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Tombusvirus-based vector systems to permit over-expression of genes or that serve as sensors of antiviral RNA silencing in plants

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ABSTRACT

A next generation *Tomato bushy stunt virus* (TBSV) coat protein gene replacement vector system is described that can be applied by either RNA inoculation or through agroinfiltration. A vector expressing GFP rapidly yields high levels of transient gene expression in inoculated leaves of various plant species, as illustrated for *Nicotiana benthamiana*, cowpea, tomato, pepper, and lettuce. A start-codon mutation to down-regulate the dose of the P19 silencing suppressor reduces GFP accumulation, whereas mutations that result in undetectable levels of P19 trigger rapid silencing of GFP. Compared to existing virus vectors the TBSV system has a unique combination of a very broad host range, rapid and high levels of replication and gene expression, and the ability to regulate its suppressor. These features are attractive for quick transient assays in numerous plant species for over-expression of genes of interest, or as a sensor to monitor the efficacy of antiviral RNA silencing.

Introduction

Agrobacterium-mediated transformation is a very common method to transfer genes of interest into plants, either for transient expression through agroinfiltration, or by regenerating transgenic plants. An alternative is to use plant viruses as gene vectors (Gleba et al., 2005, 2007; Hefferon, 2012; Komarova et al., 2010; Lindbo, 2007; Pogue et al., 2002; Sainsbury et al., 2009; Scholthof et al., 1996, 2002; Yusibov et al., 1999, 2013). However, the plant science community as a whole is still mostly unaware of the general utility of these plant virus-based vector systems; especially regarding the high levels of foreign product accumulation in a relatively short time span, taking advantage of the prolific levels of virus replication and concomitant gene expression. An additional benefit is that the same gene vector construct can be utilized for inoculation on multiple susceptible plants without the necessity for genetic transformation of each host species (Scholthof et al., 2002; Seaberg et al., 2012).

Another area in which viruses and their derived vectors have proven quite useful is in studying antiviral RNA silencing in plants, otherwise known as virus-induced gene silencing (VIGS). Instead of over-expressing genes, the latter strategy uses viruses to induce RNA silencing in plants to abrogate virus accumulation and inadvertently targeting host-encoded mRNAs homologous to gene segments inserted into the virus vector, leading to *de facto* down-regulated expression of that host gene (Burch-Smith et al., 2004).

Even though the past few decades plant viruses have become increasingly important as tools in molecular biology or biotechnology, oftentimes it is hard to predict how suitable any given plant species might be for over-expressing genes or how effective its silencing survey mechanism operates against an invading virus. For this it would be desirable to have a virus vector system that can be used in multiple plant species and for which the susceptibility to silencing can be readily monitored. *Tomato bushy stunt virus* (TBSV) is such a candidate; it has a wide host range (Martelli et al., 1988) and has shown potential for local over-expression in leaves, for VIGS of endogenous plant genes, and for studying the antiviral silencing mechanism *per se* (Omarov et al., 2007; Pignatta et al., 2007; Qiu and Scholthof, 2007; Scholthof et al., 2011; Zhong et al., 2005). The present study addressed the question whether the utility and applicability of a TBSV-derived vector system can be

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further increased to readily over-express genes, or as a sensor to survey plants for the effectiveness of antiviral silencing.

TBSV is the type member of the genus *Tombusvirus* in the *Tombusviridae* (Russo et al., 1994; Yamamura and Scholthof, 2005). The ~4.8 kb single-stranded plus-sense TBSV genome (Fig. 1) essentially functions as an mRNA for translation of two replication-associated proteins, P33 and P92 that localize to membranes for replication of the genome or subviral agents (Rubino et al., 2007; Rubino and Russo, 2012), which is regulated by a suite of *cis*-acting genomic elements and host factors (Nagy et al., 2012; Pathak et al., 2012). The capsid protein (CP) is translated from *p41* (*CP*) on subgenomic messenger RNA1 (sgRNA1), and not only is required for encapsidation, but also plays an active role in virus transport (Desvoyes and Scholthof, 2002; Qu and Morris, 2002). Additionally, for a closely related virus CP was shown to be targeted to mitochondria and chloroplasts and to be involved in fungal transmission (Hui et al., 2010; Li et al., 2013). TBSV encodes two additional proteins, P22 and P19, that are expressed via a second subgenomic mRNA (sgRNA2) which encodes two nested open reading frames (ORFs) for translation of the *p22* and *p19* genes (Hearne et al., 1990). The *p22*-encoded protein (P22) is required for cell-to-cell movement, whereas P19 is involved in host-specific systemic invasion and acts as a suppressor of post-transcriptional gene silencing (Scholthof, 2006; Yamamura and Scholthof, 2005). Furthermore, all sgRNA-translated products (CP, P22 and P19) can elicit defense responses in a host-dependent manner (Angel and Schoelz, 2013).

