Efficient viral-induced gene silencing in roots using a modified tobacco rattle virus

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Introduction

Due to their capability of eliciting a form of post-transcriptional gene silencing (termed viral-induced gene silencing or VIGS) plant viruses are increasingly used as reverse-genetics tools for functional characterization of plant genes.

Several factors affect the silencing response including host range and viral movement within the plant. The work presented here demonstrates that a modified tobacco rattle virus (TRV) vector retaining the helper-protein 2b, required for transmission by a specific vector nematode, not only invades and replicates extensively in whole plants, including meristems, but also triggers a pervasive systemic VIGS response in the roots of *N. benthamiana*, *A. thaliana* and tomato.

This sustained VIGS response was exemplified by the silencing of genes involved in root development, lateral root-meristem function and nematode resistance.

Results

A. TRV-2b vectors efficiently invade meristems and trigger a pervasive VIGS response in *N. benthamiana* and *A. thaliana*.

The relative ability of TRV-2b-GFP and TRV-Δ2b-GFP vectors to invade and spread systemically in shoots and roots of *N. benthamiana* and *A. thaliana* was investigated.

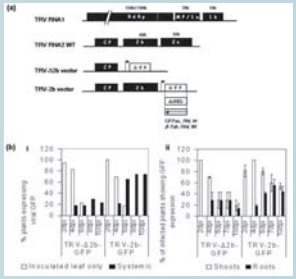
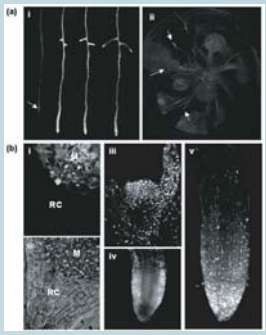


Figure 1: Invasion of roots by TRV.
a- Genome organization of TRV.
b-Movement of TRV vectors in *A. thaliana* (i, n=17-23) and *N. benthamiana* (ii, n=26-45).

A higher percentage of infected plants became systemically infected when inoculated with TRV-2b-GFP (60%-75%) compared with TRV-Δ2b-GFP (25%-30%), (Fig 1).



The pattern of TRV infection in roots was explored further using TRV-2b-GFP and TRV-2b-dsRED constructs.

Figure 2: Distribution of TRV-2b constructs in roots.
a- Invasion of the root systems by TRV-2b-GFP.
b- Shoot and root meristem invasion by TRV-2b constructs. Vibraslice section of *N. benthamiana* infected root meristem (M) (i, and corresponding transmission image ii) and shoot meristem (iii). TRV-2b-dsRED infection of *A. thaliana* root meristem, optical section (iv) and stacked image (v) showing extensive infection of meristem.

All roots meristems cell type appears to be infected except the root cap (2b-i) which was only occasionally infected in *N. benthamiana*. The outer root cap appeared to be more accessible to TRV-2b-ds RED in *A. thaliana* (2b-iv-v).

Taken together these results indicate that TRV-2b vectors spread and sustain a more prolonged systemic expression of reporter fluorescent protein than corresponding TRV-Δ2b vectors. We evaluated the efficacy of the TRV-2b vectors for silencing in roots by targeting a *GFP* transgene. GFP silencing extended to the majority of the shoot apical meristem (Fig 3a) and whole root tissues including meristems (3b-iv). A comparison of the timing of the onset of silencing in roots was explored in the GFP transgenic lines infected with TRV-2b-ds RED construct.

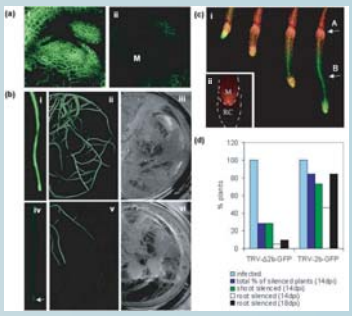


Figure 3: VIGS and recovery of viral replication in *A. thaliana* and *N. benthamiana*. Extensive VIGS of GFP transgene in a- shoot meristems and b- root meristems. c- Viral replication (TRV-2b-dsRED) suppression in newly grown root tissues and recovery in root meristems of *A. thaliana* harbouring a *GFP* transgene. Arrow A indicates the zone of constriction due to TRV invasion, arrow B new root growth exhibiting suppression of viral replication. d- Relative number of plants infected with TRV-Δ2b-GFP$_{VIGS}$ (n=17) or TRV-2b-GFP$_{VIGS}$ (n=23) vectors.

All new root tissue expressed a much-reduced level of dsRED by 8 dpi (3c-i). However TRV-2b-dsRED recovery occurs by 13dpi (3c-ii) within the root meristem.

Thus the TRV-2b vector silences and invades efficiently root meristems and appears to maintain "pockets" of infection within the root meristem, creating future opportunity for reinvasion.

