Transgenic resistance to raspberry bushy dwarf virus in *Nicotiana* species

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Raspberry bushy dwarf virus (RBDV) occurs in *Rubus* species world-wide and in some red raspberry cultivars (*R. idaeus*, *R. strigosus*) it induces yellows disease and/or crumbly fruit. In mixed infections with some aphid-borne viruses it can cause a serious degeneration in vigour and yield. Several of the recently released UK red raspberry cultivars are susceptible to the virus and in some, RBDV can induce severe crumbly fruit that greatly affects fruit quality (Fig. 1).

Because RBDV is transmitted between plants in nature through pollen, growing cultivars resistant or immune to the virus is the only means of controlling its spread and effects. Some red raspberry cultivars contain such resistance which is conferred by a single dominant gene, *Bu*. However, in recent years the presence of RBDV isolates able to overcome such resistance (RB, resistance-breaking isolates) has been identified in raspberry fields in England, including commercial raspberry and blackberry crops. The occurrence of such RB isolates, able to infect all existing raspberry and blackberry cultivars grown in commerce in the UK, is a serious threat to the cultivation of *Rubus* here and elsewhere. As there are no suitable sources of resistance to such RB isolates for use in *Rubus* breeding programmes, transgenic resistance using viral gene sequences seems the best, possibly the only, means of protecting future crops from infection.

Figure 1 Crumbly fruit in RBDV-infected Autumn Bliss raspberry.

The efficacy of such an approach to control RBDV was assessed firstly in *Nicotiana* species, as the technology for the transformation of *Nicotiana* species is much further advanced than that of *Rubus*. However, a significant impediment to this approach was that RBDV does not readily infect tobacco and some other *Nicotiana* species systemically, and does not usually induce symptoms in such infected plants. However, the discovery at SCRI of a variant of RBDV (Can-S) able to infect several *Nicotiana* species systemically and which induced noticeable symptoms in most, was a significant finding that overcame this initial difficulty.

RBDV has a bi-partite RNA genome packaged in quasi-isometric particles. RNA-1 encodes a protein with methyltransferase (mtr), NTP-binding (NTP) and RNA-dependent RNA polymerase (pol) motifs. RNA-2 encodes the coat protein (CP), which is expressed by translation of a sub-genomic messenger RNA, and a putative movement protein (MP) (Fig. 2). Of these different genes and gene sequences for possible use as transgenes, we have tested the efficacy of the CP gene in the sense and antisense orientation and in a non-translatable construction, and the pol gene sequence in the sense orientation.

Constructs were made by reverse transcription-PCR of viral RNA to produce cDNA corresponding to the required virus gene sequence. These were then cloned in pGEM-T and subsequently sub-cloned into

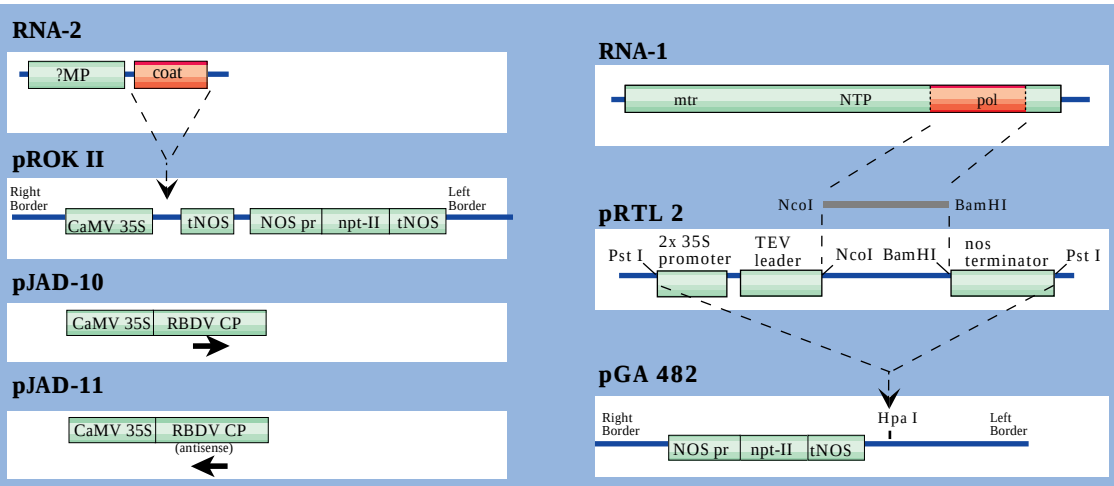

Figure 2 Diagram of the genome map of RBDV and of the cloning of RBDV genes for plant transformation. cDNA was obtained by PCR of the genome RNA-1 or RNA-2 (red) and finally inserted into pROKII (coat protein (CP) gene) or pRTL2 (polymerase gene sequences). The CP gene was in positive orientation (messenger sense) (pJAD-10) or in the inverse orientation (antisense) (pJAD-11). The polymerase gene sequences together with promoter, leader and terminator sequences were excised from pRLT-2 and cloned in pGA 482.

pROKII- or pGA-482-based vectors (Fig. 2). Vectors were introduced into disarmed *Agrobacterium tumefaciens* by triparental mating and the resulting cultures used to transform leaf pieces of plants of *Nicotiana tabacum* cv. Samsun and *N. benthamiana*. Plants regenerated from kanamycin-resistant callus were allowed to set seed, and seedlings of the F1 generation were tested for resistance to RBDV by manually inoculating expanded leaves with the Can-S isolate of the virus in sap of infected *N. clevelandii*. Inoculated leaves were assayed for RBDV in some experiments in tobacco by counting the numbers of local lesions and determining the ELISA values 7 days after inoculation. In all other experiments, inoculated leaves were assayed by ELISA 10 days after inoculation. These results were compared with those of inoculated plants that were not transformed, to assess them for any resistance to RBDV infection and invasion. More than 20 lines were obtained from transformations with each type of vector and construct, and the majority of lines showed some resistance to RBDV.