Here we describe a modified next generation TBSV *CP*-replacement vector system expressing GFP that can be applied by either RNA inoculation or through agroinfiltration. In conjunction with P19 expression, the vector yields rapid and high levels of GFP accumulation, whereas upon inactivation of *p19* it serves as a sensitive sensor to visualize the antiviral RNA silencing response. Experiments are shown for several plants species and considering the broad host range for (i) the virus (Martelli et al., 1988), (ii) *Agrobacterium* (Lacroix et al., 2006), and (iii) transcript inoculation with TBSV–GFP derivatives (Seaberg et al., 2012), the system holds high potential for a wide variety of plant species.

Results

Transcript inoculations

In the original TBSV–GFP progenitor the *CP* was partially replaced with *GFP* encoding the C3 version of the ~27 kDa green fluorescent protein (GFP) (Everett et al., 2010), while in comparison in RMJ1 (Fig. 1) ~1 kb non-expressed CP RNA sequences downstream of the 3′-end of *GFP* were removed. Transcript inoculation of the original TBSV–GFP and its derivative RMJ1 on *N. benthamiana*, followed at 4 days post-inoculation (dpi) by immunoblotting for CP showed that despite the interruption by *GFP*, CP was still produced for TBSV–GFP (Fig. S1) presumably because of recombination events that restore CP production (Qiu and Scholthof, 2007). However, because of the removal of ~1 kb *CP* sequences downstream of *GFP*, no detectable levels of CP was produced upon infection with RMJ1 (Fig. S1).

Transcripts of the different pRMJ constructs (Fig. 1B; indicated as RMJ1 when meaning RNA) were used to inoculate 3–4 week old *N. benthamiana* plants. The results show that compared to the progenitor TBSV–GFP construct, RMJ1 caused a somewhat faster appearance of green fluorescence by 2 dpi (Fig. 2A) (evident in the close up inserts). This suggests that the removal of extraneous *CP* sequences (downstream from GFP) from TBSV–GFP to construct pRMJ1, had a beneficial effect on early events during infection. In addition to an earlier accumulation, the results in Fig. 2A also suggest a more intense and dense level of green fluorescence later on for RMJ1 compared to TBSV–GFP, whereas western blot analyses for P19 (Fig. 2B) indicated that such elevated GFP levels occurred with similar levels of virus infection. Even though statistical quantitative comparisons between TBSV–GFP and RMJ1 have not been conducted, the results clearly show that RMJ1 performs very well and does not suffer from *CP*-restorative recombinations.

Mutation of the *p19* start-codon in RMJ2 did not affect the timing of GFP appearance at 2 dpi but compared to RMJ1 the levels of fluorescence were substantially lower at 3 dpi. The start codon mutation did not prohibit P19 expression, instead the levels of P19 were substantially lower than for RMJ1 (Fig. 2B). However, the

