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Exploring the limits of vector construction based on *Citrus tristeza virus*

Choaa El-Mohtar, William O. Dawson *

Department of Plant Pathology, Citrus Research and Education Center, University of Florida, Lake Alfred, FL 33850, USA

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ABSTRACT

We examined the limits of manipulation of the *Citrus tristeza virus* (CTV) genome for expressing foreign genes in plants. We previously created a vector with a foreign gene cassette inserted between the major and minor coat protein genes, which is position 6 from the 3′ terminus. Yet, this virus has 10 3′-genes with several other potential locations for expression of foreign genes. Since genes positioned closer to the 3′ terminus tend to be expressed in greater amounts, there were opportunities for producing greater amounts of foreign protein. We found that the virus tolerated insertions of an extra gene in most positions within the 3′ region of the genome with substantially increased levels of gene product produced throughout citrus trees. CTV was amazingly tolerant to manipulation resulting in a suite of stable transient expression vectors, each with advantages for specific uses and sizes of foreign genes in citrus trees.

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Introduction

There are two common methods by which foreign sequences are expressed throughout plants. The first is by integrating foreign DNA into the plant genome, which is expressed in the next generations of plants. The expression of the inserted sequences generally is permanent. Alternatively, foreign sequences can be transiently expressed in the present generation of plants using virus-based vectors. Virus vectors have been developed via manipulation of viral genomes to express foreign sequences. Most transient-expression vectors have been developed from viruses of annual, herbaceous plants and used as laboratory tools to express or over-express a gene of interest or to prevent expression of an endogenous gene by induction of RNA silencing. Additionally, some vectors are being considered to produce high-value specialty products commercially.

Perennial plants present numerous additional opportunities and challenges for virus-based vectors. Many trees are productive for 100 years or more. During the lifespan of the trees, new technologies develop and disease as well as pest pressures change. Improvement of trees by traditional transformation methods requires removing all of the present trees from the field and replanting. An exciting opportunity would be to develop virus-based vectors that could allow addition of new genes or characteristics to trees within the current generation.

The challenge is to create vectors that are stable enough to be useful. Engineering an effective vector requires a balance between different factors. The vector needs to be designed such that replication and systemic movement in the plant are reduced minimally while the level of expression of the foreign protein is maximal (Shivprasad et al., 1999). However, vector's usefulness is directly correlated with its stability. Stability is a product of reduced recombination and increased competitiveness of the vector with the resulting recombinants that have lost part or all of the inserted sequences. On the other hand, one value of virus-based vectors is that they are not infinitely stable – that they add nothing permanently to the environment.

We created a virus-based vector for citrus trees based on *Citrus tristeza virus* (CTV) (Folimonov et al., 2007). This vector, which produces moderate amounts of the foreign protein, is remarkably stable, continuing to express foreign genes for years. CTV has a monopartite RNA genome of approximately 20 kb (Karasev et al., 1995; Pappu et al., 1994) with two conserved gene blocks associated with replication and virion formation (Karasev, 2000). The replication gene block, which occupies the 5′ half of the genome, expresses proteins via a polyprotein strategy with a +1 ribosomal frame shift to occasionally express the RNA dependent RNA polymerase domain (Karasev et al., 1995). The 10 3′-genes are expressed through 10 3′-coterminal subgenomic (sg) RNAs (Hilf et al., 1995).

Building an effective vector requires understanding of the regulation of viral gene expression (Shivprasad et al., 1999). We have found that there are general rules that determine the levels of production of the different sgRNAs of CTV. First, genes located nearer the 3′ terminus are usually expressed at higher levels (Navas-Castillo et al., 1997; Satyanarayana et al., 1999; Ayllón et al., 2005). The two 3′-most genes, p23 and p20, produce the highest levels of sgRNAs. The sgRNAs for the internal p33 and

* Corresponding author. Tel.: +1 863 956 8714.
E-mail address: wodtmv@ufl.edu (W.O. Dawson).