Several lines of one CP construct in tobacco, in which no viral gene transcript was detectable, showed only

Tobacco line	No. local lesions/leaf	A_{405} values
A1	8.8	1.44
A10	34	1.53
A17	8	1.31
A26	4.7	1.01
Control	85.6	1.71

Table 1 Mean numbers of local lesions per leaf and A_{405} values in ELISA for RBDV in four lines of tobacco transformed with the CP gene of RBDV.

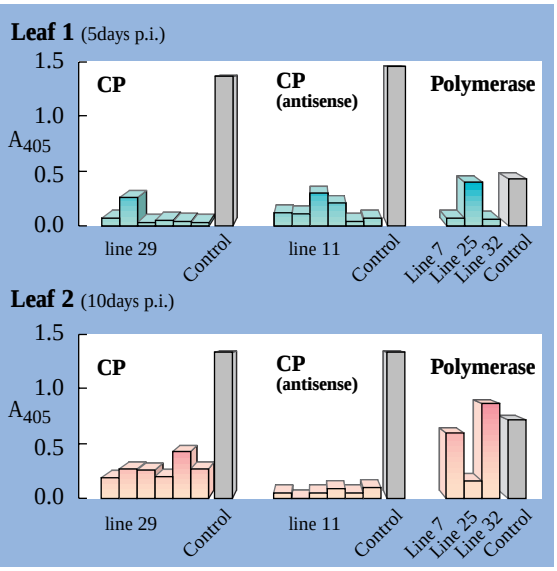

Figure 3 The effect of transformation with RBDV sequences on virus titre as evidenced by A_{405} values in ELISA for RBDV of inoculated tobacco leaves assayed 5 and 10 days post-inoculation with RBDV. The histograms show six R1 plants of CP gene-transformed (29) and CP-antisense-transformed (11) and three individual R0 plants transformed with the pol gene sequence (7, 25, 32).

Figure 4 Symptom-bearing and symptomless *Nicotiana benthamiana* plants inoculated with Can-S.

10-35% of the number of local lesions present on comparable control plants but ELISA values were 65-94% of controls (Table 1). This suggests that these transformants may have some resistance to inoculation with RBDV but little or no resistance to its multiplication once plants are infected.

This work has demonstrated the effectiveness of transformation with the RBDV-CP gene or -pol gene sequences to induce resistance to RBDV infection in *Nicotiana* species. Furthermore, it demonstrates that this resistance can be conferred whether the CP gene is in a translatable or non-translatable form, and whether it occurs in the sense or anti-sense orientations. These results are encouraging and, if equally successful when applied to *Rubus* plants, offer the prospect of providing possibly the best means of protecting future *Rubus* crops against this difficult-to-control pathogen.

The increasing importance and control of wingless weevils as pests in temperateWorld horticulture

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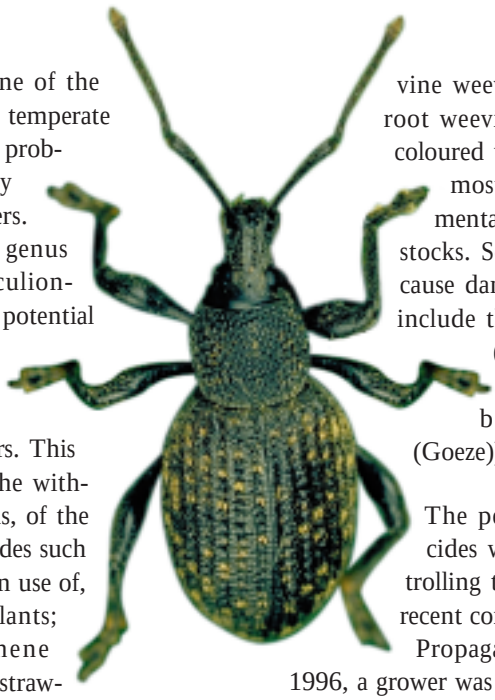

Wingless weevils are now one of the most troublesome pests of temperate horticulture, causing considerable problems to fruit, ornamental nursery and forest nursery stock producers. Wingless weevils belong to the genus *Otiorhynchus* (Coleoptera: Curculionidae) and have been recognised as potential pests of a wide range of crops for many years, but they have become particularly troublesome in horticulture in the last 15-20 years. This follows, amongst other factors, the withdrawal, on environmental grounds, of the persistent organochlorine insecticides such as aldrin and DDT; the increase in use of, and trade in container grown plants; and the reliance on polythene mulches, especially in soft fruit (strawberry and blackcurrant) production. Three species, the vine weevil (*O. sulcatus* (F.)), strawberry root weevil (*O. ovatus* (L.)) and the clay-coloured weevil (*O. singularis* (L.)), are the most damaging to fruit, hardy ornamentals and in commercial tree nursery stocks. Several other *Otiorhynchus* species cause damage to soft fruit crops and they include the red-legged weevil (*O. clavipes* (Bonsdorff)), and *O. rugifrons* (Gyllenhal)) and the rough strawberry weevil (*O. rugosostriatus* (Goeze)).

The persistent organochlorine insecticides were particularly effective in controlling this group of insects. Indeed, at a recent conference of the International Plant Propagators Society in Cork in August 1996, a grower was quoted as saying 'Life after aldrin is difficult and expensive....Control of vine weevil is