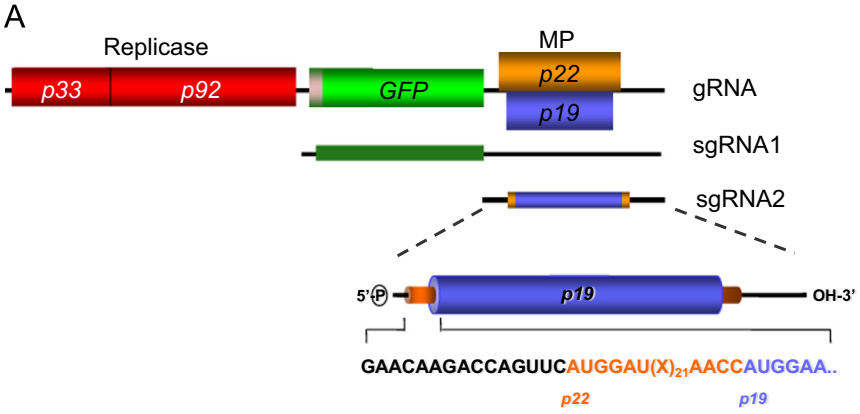

Fig. 1. Properties of modified *Tomato bushy stunt virus* (TBSV) GFP expressing RMJ and derivatives used in this study. (A) The top shows the ~4.8-kb single-stranded (ss) message-sense genomic RNA (gRNA); black lines indicate non-translated regions. The nomenclature of open reading frames (ORFs) (boxed regions) is derived from the predicted molecular weight (in kDa) of the products with functions indicated; MP, movement protein. The 5′-end of the coat protein gene (*CP*) (shaded) is fused in frame-to *GFP* (green), which is translated from subgenomic RNA1 (sgRNA1). Features of sgRNA2 are shown in some more detail to illustrate that *p22* and *p19* are expressed from different ORFs allowing for gene-specific mutations. *Note*: as indicated in the text, in the progenitor TBSV–GFP construct (Everett et al., 2010) an additional ~1 kb stretch of *CP* RNA sequences remained downstream of *GFP*, and this has been removed in RMJ1. (B) Summary that pRMJ1 contains wild-type *p19* and effect of mutations introduced in pRMJ2 and pRMJ3.

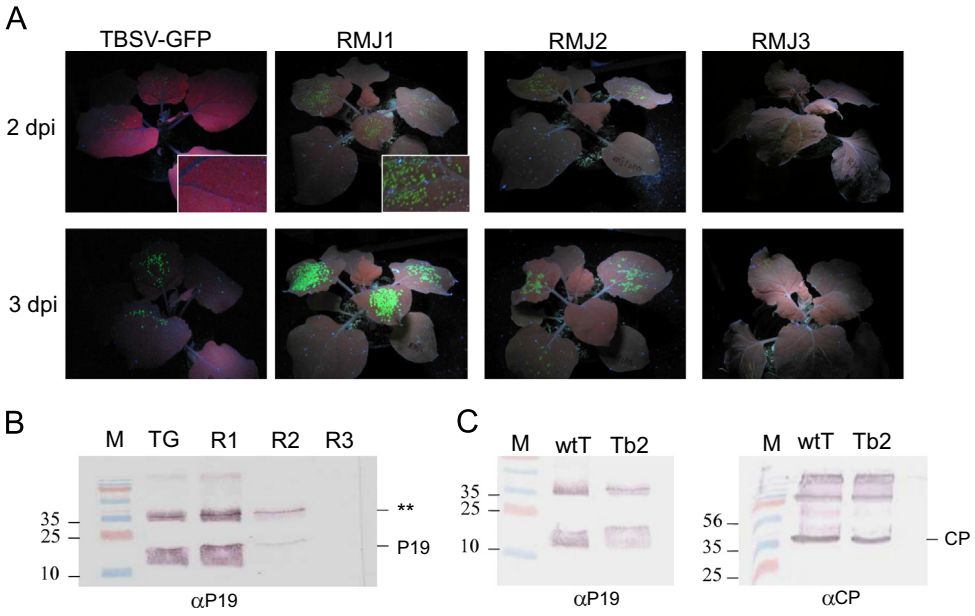

Fig. 2. Inoculation of TBSV constructs on *N. benthamiana*. (A) Images were captured at 2 and 3 days post-inoculation (dpi); the inserts on the lower right-hand for TBSV–GFP and RMJ1 at 2 dpi are close up images of leaf sections. (B) Alkaline phosphatase-mediated immunoblot detection at 3 dpi of P19 showing the P19 monomer and dimer (**); P19 is functional as a dimer and because of its is SDS-recalcitrance it is commonly observed with western blots upon SDS-PAGE (Scholthof, 2006). Inoculations were performed with RMJ1 (R1), RMJ2 (R2), or RMJ3 (R3). (C) Alkaline phosphatase-mediated immunoblot detection of P19 (αP19) or CP (αCP) in *N. benthamiana* plants 4 dpi with either wild-type TBSV (wtT) or its derivative with the same mutation as RMJ2 and referred to as Tb2. The upper bands recognized by αCP represent non-denatured oligomers. Size markers (M) are indicated in kDa.

