Technical advance

The reliability of virus-induced gene silencing experiments using tobacco rattle virus in tomato is influenced by the size of the vector control

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SUMMARY

The tobacco rattle virus (TRV)-based vector pYL156 is the most widely used vector for virus-induced gene silencing (VIGS) in solanaceous plants. We show here that vacuum agroinfiltration of the pYL156 empty vector causes extensive stem lesions, foliar necrosis, stunted growth and delayed flowering in tomato, whereas plants infiltrated with pYL156-based silencing constructs that carry inserts of ~250 bp or more show few symptoms of virus infection. This difference in plant health influences plant susceptibility to insects, a trait measured in many VIGS experiments, and could potentially bias the results of other bioassays designed to assess the phenotypic consequences of VIGS. When plants were infiltrated with the pYL156:*GUS* construct, which carries a 396-bp fragment of the β -glucuronidase gene that has no significant homology with any endogenous gene in tomato, they showed similar growth and development as buffer-infiltrated controls, and exhibited significantly fewer viral symptoms than the empty vector. Furthermore, pYL156:*GUS* did not influence plant susceptibility to aphids. Reverse transcription-polymerase chain reaction (RT-PCR) results indicated that the *GUS* insert may cause a modest delay in virus movement within the plant, but does not limit TRV replication in infected tissue. Therefore, the pYL156:*GUS* construct represents an improved negative control for TRV-induced gene silencing.

VIRUS-INDUCED GENE SILENCING

Virus-induced gene silencing (VIGS) is an important reverse genetics tool for the analysis of gene function in many different plant species (Burch-Smith *et al.*, 2004; Senthil-Kumar *et al.*,

2007). Vectors that have been used for VIGS in solanaceous plants include those derived from tobacco mosaic virus (TMV), potato virus X (PVX), tomato golden mosaic virus (TGMV) and tobacco rattle virus (TRV) (Ratcliff *et al.*, 2001). Compared with TMV, PVX and TGMV, TRV is a superior vector because it is able to spread more vigorously throughout the entire plant, including meristem tissue, and its impact on plant health is relatively mild compared with the other viruses (Ratcliff *et al.*, 2001). TRV is a single-stranded, positive-sense RNA virus with a bipartite genome. One component of the genome, TRV2, has been engineered to carry a multiple cloning site for VIGS, whereas TRV1 encodes proteins required for virus replication and movement (Liu *et al.*, 2002a). TRV-based VIGS vectors have been successfully used in a broad range of solanaceous plants (e.g. tomato, potato, pepper, petunia, black nightshade and wild tobacco species), as well as *Arabidopsis*, opium poppy, California poppy and columbine (Brigneti *et al.*, 2004; Burch-Smith *et al.*, 2004, 2006; Chen *et al.*, 2009; Godge *et al.*, 2008; Gould and Kramer, 2007; Hartl *et al.*, 2008; Hileman *et al.*, 2005; Liu *et al.*, 2002a, b; Lu *et al.*, 2003a, b; Ryu *et al.*, 2004; Senthil-Kumar *et al.*, 2007; Wege *et al.*, 2007).

VIGS IN TOMATO

Tomato, *Solanum lycopersicum* (Solanaceae), is an economically important crop and a widely used model to study mechanisms of plant defence against disease and herbivory. A large number of expressed sequence tags (ESTs) are available for this species, and a draft of the genome sequence is available through the SOL Genomics Network (Mueller *et al.*, 2005; Yano *et al.*, 2007). Genetic transformation of tomato is also feasible; however, the efficiency of transformation is poor and regeneration of transformed plants is slow and labour-intensive. Therefore, VIGS is particularly important for the analysis of gene function in this species. For example, the TRV-based VIGS approach has been used to identify several genes required for

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plant defences against pests, including aphids (Bhattarai *et al.*, 2007; Liu *et al.*, 2002a, 2004; Lu *et al.*, 2003a).