B. TRV-2b VIGS vectors for silencing in root tissues.

In order to investigate the efficacy of the TRV-2b VIGS vector in silencing endogenous genes, TRV-2b VIGS constructs harbouring cDNA of genes whose functions are associated to root development (*iron-regulated metal transporter* [*irt*], *transparent testa glabra* [*ttg*], *root hairless* [*rhl*], *root meristemless* [*rml*], β-*tubulin*) and resistance to root-pathogen (root-knot nematode resistance gene *Mi* from tomato) have been engineered.

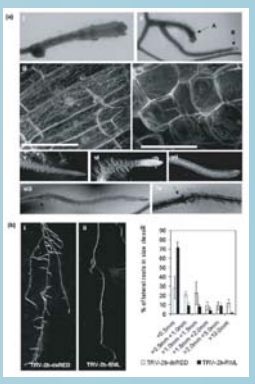
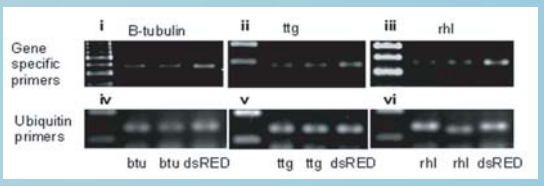


Figure 4:
a) VIGS of *beta-tubulin* (change in cell shape and microtubule structure on α-*tubulin::GFP* transgenics: i, ii, iv), *transparent testa glabra* (ectopic root hair **[1]**, vi), *root hairless* (no root hair **[2]**, vii), *iron-regulated metal transporter* (extended root hair **[3]**, viii-ix).
b) VIGS of *root meristemless* (reduction of lateral root size **[4]**, i-ii-iii). Unsilenced control (iii, v).

Figure 5: Semi-quantitative RT-PCR on *A. thaliana* silenced and control roots using β-*tubulin* (TUB8) (i), ttg1 (ii) and *rhl1* (iii) specific primers, showing reduced expression in plant inoculated with VIGS vectors harbouring the β-*tubulin* (i), *ttg* (ii) or *rhl* (iii) sequences compared with control plants infected with TRV-2b-dsRED virus (right lane on each panel). NB: RNA was extracted from the whole roots. Semi-quantitative RT-PCR using *ubiquitin* primers as internal control for samples used in gene specific RT-PCRs (iv: β-*tubulin*, v: *ttg*, vi: *rhl*,).



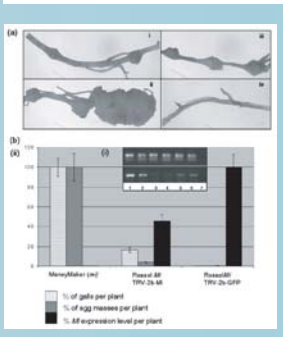


Figure 6: VIGS of nematode resistance gene *Mi* (confers resistance to root-knot nematodes, **[5]**) in tomato. *Mi* resistance–breaking phenotype in tomato (cv Rossol) silenced roots.
a- Small and large galls observed on susceptible MoneyMaker (*mi*) plants (i, ii). Galls observed on silenced *Mi* plants (iii). No galls were observed in all control unsilenced Mi plants (iv).
b- Semi-quantitative (i: lane 1: unsilenced, lane 2-6: independent silenced plants, 7: non-template control; upper panel *pds* PCR product, lower panel *Mi* PCR product) and quantitative Real-time RT-PCR determination of Mi mRNA levels (ii: % Mi expression levels) in control and silenced plants. The averaged percentage of galls and egg masses per plant from two independent experiments is presented.

VIGS responses of the target genes phenocopy previously described mutant alleles [1]-[5]. The silencing effect was analysed at the RNA level. In all cases significant reduction of target mRNA was observed in silenced tissue (Fig 5). The sustained silencing state was still observed by 3 months post infection with the VIGS construct in the case of the nematode assay (Fig 6). This indicate that TRV-2b VIGS response in roots can be maintained and such approach can be used for functional characterization of genes involved in nematode resistance.

Conclusion

These results demonstrate that the TRV-2b vector displays an increased infectivity and meristem invasion, both key requirements for efficient VIGS-based functional characterization of genes in root tissues.

The fact that the presence of the 2b helper protein (a 40-kDa protein harbouring a predicted central coiled-coil domain required for transmission by a specific vector nematode) does not modify symptomatology but is required for extensive shoot and root invasion suggests that the TRV helper-protein 2b may confer tropism to the viral invasion process.

Therefore our data suggest that the TRV helper-protein 2b may have an essential role in the host regulatory mechanisms that control TRV invasion.

Reference / Acknowledgements:
[1] Galway *et al* (1994) *Devel. Biol.* 166, 740-754. **[2]** Schneider *et al* (1998) *Plant Phys.* 12, 2013-2021. **[3]** Cheng *et al* (1995) *Plant Physiol.* 107, 365-376. **[4]** Schmidt and Schikora (2001) *Plant Physiol.* 125, 2078-2084. **[5]** Milligan *et al* (1998) *Plant Cell*, 10, 1307-1319. This work was supported by Large Scale Biology Corporation and the Scottish Executive Environment and Rural Affairs Department.