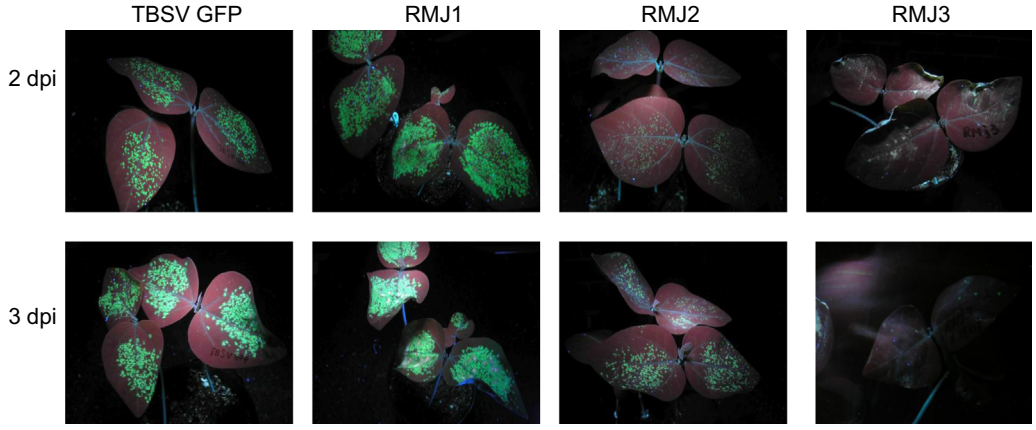

Fig. 3. Visualization of GFP expression upon inoculation of vector constructs on cowpea. Images were captured at 2 and 3 dpi.

difference is probably also partly due to the overall reduced accumulation (based on GFP levels in Fig. 2A). To illustrate that the start-codon mutation specifically down-regulated P19 levels, expression was normalized by incorporating the same mutation as in RMJ2 into the wild-type TBSV background to yield pTb2, followed by comparing P19 and CP levels in infected plants (Fig. 2C). This showed that when compared to wild-type TBSV infections, *N. benthamiana* infected with Tb2 accumulated similar levels of the 42-kDa CP while amounts of P19 were reduced (Fig. 2C). Combined, these results suggest that the reduced levels of P19 associated with RMJ2 is insufficient to entirely block silencing, resulting in interference with the progression of the infection (Fig. 2A), similar to what we observed previously when the presence of defective interfering RNAs (DIs) down-regulated P19 levels (Qiu et al., 2002). When P19 expression was eliminated upon introduction of two premature stop-codons in pRMJ3, little or no GFP fluorescence was detected (Fig. 2). This is directly due to the rapid effects of silencing since expression can be restored by expressing P19 or unrelated suppressors *in trans* (Scholthof et al., 2011), and as shown further below in the agroinfiltration section.

Inoculations of the RMJ series on cowpea (*Vigna unguiculata*) (Fig. 3), as well as RMJ1 or RMJ3 inoculations of tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), and lettuce (*Lactuca sativa*) (Fig. S2) yielded essentially the same results as for *N. benthamiana*, indicating that the differences observed for the different constructs are consistent across platforms. Combined with the broad host range for TBSV-mediated GFP expression (Seaberg et al., 2012), the pRMJ1 construct represents currently the most robust TBSV-derived vector for generating and inoculating transcripts to allow quick gene expression analyses in plant species of several families. Likewise, RMJ3 provides an ideal sensor to analyze different plant species for the effectiveness of their antiviral silencing response.

Agroinfiltration

Agrobacterium-mediated transfer of DNA upon infiltrating leaves (agroinfiltration) to launch virus infections in plants has become a very useful, convenient, and important tool (Gleba et al., 2005, 2007, 2014; Lindbo, 2007). This technique avoids the

necessity to generate transcripts *in vitro*, instead the T-DNA is efficiently transferred from *Agrobacterium* to the plant nucleus, resulting in a high number of infected cells upon inoculation. To test the utility of the RMJ1 and RMJ3 vectors in conjunction with agroinfiltration, binary constructs pT31 (expressing RMJ1) and pT33 (expressing RMJ3) were generated and used to transform *Agrobacterium*. These cultures were used for infiltration of *N. benthamiana* plants for which GFP fluorescence was monitored at 2–4 day intervals. The results show that GFP expression from pT31 was readily evident and abundant (Fig. 4A).