SYMPTOM DEVELOPMENT IN TOMATO IN RESPONSE TO THE pYL156 EMPTY VECTOR

This study aimed to improve the methods for use of a TRV-based VIGS vector (pYL156) widely employed to study aphid resistance and other phenotypes in tomato. VIGS vectors can, in some cases, cause symptoms of viral infection in plants, and these symptoms can be a confounding variable in experiments to discern gene function, particularly if the severity of the symptoms varies between experimental constructs and empty vector controls (Hartl *et al.*, 2008). Therefore, the first goal of this study was to assess the extent of symptoms induced in tomato by pYL156. The pYL156 vector was obtained from Dr Dinesh-Kumar (Yale University, New Haven, CT, USA), and a silencing construct carrying a 408-bp fragment of the *phytoene desaturase* (*PDS*) gene was obtained from Dr Kyle Willis (US Department of Agriculture/Agricultural Research Service, Washington DC, USA). Silencing of this gene results in photobleaching, providing a convenient visual indicator of the effectiveness of VIGS (Rotenberg *et al.*, 2006). We also developed an additional silencing construct carrying a 386-bp fragment of *NPR1* (*Nonexpressor of PR Genes 1*), a key regulator of plant defences against pathogens that has been targeted in previous VIGS experiments in tomato and *Nicotiana benthamiana* (Liu *et al.*, 2002a). [Please see Table S1 (Supporting Information) for the primers used to clone this fragment.] Constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation, and 2-week-old tomato plants (*S. lycopersicum* cv. Motelle) were vacuum infiltrated with buffer alone, or with *A. tumefaciens* carrying the empty vector (pYL156) or a silencing construct [pYL156::PDS (408 bp) or pYL156::NPR1]. Plants receiving the empty vector or a silencing construct were also co-infiltrated with an *A. tumefaciens* culture carrying TRV1, and agroinfiltration was performed as described previously (Ekengren *et al.*, 2003). Vacuum infiltration was used to introduce the virus vector into plants, because we found that it resulted in more efficient silencing than the leaf injection method, as also reported by Ekengren *et al.* (2003). Plants were maintained at 20 °C with a light (16 h) : dark (8 h) photoperiod, and examined for symptoms of virus infection 8 days post-infiltration (dpi). All tomato plants infiltrated with the empty vector pYL156 displayed severe symptoms of virus infection, including dead or malformed leaves and necrotic stem lesions (Fig. 1A,B). Furthermore, compared with buffer-treated controls, their growth was permanently stunted (Fig. 1C,D). In comparison, few symptoms were observed in plants infiltrated with the two different silencing constructs: pYL156::PDS (408 bp) and pYL156::NPR1. Stem and leaf lesions were either absent or very small in plants infiltrated with either of the

Fig. 1 Symptom development in tomato plants treated with tobacco rattle virus (TRV). Two-week-old tomato seedlings (cv. Motelle) were vacuum infiltrated with TRV1 plus the pYL156 empty vector, pYL156::NPR1 or pYL156::PDS (408 bp). Additional control plants were infiltrated with inoculation buffer only. Eight days after infiltration, plants inoculated with pYL156 displayed numerous dead leaves (A), stem lesions (B), and reduced height (C) and width (D) relative to the other three treatment groups.

silencing constructs, and the growth rate of these plants was comparable with that of buffer-infiltrated controls (Fig. 1C,D). Similar results were obtained with silencing constructs targeting three additional unrelated genes in three genetic backgrounds (cultivars Motelle, Moneymaker and Castlemart; data not shown); in all cases, plants infiltrated with the empty vector were severely stunted compared with plants infiltrated with VIGS constructs. This is the first report that the empty vector pYL156 induces severe viral symptoms in tomato.

