response (HR) and the confinement of TMV to cells surrounding the initial site of infection. This response also leads to a systemic acquired resistance (SAR) mediated by salicylic acid (SA). At temperatures above 28°C, the HR and the restriction response associated with the *N* gene are inactive, and so TMV spreads throughout the plant. Lowering the temperature of incubation to below 28°C allows activation of the *N* gene resulting in necrosis in all tissues containing TMV. Physiological and cellular events that take place during the induction of the HR were described recently by Wright and Santa Cruz (Ann. Rep. 99/2000, 136-139).

During an analysis of transgenic tobacco expressing RNA 1 of *Cucumber mosaic virus* (CMV) and showing resistance to CMV, it was observed that *N* gene-mediated resistance to TMV was retained in these plants at temperatures below 28°C.

However, at higher temperatures, when the *N* gene was inactive, TMV appeared to show greater spread into leaves and an increase in accumulation of TMV in the CMV RNA 1 transgenic plants. To determine whether this increase was due to increased replication, movement or both, non-transgenic tobacco plants as well as CMV RNA 1 transgenic tobacco plants were infected with TMV expressing the gene encoding the green fluorescent protein (GFP). These experiments showed that an additional mechanism of resistance (associated with the *N* gene), limiting TMV movement was still active at higher temperatures. This mechanism could be overcome by expression of CMV RNA 1.

Figure 1 Infection by TMV-GFP at 25 °C and at 4 days post inoculation resulted in visible necrotic lesions in inoculated leaves of tobacco transgenic for CMV RNA 1 (right leaf), that were absent in inoculated leaves of non-transformed tobacco (left leaf).

CMV RNA 1 enhances cell-to-cell movement of TMV-GFP in *N* gene tobacco

Infection of tobacco (cv. Samsun NN) expressing CMV RNA 1, by TMV containing the gene encoding the GFP (TMV-GFP), at 25°C, resulted in the appearance of visible necrotic local lesions in the inoculated leaf (Fig. 1, right leaf). Surprisingly, such macroscopic lesions were not visible in tobacco (cv. Samsun NN) that was not transgenic (Fig. 1, left leaf). Since

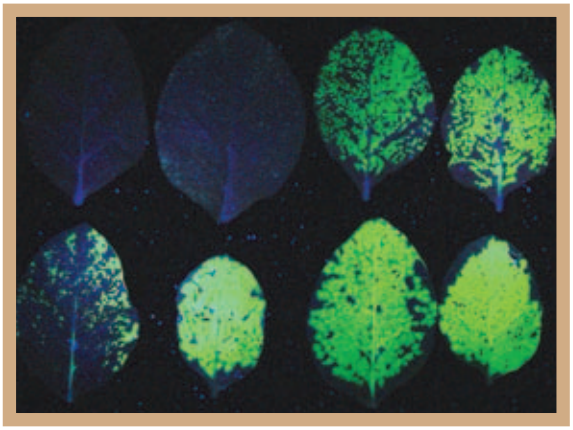

Figure 2 GFP fluorescence (under a UV lamp) in tobacco leaves 4 days after their inoculation with TMV-GFP, kept at the *N* gene-active temperature of 25 °C (upper row) or at the *N* gene-inactive temperature of 33 °C (lower row). From left to right: inoculated leaves of non-transformed tobacco NN, CMV RNA 1-transgenic tobacco NN, non-transformed tobacco nn, and CMV RNA 1-transgenic tobacco nn (indicated as NN, NN-TG, nn and nn-TG, respectively).

wildtype TMV gave necrotic lesions of comparable sizes in the two types of plants, this retarded local movement appears to be a consequence of slower movement of some TMV vectors in *N* gene-containing tobacco. The enhanced effect by the CMV RNA 1 transgene on the cell-to-cell movement of TMV-GFP in tobacco containing the *N* gene, also occurred at 33°C, where the ability of the *N* gene to induce an HR and restrict cell-to-cell movement of TMV is known to be inactive (Fig. 2, compare lower left two leaves).