Upon infiltration of *N. benthamiana* with pT33, GFP expression was low or absent (Fig. 4A). To verify that this was not due to unintended mutations but instead to induction of RNA silencing, the now commonly accepted practice was applied of co-infiltrating pT33 with a construct expressing P19 (Saxena et al., 2010; Voinnet et al., 2003). The results show (Fig. 4A) that this restored GFP expression for pT33 to levels comparable to those obtained with pT31 alone. Western blot analyses (Fig. 4B) confirmed the imaging data (Fig. 4A) for GFP expression, and these also demonstrated the absence of P19 expression from pT33 and the restoration of P19 expression upon co-infiltration of a P19 expressing construct. Agroinfiltrations with pT31 and pT33 onto tomato (Fig. S3) also showed strong GFP expression for pT31 and poor expression for pT33. Together this shows the utility of pT33 for rapid visualization of antiviral silencing across plant platforms.

Discussion

Presently the most commonly used viral vectors are based on *Tobacco mosaic virus* (TMV), *Potato virus X* (PVX), *Cowpea mosaic virus* (CPMV), and select geminiviruses (Gleba et al., 2014), that are for the most part used in a few very select host species (*e.g.*, cowpea or *N. benthamiana*). To have a truly versatile and readily applicable virus vector-based transient gene expression system that can be used in a wide variety of plants could be a substantial added benefit. TBSV has a wide host range spanning 180 families (Russo et al., 1994; Yamamura and Scholthof, 2005) and many of those are susceptible to infection with TBSV *CP*-replacement vectors (Seaberg et al., 2012). No biological vector is known for the virus (Martelli et al., 1988), making TBSV a suitable and biosafe vector for rapid transient assays.

Upon infection of *N. benthamiana* with progenitor TBSV–GFP (Everett et al., 2010) transcripts, CP was detected (Fig. S1) presumably because of the rapid onset of recombinants that had restored the ability to produced a (slightly truncated) CP. This precisely mirrors our observations with a *CP* mutant harboring a small out-of-frame deletion between *Not*I and *Bal*I (Desvoyes and Scholthof, 2002), the same sites used to insert GFP for TBSV–GFP (see "Materials and methods"). The removal of ~1 kp of *CP* RNA nucleotides downstream of *GFP* in TBSV–GFP to yield RMJ1, disabled the opportunity for *CP* restoration and consequently no CP was detected (Fig. S1). Thus, we have developed a new modified TBSV vector backbone in which essentially the whole coat protein-encoding region is replaced with *GFP* (Fig. 1) that does not have the ability to restore CP synthesis. The present configuration does result in a fusion of ca. 20-25 N-terminal CP sequences (depending on which of the methionine codons is used as a start) to GFP. Preliminary findings suggest that this fusion of RNA and/or protein enhances expression levels (data not shown), but this should not affect the utility because incorporation of specific protease cleavage sites can ensure *in vivo* or *in vitro* production of authentic proteins if so desired, as is also the case for most bacterial, or eukaryotic expression systems currently available.

The consequence of the *CP* substitution for infections with RMJ1 is that in comparison to the rapid onset of local GFP expression in RMJ1-inoculated or pT31-infiltrated leaves, systemic

