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Efficiency of VIGS and gene expression in a novel bipartite potexvirus vector delivery system as a function of strength of TGB1 silencing suppression

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ABSTRACT

We have developed plant virus-based vectors for virus-induced gene silencing (VIGS) and protein expression, based on *Alternanthera mosaic virus* (AltMV), for infection of a wide range of host plants including *Nicotiana benthamiana* and *Arabidopsis thaliana* by either mechanical inoculation of *in vitro* transcripts or *via* agroinfiltration. *In vivo* transcripts produced by co-agroinfiltration of bacteriophage T7 RNA polymerase resulted in T7-driven AltMV infection from a binary vector in the absence of the *Cauliflower mosaic virus* 35S promoter. An artificial bipartite viral vector delivery system was created by separating the AltMV RNA-dependent RNA polymerase and Triple Gene Block (TGB)123-Coat protein (CP) coding regions into two constructs each bearing the AltMV 5′ and 3′ non-coding regions, which recombined *in planta* to generate a full-length AltMV genome. Substitution of TGB1 L(88)P, and equivalent changes in other potexvirus TGB1 proteins, affected RNA silencing suppression efficacy and suitability of the vectors from protein expression to VIGS.

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Introduction

Several plant viruses, including tobamoviruses (e.g. *Tobacco mosaic virus*; TMV), potexviruses (e.g. *Potato virus X*; PVX), potyviruses (e.g. *Zucchini yellow mosaic virus*), *Alfalfa mosaic virus*, and *Cucumber mosaic virus*, have been used for expression of plant-produced recombinant proteins. These viral vectors have been used to produce many different kinds of proteins in plants including allergens, antibodies or antibody fragments, and vaccine candidates (e.g. Chapman et al., 1992; Donson et al., 1991; Hsu et al., 2004; Yusibov et al., 1997; Zhao et al., 2000). Another important application of plant viral-based vector systems is in studies on host gene function. With more plant genomic information available, a high-throughput tool is required. Virus-induced gene silencing (VIGS) using viruses such as *Tobacco rattle virus* (TRV) and *Barley stripe mosaic virus* is an exceptional reverse genetics tool that can be employed to generate mutant phenotypes for determining function of unknown genes (e.g. Holzberg et al., 2002; Ratcliff et al., 2001).

Effective viral-based protein expression vectors require the ability to produce target proteins stably through high accumulation of viral genomic RNA and/or subgenomic RNA. To be an effective VIGS vector, replication and accumulation of viral RNA(s) should be high enough to produce a threshold level of the replicative form dsRNA molecules that enhance silencing, which has been reported to be a major factor for VIGS (Lacomme et al., 2003), but lacking an effective suppressor of RNA silencing.

Agroinfiltration (e.g. Leiser et al., 1992), biolistic delivery of plasmid (e.g. Gal-On et al., 1997) or *in vitro* transcripts (e.g. Jakab et al., 1997), and mechanical inoculation of either plasmids (e.g. Dessens and Lomonossoff, 1993) or *in vitro* transcripts (e.g. Domier et al., 1989) are the main methods for delivery of viral nucleic acids into plant cells. Agroinfiltration is relatively easy to apply with low cost; however, not all plant species are susceptible to agroinfiltration. In contrast, most plant species can be infected by biolistic delivery or mechanical inoculation with plasmids or *in vitro* transcripts, but the techniques are more laborious and expensive than agroinfiltration. Each method has advantages and disadvantages, but since most virus-based vectors are constructed with a single promoter sequence, it has not typically been possible to use both DNA and RNA delivery methods with the same vector.

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Alternanthera mosaic virus (AltMV) has a natural host range that includes a large number of ornamental plants, including several *Phlox* species (Hammond et al., 2006a,b), and *Portulaca* (Baker et al., 2006; Ciuffo and Turina, 2004; Hammond et al., 2006a,b), as well as *Scutellaria*, *Crossandra* (Baker et al., 2006), and *Angelonia* (Lockhart and Daughtrey, 2008). AltMV is a potexvirus most closely related to *Papaya mosaic virus*, and was originally reported from a weed species, *Alternanthera pungens*, in Australia (Geering and Thomas, 1999). We have previously reported the full 6607 nt sequence of a phlox isolate, AltMV-PA (Hammond et al., 2006a). A second isolate, AltMV-SP, has been shown to be a mixture of two distinct strains, one severe and one mild; the predicted amino acid sequences of the mild and severe strains differed at multiple positions in the RNA-dependent RNA polymerase (RdRp) affecting viral RNA replication, and at one position in TGB1 affecting suppression of RNA silencing (Lim et al., 2010).

Many plant viruses have been modified for either protein expression or gene silencing. However, a single plant virus-based vector has been unable to effectively fulfill both functions because of the conflicting requirements for strong or weak RNA silencing suppression, for protein expression and VIGS, respectively. Perhaps the best examples to date are PVX (Chapman et al., 1992; Ruiz et al., 1998) and *Bean pod mottle virus* (BPMV) (Zhang and Ghabrial, 2006; Zhang et al., 2009), although PVX is far more widely used for protein expression, and BPMV has not yet been widely adopted. Here, we report using AltMV to develop virus-based vectors whose function can be easily changed from protein expression to VIGS and that can also be introduced into plants by either agroinfiltration or, in plant species recalcitrant to *Agrobacterium*-mediated inoculation, by mechanical inoculation of *in vitro* transcripts. Also, bacteriophage T7 RNA polymerase and reverse genetic systems were applied for developing this adaptable new plant viral vector, applicable to *Arabidopsis* and soybean in addition to *Nicotiana benthamiana* and a number of ornamental species. We have further examined the factors affecting TGB1 function, and have adapted the vector as a bipartite launch system, significantly simplifying gene insertion at the multiple cloning site and allowing higher throughput for gene expression or VIGS studies.