IMPACT OF INSERT SIZE ON SYMPTOM DEVELOPMENT AND GENE SILENCING

The fact that the empty vector caused far more extensive symptoms of virus infection than our silencing constructs suggested that symptom development may be influenced by the size of the viral vector. The inserts tested ranged in size from 300 to 500 bp, resulting in recombinant TRV2 constructs of 2671 to 2871 bp, whereas the empty vector is 2371 bp in size. To explore the influence of insert size on viral symptom development, we compared plants infiltrated with three VIGS constructs that all targeted the same transcript, *PDS*, but contained inserts of different sizes (96, 249 or 408 bp; see Table S1 for information on the cloning of these inserts). Plants were infiltrated as described above, and observed at 12 dpi. Data were analysed by one-way analysis of variance (ANOVA) using JMP Version 7.0 (SAS Institute Inc., Cary, NC, USA). In cases in which ANOVA identified a significant difference among treatments, mean separations were then performed using Student's *t*-tests ($P < 0.05$). Stem lesions were observed on all plants infiltrated with the 96-bp *PDS* construct, but not on buffer-treated controls or on plants infiltrated with either of the larger constructs. Furthermore, compared with plants infiltrated with pYL156::*PDS* (249 bp), pYL156::*PDS* (408 bp) or buffer alone, plants infiltrated with the smaller pYL156::*PDS* (96 bp) construct were reduced significantly in height, width and leaf number, and had a larger number of dead

leaves ($P < 0.0001$; Fig. 2A). We also compared the extent of photobleaching induced by the three constructs to assess their efficacy in inducing gene silencing. For all three silencing constructs, all inoculated plants (seven plants per construct) showed signs of *PDS* silencing. A single mature leaf per plant at a fixed leaf position (sixth leaf from the youngest emerging leaf) was scored using a ranking system to assess the proportion of the leaf area that showed photobleaching (rank 1, no bleaching; 2, <25% of leaf area bleached; 3, 25–50% bleached; 4, 50–75% bleached; 5, >75% bleached). It was not possible to score the plants infiltrated with the 96-bp *PDS* construct because widespread chlorosis in these plants made it difficult to outline the bleached regions of the leaf; however, we observed a clear distinction between the plants infiltrated with the 249- and 408-bp constructs (Fig. 2B). For all plants infiltrated with pYL156::*PDS* (249 bp), less than 50% of the leaf area was bleached, whereas in plants infiltrated with pYL156::*PDS* (408 bp), all but two plants displayed *PDS* silencing in more than 50% of the leaf (average rank, 4). Photobleaching rankings for the larger construct were significantly higher [average rank for pYL156::*PDS* (249 bp), 3; average rank for pYL156::*PDS* (408 bp), 4; χ^2 test, $P = 0.005$]. These data support the hypothesis that viral symptoms decrease with increasing construct size, and indicate that insertions of approximately 400 bp are preferable for limiting symptom development and promoting effective gene silencing.

Fig. 2 Impact of insert size on viral symptom development and the efficiency of gene silencing. Two-week-old tomato seedlings (cv. Motelle) were vacuum infiltrated with buffer alone or co-infiltrated with tobacco rattle virus (TRV1) and one of three VIGS constructs of varying size (96, 249 or 408 bp) that all target the same transcript, *phytoene desaturase* (*PDS*) (seven to eight plants per treatment). Twelve days after inoculation, plants were examined to assess their growth (plant height, width and number of total leaves) and symptom development (number of dead leaves and stem lesion size) (A), as well as the extent of photobleaching resulting from the silencing of *PDS* (B).