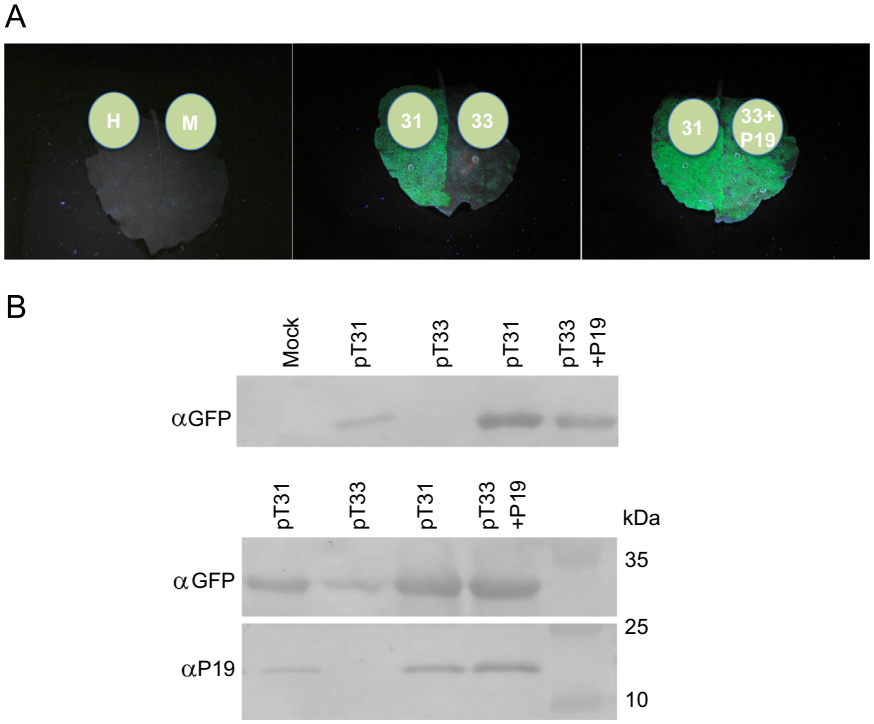

Fig. 4. Agroinfiltration of bacterial cultures harboring TBSV constructs, on 5-week old *N. benthamiana*. (A) Half-leaf assays were performed with either Healthy/Mock treated (left; H, M), infiltration with pT31/pT33 (middle; 31, 33), infiltration with pT31/pT33+p19 (right; 31, 33+P19), as further illustrated with the inset green circles. Images were captured at 6 days post-infiltration (dpi). (B) Alkaline phosphatase-mediated immunoblot detection of GFP and P19 from plants treated as in A. Results of two experiments are shown for GFP detection to illustrate no or low levels of GFP for pT33 infiltrated leaves and to incorporate a Mock control for GFP (top panel) and size markers for comparison (middle panel). Size markers in kDa are present in the unmarked lanes on the right, on the middle and lower panels.

infections developed slowly (data not shown). This agrees with findings that the CP of TBSV contributes to efficient systemic infection (Desvoyes and Scholthof, 2002; Qu and Morris, 2002). Even when segmented systemic invasion eventually occurs in absence of CP, systemic foreign gene expression is only sporadically observed in upper non-inoculated tissues due to recombination events that partially or entirely remove *GFP* or any other foreign insert (Everett et al., 2010; Qiu and Scholthof, 2007; Scholthof, 1999; Scholthof et al., 1993). Accordingly, even though the removal of *CP* sequences in RMJ1 prevented CP restoration, this did not seem to prohibit the inherent capacity to rapidly accumulate foreign insert deletions. Nevertheless, the experiments with RMJ1 (Figs. 2 and 3) and pT31 (Fig. 4) illustrate that this TBSV vector system is very useful to quickly obtain high levels of transient expression in inoculated leaves of many plant species.

The advantage of using the agroinfiltration method with pT31 is evident by the more uniform distribution of green fluorescence (Fig. 4A) compared to the individual foci obtained with RMJ1 transcript inoculations (Figs. 2 and 3). Evidently, upon agroinfiltration many more infection foci are established compared to transcript inoculation. However, *N. benthamiana* and cowpea are known to be very susceptible to TBSV-vector transcript inoculations (Everett et al., 2010; Scholthof, 1999; Seaberg et al., 2012), and thus the results in Figs. 2 and 3 are still quite acceptable. However, the plant research community is likely better served by the now commonly accepted agroinfiltration technique compared to the more specialized transcript synthesis and inoculation. Perhaps more importantly, plants other than *N. benthamiana* and cowpea are sometimes recalcitrant to transcript inoculation (Seaberg et al., 2012) and thus agroinfiltration would be an attractive alternative.

Studies are ongoing to compare the performance of the RMJ1/pT31 system in different hosts with that of other plant RNA virus-based gene vector systems. Preliminary comparisons have been performed by monitoring the local expression levels in leaves agroinfiltrated with an optimized TMV-based CP-defective vector (Lindbo, 2007). The results (not shown) indicate that the level of expression from this TMV vector in *N. benthamiana* may be higher than what is obtained with pT31 but considering the broad host range for local infection for TBSV it will be interesting to compare the TBSV and TMV systems in a variety of hosts.