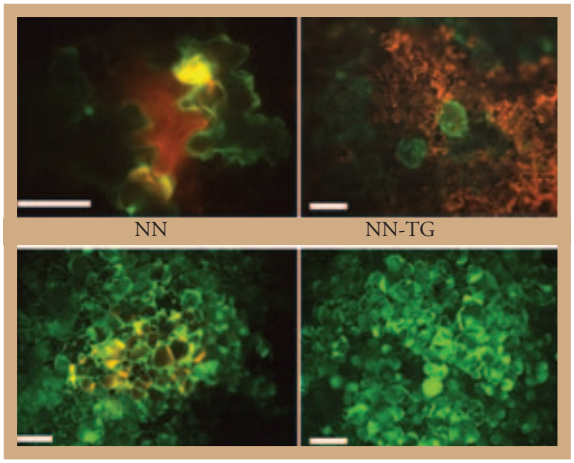

Figure 3 GFP fluorescence (by UV microscopy) in tobacco leaves 4 days after their inoculation with TMV-GFP, kept at the *N* gene-active temperature of 25 °C (upper row), or at the *N* gene-inactive temperature of 33 °C (lower row). From left to right: infection loci in non-transgenic tobacco NN, and CMV RNA 1-transgenic tobacco NN, (indicated as NN and NN-TG, respectively). An FITC/Rhodamine filter was used in which GFP fluorescence is green, while autofluorescence from dead tissue is orange. Healthy tissue appears as a dark background. Bars represent 100 µm.

TMV-GFP shows very limited cell-to-cell movement in *N* gene tobacco The accumulation of TMV or TMV-based vectors was found to be similar in isolated tobacco mesophyll cells (data not shown). Therefore, the differences observed here are due to effects on cell-to-cell movement rather than on intracellular accumulation of viral RNA *per se*. This was confirmed by examining the cell-to-cell movement of TMV-GFP using fluorescence microscopy (Fig. 3). In tobacco cv. Samsun NN at 25°C, movement of TMV-GFP was limited to a few cells, resulting in a microscopic lesion (Fig. 3, upper left panel). By contrast, in CMV RNA 1 transgenic Samsun NN tobacco, TMV-GFP spread to a larger number of cells, but further movement was restricted by activation of the *N* gene (Fig. 3, upper right panel). At 33°C, TMV-GFP showed cell-to-cell movement to numerous cells in both types of plants (Fig. 3, lower panels), indicating that the severely restricted movement of TMV-GFP at 25°C was due to effects associated with the *N* gene.

CMV RNA 1 enhances long-distance movement of TMV-GFP in *N* gene tobacco At 33°C, TMV can systemically infect both non-transgenic as well as CMV RNA 1 transgenic plants (not shown). However, at this temperature, movement of TMV-GFP was mostly restricted to within the inoculated leaves, and to the main veins of one or a few leaves above the inoculated leaves in tobacco cv. Samsun NN (Fig. 4, right panel, top row). By contrast, in Samsun NN tobacco plants transgenic for CMV RNA 1, systemic infection of leaves above the inoculated leaf occurred with TMV-GFP spreading throughout the invaded leaves (Fig. 4, right panel, second row). Thus, there appears to be some factor in tobacco plants restricting the movement of TMV-GFP even at temperatures when other aspects of *N* gene-mediated resistance are inactive.

CMV RNA 1-mediated cell-to-cell movement of TMV-GFP is *N* gene associated To ascertain whether the *N* gene was associated with the restriction of TMV-GFP in non-transgenic tobacco, plants of the tobacco cultivar Samsun nn, containing the *n* allele, were tested for their ability to restrict the cell-to-cell movement of TMV-GFP. Such plants were also made transgenic for CMV RNA 1, to compare the