Results

Transient expression of eGFP directed by bacteriophage T7 RNA polymerase co-expressed by agroinfiltration

Infectious transcripts of many positive-sense RNA viruses have been produced using the bacteriophage T7 RNA polymerase (e.g. Domier et al., 1989). Reverse genetics systems for either positive or negative-strand viruses are also often based on transcription of viral RNA by the T7 RNA polymerase (de Wit et al., 2007; Naylor et al., 2004; Zheng et al., 2009), and a hybrid baculovirus-T7 RNA polymerase system has been used for transient expression in mammalian cells (Yap et al., 1997). We wished to determine whether the 35S and T7 promoters could be combined in a single construct that could be used for both *in vitro* and *in vivo* transcription, and whether the T7 promoter could be utilized *in planta* as well as *in vitro*. Therefore, we expressed T7 RNA polymerase from a binary vector (Fig. 1A). The expressed T7 RNA polymerase was detected as a 98 kDa band on a western blot (data not shown). The ability of T7 RNA polymerase to direct gene expression *in planta* was evaluated by co-infiltrating *N. benthamiana* leaves with pCAM-T7RNAP with pGD-T7eGFP or pGD-PFGe7T, respectively containing eGFP coding sequences inserted in sense and antisense orientations relative to the CaMV 35S and bacteriophage T7 promoters. Infiltration without pCAM-T7RNAP served as a control. In leaves infiltrated with pGD-T7eGFP either with or without pCAM-T7RNAP, eGFP fluorescence was detected in almost every cell (Fig. 1B). In leaves infiltrated with pGD-PFGe7T alone, no eGFP expression was detected, but in leaves infiltrated with both pGD-PFGe7T and pCAM-T7RNAP, eGFP expression was detected in 19 out of 200 epidermal cells examined by LSCM.

Co-expression of T7 RNA polymerase enhances infectivity of agroinfiltrated full-length clones of AltMV

Previously, we produced full-length clones of AltMV that were used to synthesize infectious *in vitro* transcripts for mechanical inoculation of plants (Lim et al., 2010). Full-length AltMV cDNA including the fused T7 promoter was cloned into pGD-T7ttr and pGD (Δ35S)-T7ttr binary vectors with the T7 RNA polymerase terminator sequence downstream of AltMV to produce pGD-AltMV and pGD (Δ35S)-AltMV (Fig. 2). *In vitro* transcripts from pGD-AltMV were highly infectious, yielding symptoms at 7 dpi (Table 1). When plants were agroinfiltrated with pGD(Δ35S)-AltMV without pCAM-T7RNAP, no plants were infected; however, when pGD(Δ35S)-AltMV was co-infiltrated with pCAM-T7RNAP, all plants were infected resulting in symptom development within 7–10 dpi (Table 1). When plants were agroinfiltrated with pGD-AltMV without pCAM-T7RNAP, most (66–80%) plants were infected, showing symptoms in 15–25 days, whereas in the presence of pCAM-T7RNAP, all plants were infected and developed symptoms in 7–10 dpi (Table 1, and data not shown). Thus co-expression of T7 RNA polymerase resulted in infectivity and symptom expression similar to that of transcripts, and enhanced infectivity from the 35S promoter.

We note that the presence of 54 non-viral nucleotides upstream of the T7 promoter in the 35S transcript may have reduced initial infectivity of transcripts from the 35S promoter. However, PCR performed on the plasmid template, and on total RNA extracted from systemically infected leaves of plants infected by agroinfiltration of pGD-AltMV in the presence of pCAM-T7RNAP clearly indicated lack of non-AltMV 5′ sequence in the progeny virus population (Fig. 2B).

Replication of a defective AltMV genome by AltMV RdRp variants in a transient expression assay

To test the ability of the RdRp region to support replication *in trans* using agroinfiltration, we separated the RdRp (with non-viral 5′ and 3′ UTR) and 5TGB123-eGFP-CP3C (Fig. 3A; with AltMV 5′ and 3′ UTR), and inserted each into the pGD vector; RdRp constructs were amplified from each of AltMV 3-1, 3-7, 4-1, and 4-7, which differ in replication efficiency caused by a limited number of aa differences (Lim et al., 2010). Each RdRp construct was co-infiltrated with pGD-5TGB123-eGFP-CP3C; the empty pGD vector and pGD-5TGB123-eGFP-CP3C were separately infiltrated as controls. All infiltrations also included pGD-p19, but not pCAM-T7RNAP, so that eGFP expression would reflect relative replication levels of 5TGB123-eGFP-CP3. To evaluate differences in the RdRp function efficiency of the different constructs, we analyzed eGFP expression controlled by each RdRp in the same leaf tissue (Fig. 3B, upper) by LSCM at uniform laser power; the relative level of eGFP expression was clearly 3-7>4-7>3-1>4-1 (Fig. 3C), in agreement with prior observations on symptom expression and quantitative reverse transcription PCR (Lim et al., 2010). Expression of eGFP was limited to the agroinfiltrated area. A western blot of leaf extracts demonstrated that expression of the CP was also correlated with replication of 5TGB123-eGFP-CP3C *in trans* by 35S-driven RdRp (Fig. 3B, lower). As the $RdRp_{3-7}$ construct clearly resulted in the highest level of expression of GFP from the duplicated sg-promoter, pGD-5$RdRp3_{3-7}$ was selected as the basis for development of a bipartite AltMV launch system.

T7 RNA polymerase is required for infectivity of the AltMV bipartite vector

We were interested in whether a bipartite vector could be generated that would maintain the separation of the genome functions. First we