The start codon mutation introduced in RMJ2 resulting in an CTG instead of ATG yields a viral RNA that still synthesizes a detectable level of P19 expression (Fig. 2) indicating that this noncanonical start codon is recognized for initiation of translation. The reduced levels of P19 compromise the progression of the infection as evidenced by diminished GFP expression, indicative of a direct correlation between P19 dosage and function. This agrees entirely with previous findings in which reduced levels of P19 in infected plants interfered with the effectiveness of invading certain hosts and reduced the symptom severity (Scholthof et al., 1999). Furthermore, in presence of DIs (Hillman et al., 1987; Rubio et al., 1999), P19 levels are also reduced and symptoms mild, all because the lower P19 levels interfere with its ability to effectively suppress silencing (Havelda et al., 2005; Qiu et al., 2002; Szittya et al., 2002). This is likely related to the biochemical role of P19 in having to sequester the abundantly circulating Dicer-produced short-interfering RNAs (siRNAs) to prevent RISC-programming (Lakatos et al., 2004; Omarov et al., 2006), and this can only be achieved when P19 is produced at high levels during infection (Scholthof, 2006). The results with RMJ2 not only confirm this, but are in fact the first time that this dosage correlation is directly visualized with diminished virus-controlled GFP expression compared to the wild-type P19-expressing control (RMJ1), and already evident at 2–3 dpi.

The premature translational stop for *p19* in RMJ3 and its equivalent pT33 resulted in the rapid onset of silencing with GFP expression severely reduced or undetectable at 3 dpi (Fig. 4). The start-codon and stop-codon mutations introduced in RMJ3 were predicted to yield a low level (like in RMJ2) of a C-terminal truncated (due to premature stop) P19. But apparently the combination of mutations results in severe destabilization and rapid degradation of P19 because it is not detectable (Figs. 2 and 4B). This is in agreement with the interpretation of structural data suggesting that the otherwise stable P19 dimers (Omarov et al., 2006; Park et al., 2004; Vargason et al., 2003) are formed through protein–protein interactions involving the C-terminal portion of P19 (Vargason et al., 2003). The combined results illustrate that in the absence of the C-terminal portion, P19 is a very unstable and not functional.

GFP expression upon infection with pT33 could be restored and maintained by co-infiltration with a separate construct expressing P19 (Fig. 4). This is consistent with recent results showing that the rapid silencing of a transcript-inoculated TBSV *CP*-replacement vector in absence of P19 can in fact not only be alleviated by *in trans* co-expression of P19, but also with several heterologous virus suppressors (Scholthof et al., 2011). This is evidence that the rapid decline of GFP expression associated with RMJ3 and T33 is a direct consequence of silencing. Compared to the aforementioned TMV-vector system (Lindbo, 2007), the utility of the suppressor-inactivated TBSV-T33 vector to rapidly monitor the onset of silencing is unique because for TMV and related viruses, the suppressor is embedded within the replicase (Ding et al., 2004; Kubota et al., 2003), precluding its removal.

It is interesting to note that in absence of a functional P19, TBSV expressing its intact CP is able to initiate a systemic infection at 4–5 dpi prior to the silencing-mediated recovery (Chu et al., 2000; Hsieh et al., 2009; Omarov et al., 2006; Scholthof et al., 1995). This contrasts with the rapid onset of silencing and clearance observed for RMJ3 and T33 (Figs. 2, 3 and 4) in which *CP* is replaced with *GFP*. Evidently the virus spread-accelerating affect of the CP (Desvoyes and Scholthof, 2002; Qu and Morris, 2002) avoids or outpaces the full impact of silencing, allowing a rapid invasion to occur. It is also likely that encapsidated RNA is protected from the silencing survey and thus a constant albeit diminished level of virus load is maintained, agreeing with observations that even in systemically invaded plants that appear recovered, viral RNA often remains detectable (unpublished results). Thus, it turns out that the combined loss of CP and P19 expression yields a TBSV vector system that serves very effectively as a sensitive sensor of antiviral silencing that is available for rapidly screening plant species or mutants for their ability to activate antiviral silencing.

Conclusion

The described next generation TBSV vector systems can be applied by either RNA inoculation or through agroinfiltration on different to rapidly test foreign gene expression and to monitor the efficiency and effectiveness of the antiviral response. The additional utility of the TBSV vector platform compared to other virus-based vector systems resides in the combination of its high rate of replication and concomitant levels of gene expression, and the vast host range of the virus that permits utilization for quick transient assays in numerous plant species, as illustrated in this report for *N. benthamiana*, tomato, pepper, cowpea and lettuce.