COMPARISON OF SYMPTOMS INDUCED BY pYL156 AND pYL156::GUS, AN ALTERNATIVE VECTOR CONTROL

To develop an appropriate vector control for silencing constructs that carry inserts of approximately 400 bp, we developed the pYL156::GUS construct by inserting a 396-bp fragment of the β -glucuronidase gene (*GUS*) of *Escherichia coli* into the pYL156 multiple cloning sites (Fig. 3A; see Table S1 for the primers used in cloning). This construct also allowed us to test the effects of DNA insertion on symptom development independent of any effects that might result from gene silencing. The *GUS* reporter gene is not present in the tomato genotypes used for this study, and the cloned fragment does not bear significant homology with any endogenous tomato sequences reported in GENBANK and the SOL Genomics Network (<http://sgn.cornell.edu>). Therefore, this construct is not expected to cause gene silencing. It is also similar in size to most published TRV-based silencing constructs, which typically carry inserts of 300–500 bp (Burch-Smith *et al.*, 2004; Ratcliff *et al.*, 2001). Two-week-old tomato seedlings (cv. Motelle) were vacuum infiltrated with inoculation buffer alone or co-infiltrated with TRV1 and either the pYL156 empty vector or pYL156::GUS (16 plants per treatment). At 12 dpi, symptom development was significantly greater on tomato plants treated with the pYL156 vector compared with plants treated with pYL156::GUS or inoculation buffer (Fig. 3B). Statistical analysis demonstrated that there were significant differences in plant height, width, number of total leaves per plant, number of dead leaves per plant, stem lesion size, above-ground weight and flowering time of the first flower to open ($P < 0.0001$; Fig. 3C,D). Plants infiltrated with the pYL156 vector showed reduced size and weight, extensive stem lesions, necrotic leaves and delayed flowering compared with the other two treatments, whereas there were no significant differences ($P > 0.10$) between plants infiltrated with pYL156::GUS and the inoculation buffer in any variable other than stem lesion size, which was marginally greater in pYL156::GUS plants ($P = 0.0865$). Buffer-treated controls showed no lesions, whereas three pYL156::GUS plants showed small (<4.2 cm) stem lesions. Furthermore, differences in plant size and health persisted throughout the 40-day observation period (data not shown). Taken together, these results demonstrate that, unlike the pYL156 vector, pYL156::GUS does not significantly impair tomato plant development. This is consistent with a previous report indicating that the pYL156 empty vector decreased the growth of black nightshade, but that control vectors carrying a piece of noncoding DNA did not affect plant growth (Hartl *et al.*, 2008).

Fig. 3 Comparison of symptoms in tomato plants treated with the pYL156 empty vector or pYL156::GUS. Two-week-old tomato seedlings (cv. Motelle) were vacuum infiltrated with inoculation buffer only or with tobacco rattle virus (TRV1) plus the pYL156 empty vector or the pYL156::GUS construct, which was created by inserting a 396-bp DNA fragment of the *Escherichia coli* β -glucuronidase gene (*GUS*) into the multiple cloning site (MCS) (A). Twelve days after inoculation, eight plants per treatment were sampled to compare plant height, width, number of total leaves, number of dead leaves, stem lesion size and above-ground weight (B, C and D). An additional eight plants per treatment were retained and monitored daily to test flowering (date at which the first flower per plant opened).

INFLUENCE OF pYL156 AND pYL156::GUS ON APHID RESISTANCE

The next objectives of this study were to determine whether symptoms caused by the pYL156 vector can influence important biological traits, such as aphid resistance, that are of interest in VIGS experiments, and to assess whether pYL156::GUS alleviates this problem. To this end, we performed two bioassays to measure the population growth of the potato aphid (*Macrosiphum euphorbiae*) on two tomato cultivars infiltrated with pYL156, pYL156::GUS or buffer alone. Two-week-old tomato plants (cv. Moneymaker for assay 1) were vacuum infiltrated with inoculation buffer alone or co-infiltrated with TRV1 and either the pYL156 empty vector or pYL156::GUS (eight plants per treatment). Four weeks after infiltration, plants were inoculated with potato aphids using lightweight mesh clip cages (3.5 cm in diameter) that restrict aphids to a single leaflet. Aphids were confined to newly formed leaflets without any lesions (four cages per plant; five young adult aphids per cage). On the fifth day post-inoculation, the numbers of surviving adults and offspring were counted. A second bioassay was also performed on the aphid-resistant cultivar Motelle (four to six plants per treatment; two to five cages per plant). Aphid numbers on Motelle were scored on the fourth day after inoculation because of high aphid mortality on this genotype. Prior to statistical analysis by ANOVA, the data were log-transformed to normalize the variances. On the susceptible cultivar Moneymaker, there was a highly significant difference in aphid population growth among the three treatment groups (adult numbers, $P = 0.0011$; juvenile numbers, $P = 0.0015$; total aphid numbers, $P = 0.0014$). Aphid numbers were lower on plants treated with pYL156 than on plants infiltrated with pYL156::GUS or buffer ($P < 0.01$) for adults, juveniles and total aphids, whereas there were no significant differences in aphid numbers between pYL156::GUS and buffer controls ($P > 0.1$ for all three variables) (Fig. 4A). Similar trends were also observed on the aphid-resistant cultivar Motelle (Fig. 4B). Although adult survival was negligible and offspring numbers were much lower on this resistant cultivar than on Moneymaker, the numbers of offspring and total aphids on plants infiltrated with pYL156 were less than half the numbers observed on plants infiltrated with pYL156::GUS, and these differences were statistically significant at $\alpha = 0.1$ (adult numbers, $P = 0.84$; juvenile numbers, $P = 0.071$; total aphid numbers, $P = 0.086$). These results demonstrate that the pYL156 vector influences plant susceptibility to aphids in both resistant and susceptible tomato genotypes and, as a result, could bias the outcome of aphid bioassays when used as a negative control for VIGS experiments. By artificially reducing aphid numbers on the negative control, pYL156 could cause bioassays to overestimate the degree to which other silencing constructs compromise plant defences and increase aphid numbers. Given that this vector has extensive negative effects on