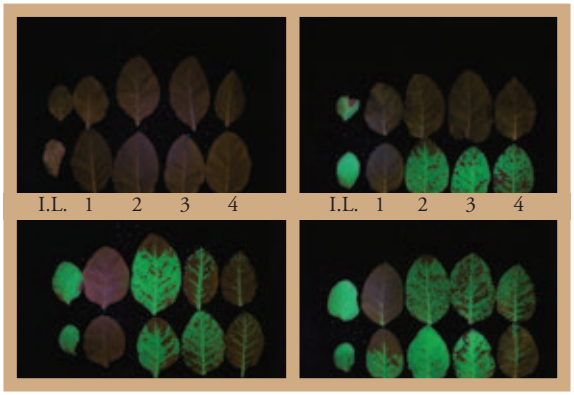

Figure 4 GFP fluorescence (under a UV lamp) in tobacco plants 14 days after their inoculation with TMV-GFP, kept at the *N* gene-active temperature of 25°C (left panel), or at the *N* gene-inactive temperature of 33°C (right panel). From top to bottom: non-transformed tobacco NN, CMV RNA 1-transgenic tobacco NN, non-transformed tobacco nn, and CMV RNA1-transgenic tobacco nn (indicated as NN, NN-TG, nn and nn-TG, respectively). Each row of leaves shows the detached inoculated leaf (indicated as I.L.) followed by the detached, ascending, four consecutive leaves (numbered 1 to 4).

effects of the CMV RNA 1 transgene on TMV-GFP movement in the two lines (Samsun NN vs. Samsun nn). In fact, in the absence of the *N* gene, TMV-GFP moved efficiently from cell to cell in the presence or absence of the CMV RNA 1 transgene (Fig. 2 upper right two leaves). This was true at 25°C and at 33°C (Fig. 2, upper right two leaves vs. lower right two leaves). Thus, the restriction on cell-to-cell movement exhibited in Samsun NN plants at 25°C and 33°C (Fig. 1, and Fig. 2, left leaves), was associated with the presence of the *N* gene.

CMV RNA 1-mediated long-distance movement of TMVGFP is *N* gene associated The above Samsun nn lines were also assessed at both 25°C and 33°C to determine whether the inhibition of long-distance movement of TMV-GFP was associated with the *N* gene, or with a gene(s) not linked to the *N* gene. Interestingly, both non-transgenic and CMV RNA 1-transgenic Samsun nn tobacco supported the long-distance movement of TMV-GFP, and at 25°C as well as at 33°C (Fig. 4, both panels, lower two rows). Thus, the restrictions to both cell-to-cell and long-distance movement of TMV-GFP are associated with the presence of the *N* gene.

It is possible that the temperature-independent resistance observed against TMV movement might not be due to an effect of the *N* gene itself, but rather to one of the genes closely linked to the *N* gene that were co-introgressed from *N. glutinosa*. To test this possibility, tobacco plants from an nn cultivar made transgenic for the *N* gene alone[1] have been tested for their resistance to the cell-to cell movement of TMV-GFP. These plants showed the same restrictions of movement observed in tobacco cv. Samsun NN (cf. Fig. 1). Thus, this resistance phenotype is associated with the *N* gene and not specifically to genes linked to the *N* gene.

Resistance to TMV-GFP movement occurs independent of the SAR pathway The effects of the CMV RNA 1 transgene could be due to an interaction with either the *N* gene itself or some factor downstream of the pathway activated by the *N* gene. SAR, induced by TMV infection, is activated by an SA-dependent pathway. To determine whether the restriction to the movement of TMV-GFP was associated with this pathway, transgenic Samsun NN tobacco plants expressing the bacterial *nahG* gene, which results in SA degradation, were infected with TMV-GFP. When such plants are infected with wildtype TMV, they develop necrotic lesions, but the SA-mediated restriction response is not activated, and so TMV continues to move slowly through the plant, inducing necrosis in infected tissues. In the Samsun NN, *NahG*-expressing tobacco, TMV-GFP was confined to a micro-lesion, as was observed in non-transgenic Samsun NN tobacco (cf. Fig. 3). Thus, it appears that the resistance associated with the *N* gene is triggered by a pathway different from that associated with SA production. It would be interesting to determine whether similar domains of the *N* gene are associated with the SA-mediated resistance response and the novel, CMV RNA 1-suppressible resistance response. Testing transgenic plants expressing various *N* gene mutants[2] as well as an alternatively spliced *N* gene transcript[3] could lead to a clearer understanding of the nature of the elictor of the two distinct resistance responses. This may also provide more information on how CMV RNA 1, or the encoded 1a protein, is able to suppress a resistance mechanism that has a limited effect on the movement of wildtype TMV, but a strong effect on the movement of a TMV vector.

References

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