Materials and methods

Although components of the described vector system have been used in some of our latest studies (Manabayeva et al., 2013; Scholthof et al., 2011; Seaberg et al., 2012) an integral description

of their construction, development, molecular properties, mutations, and side-by-side comparison has not yet been reported. A progenitor construct was based on a full-length TBSV cDNA in which the *CP* was partially replaced with *GFP* encoding the C3 version of the ~27 kDa green fluorescent protein (GFP) (Everett et al., 2010). The *GFP* cDNA was inserted in-frame between the *Not*I and *Bal*I sites within TBSV cDNA resulting in a fusion of *GFP* to the 5′-*CP* fragment and the presence of ~1 kbp non-expressed CP RNA sequences downstream of the 3′-end of *GFP* at the *Bal*I site. To remove these extraneous, potentially recombination-prone sequences (Qiu and Scholthof, 2007), using standard reactions (Hsieh et al., 2009), site-directed mutagenesis was performed to introduce a *Bgl*II site at the 3′-end of *GFP* and another ~150 bp upstream of the start of *p22*, followed by digestion with *Bgl*II and relegation to remove the intervening segment to yield pRMJ1. This plasmid was used for site-directed mutagenesis to change the ATG start codon of *p19* to CTG, to result in pRMJ2; likewise by using the wild-type TBSV cDNA clone as substrate the same mutagenesis yielded pTb2. Again by using site-directed mutagenesis, pRMJ2 was used as template for site-directed mutagenesis to change the lysine codon at nucleotide position 421 to a stop-codon by change of A to T, without disturbing the amino acid sequence encoded by the overlapping *p22*. All above constructs (Fig. 1) were sequenced and it was found that an additional serendipitous stop-codon was introduced in pRMJ3 immediately adjacent and downstream of the target codon. The plasmids were linearized with *Sma*I followed by T7 polymerase-mediated *in vitro* transcription to yield viral RNA that was inoculated onto plants (Scholthof et al., 1995).

To generate constructs compatible with agroinfiltration, an intermediate vector, pJL54, was constructed. The binary vector pJL54 is a modification of pJL22 (Liu and Kearney, 2010) that is a minibinary vector which contains (between its left and right T-DNA borders) a CaMV 35S promoter, and a multiple cloning site starting at the transcription start site of the 35S promoter, followed by a poly(A) signal. To generate pJL54 the ribozyme sequence from pJL36 (Lindbo, 2007) was inserted upstream of the poly(A) signal of pJL22. A TBSV cDNA with a *C3-GFP* replacing the *CP* sequence, similar to that described previously (Scholthof, 1999), was inserted between the 35S promoter and the ribozyme to yield pJL54TBGFP. This served as the backbone for generation of the binary vectors harboring the pRMJ1-3 analogs, by substituting the *Stu*I-*Xma*I fragment of pJL54TBGFP with the homologous segments of pRMJ1, pRMJ2, or pRMJ3, to yield pHJR3-1 (referred to as pT31), pHJR3-2 (pT32; for informational purposes only, not used in this study), and pHJR3-3 (pT33), respectively.

Binary vectors were used to transform *A. tumefaciens* strain GV3101, and prepared for agroinfiltration essentially as described (Scholthof et al., 2011) with some modifications. Briefly, *Agrobacterium* cultures containing pT31, pT33 and P19-expressing constructs were grown overnight (16–22 h) under constant shaking (200 rpm) at 28 °C in 5 ml Luria broth (LB) media with kanamycin at 50 mg/L. *Agrobacterium* were collected by centrifugation at 3500 rpm for 20 min and pelleted cells suspended at 0.5 optical density (O.D.) in 1 × infiltration media (10 mM MgCl, 10 mM MES and 150 mM Acetosyringone) with an incubation of 3 h. The use of the construct and cultures expressing P19 (pKYLX-p19) was described previously (Saxena et al., 2010). After incubation, *Agrobacterium* treatments pT31, pT33, and PT33 plus P19 at a 1:1 ratio, were infiltrated with a single use syringe at the abaxial side of leaves.

SDS-PAGE and immunoblot (western) analyses were conducted as in earlier studies (Desvoyes and Scholthof, 2002; Omarov et al., 2006). Plants were grown at 25 °C with 16 h constant light and 60% humidity. GFP imaging was performed as previously described (Saxena et al., 2010; Scholthof et al., 2011).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.12.031.

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