Fig. 4 Comparison of aphid population growth on tomato plants infiltrated with the pYL156 empty vector or pYL156::GUS. The effects of pYL156 and pYL156::GUS on potato aphid infestation were tested in two genetic backgrounds: the aphid-susceptible cultivar Moneymaker (A) and the aphid-resistant cultivar Motelle (B). Adult aphids were confined to individual leaflets using small clip cages (five adults per cage; two to four cages per plant), and the number of surviving adults and offspring was monitored 4–5 days after inoculation. The average number of aphids per cage per plant is reported $\pm$ the standard error of the means.

plant growth and development (Figs 1 and 3), it is likely that it would also influence other important phenotypes analysed in VIGS studies. In contrast, the pYL156::GUS construct does not influence susceptibility to aphids, causes fewer symptoms of virus infection and represents an improved negative control.

REPLICATION OF pYL156 AND pYL156::GUS IN PLANTS

Potentially, symptom severity could differ between the pYL156 vector and pYL156::GUS as a result of an effect of DNA insertion on virus replication and/or movement (Burch-Smith *et al.*, 2004; Liu and Page, 2008). To compare the replication of TRV with and without a DNA insertion, 2-week-old tomato seedlings (cv. Motelle) were vacuum infiltrated with TRV1 and either the

pYL156::GUS or pYL156 empty vector (nine plants per treatment). At 5, 10 and 15 dpi, an infiltrated leaflet at a standardized leaf position per plant was collected (terminal leaflet of leaf 2 on day 5, terminal leaflet of leaf 3 on day 10 and terminal leaflet of leaf 4 on day 15; with the first true leaf counted as leaf 1). There were three biological replications per treatment per time point, and each replicate consisted of tissue pooled from three plants. Real-time reverse transcription-polymerase chain reaction (RT-PCR) was used to quantify transcripts encoding the TRV movement protein, and was performed with the QuantiTect SYBR Green PCR kit (Qiagen, Inc., Valencia, CA, USA). The tomato ribosomal protein L2 gene (*RPL2*) was used as the housekeeping gene control for this study (Table S1). The data were analysed using the relative standard curve method (User Bulletin #2 ABI Prism 7700 Sequence Detection System, 1997). There were no significant differences in transcript abundance between the two treatments at any time point ($P > 0.1$), although the relative expression value of the TRV movement protein at 15 dpi was higher in plants treated with pYL156::GUS than in those treated with the empty vector (Fig. S1A, see Supporting Information). These results indicated comparable levels of virus replication in infiltrated tissue in the two treatment groups.

MOVEMENT OF pYL156 AND pYL156::GUS IN PLANTS

Two additional experiments were performed to assess the movement of pYL156 and pYL156::GUS within tomato plants. In the first experiment, the virus was introduced into seedlings using vacuum infiltration (six plants per treatment), and tissue was collected from each plant at 10, 20 and 30 dpi from an infiltrated leaf and a newly formed leaf at a standardized position (terminal leaflet of leaf 7 on day 10, terminal leaflet of leaf 9 on day 20 and terminal leaflet of leaf 11 on day 30; with the first true leaf counted as leaf 1). TRV movement protein was detected by standard RT-PCR in both infiltrated leaves and newly formed foliage (i.e. foliage that was formed after the infiltration process had taken place). RT-PCR results demonstrated that, at 10 and 20 dpi, pYL156 and pYL156::GUS had moved into newly formed foliage in all six replicate plants per treatment. However, a difference was observed between treatments at the 30-day time point; although the 11th leaf of all plants (6/6) infiltrated with pYL156 tested positive for the virus, RNA encoding the viral movement protein was not detected in new leaves in one-third (2/6) of plants infiltrated with pYL156::GUS. These results suggest that DNA insertion may inhibit virus movement. To examine virus movement at earlier time points in the infection process, a second experiment was performed in which TRV1 plus either the pYL156 vector or pYL156::GUS was introduced into a single leaflet per plant (terminal leaflet of the first true leaf) by syringe injection (six plants per treatment). Virus spread was

monitored by the detection of transcripts encoding TRV movement protein using standard RT-PCR at 3, 5, 7, 10 and 20 dpi at three positions: in uninjected leaflets within the inoculated leaf (inoculated position); in a younger but mature leaf (middle position); and in the youngest expanded leaf on the plant (top position) (Fig. S1B,C). Each sample consisted of tissue pooled from six biological replicates. From 5 dpi onwards, the virus was detected in all samples infiltrated with pYL156. In contrast, virus spread was slower and patchier in plants infiltrated with pYL156::GUS; it was not until 15 dpi that the virus was uniformly detected at all leaf positions. The virus appeared to move more rapidly to newly expanded leaves than to older leaves or to other leaflets within the same leaf. These results demonstrate that the pYL156 empty vector moves more rapidly within the plant than the larger pYL156::GUS construct.

CONCLUSIONS

We report here that the pYL156 empty vector can cause extensive damage to tomato plants, whereas silencing constructs that carry inserts of 250 bp or more cause relatively few symptoms of virus infection. As a result, symptoms of virus infection can bias experimental results when the empty vector is used as a negative control in aphid bioassays or in measurements of plant growth and development. In contrast, the pYL156::GUS construct, which is similar in size to the most effective VIGS constructs but should not influence the expression of any endogenous genes in tomato, does not have an impact on plant growth, development or aphid resistance. Virus replication in infected tissues was similar for the empty vector and pYL156::GUS, although virus movement was slower for the larger pYL156::GUS construct. This construct represents an improved negative control for TRV-based gene silencing experiments that more accurately mimics infection by silencing constructs.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 The replication and movement of pYL156 and pYL156::GUS in tomato plants. Real-time reverse transcription-polymerase chain reaction (RT-PCR) was used to assess the replication efficiencies of the pYL156 vector and pYL156::GUS in infiltrated leaves of tomato seedlings (cv. Motelle) at 5, 10 and 15 days after vacuum inoculation. The transcript abundance of the tobacco rattle virus (TRV) movement protein (MP) gene was calculated by the relative standard curve method normalized to the tomato housekeeping gene *RPL2*, and averages are reported $\pm$ the standard error of the means (A). To measure the movement of the pYL156 vector and pYL156::GUS in tomato plants, tomato seedlings were inoculated with TRV1 and either the pYL156 empty vector (B) or pYL156::GUS (C) by syringe injection into a single leaf per plant. RT-PCR was then used to detect the movement of the virus to leaflets at three positions within the plant: in uninjected leaflets within the inoculated leaf (inoculated position, I), in a younger but mature leaf (middle position, M) and in the youngest expanded leaf on the plant (top position, T). Each sample consisted of tissue pooled from six replicate plants per treatment. The far left lane contains a 1-kB plus DNA ladder (L).

Table S1 Primers of inserted fragments in pYL156 vector and real-time reverse transcription-polymerase chain reaction (RT-PCR).

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