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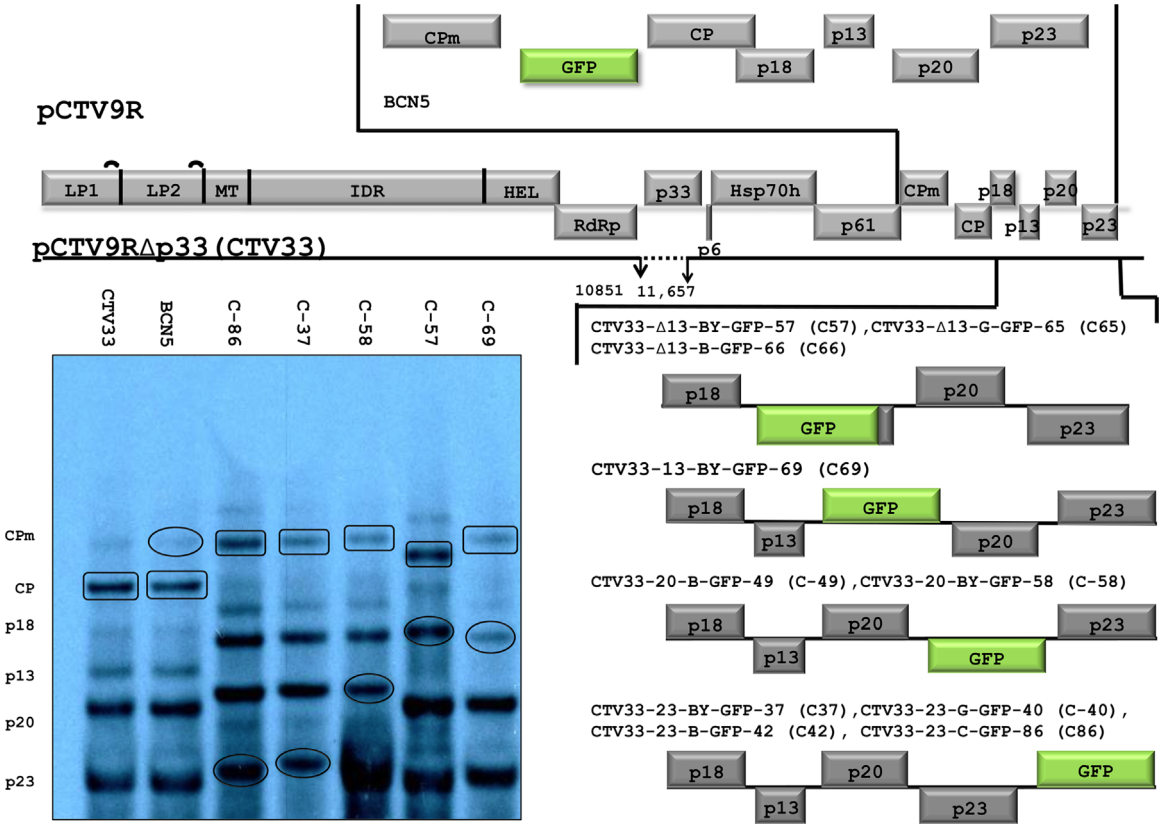

Fig. 1. Insertion of a GFP gene cassette into several locations of the CTV genome: between CPm and CP genes; replacement of the p13 gene; between the p13 and p20 genes; between the p20 and p23 genes; or behind the p23 gene. A schematic representation of the base constructs, full-length CTV9R and the deletion mutant CTV9RΔp33 (represented by the line under the genome diagram showing the location of the deletion). Boxes represent open reading frames with encoded protein domains or proteins labeled within. Northern hybridization analysis of CTV9RΔp33 and representatives of each type of vector construct from transfected *N. benthamiana* protoplasts. The CP mRNA is boxed and the GFP mRNA is circled.

p6 genes, which are farthest from the 3′ terminus, are barely produced (Ayllón et al., 2005). Also, when genes are moved closer to the 3′ terminus, expression levels increase dramatically. Positioning the lowly expressed p33 gene near the 3′ terminus results in a level of expression comparable to the highest expressed genes (p20 and p23) (Satyanarayana et al., 1999; Ayllón et al., 2005). However, there are exceptions. The CP gene, located at position 5 from the 3′ end, is expressed higher than the p13 and p18 genes, located at positions 3 and 4. The second rule is that genes that have open reading frames (ORFs) with an upstream nontranslated (NTR) region are generally expressed higher than genes with ORFs that overlap with the preceding ORF (Pappu et al., 1994; Hilf et al., 1995). The p23, p20, p13, p25, p6, and p33 ORFs have upstream NTRs. With the exception of the 5′-most genes (p33 and p6), these are the more highly expressed genes. The cis-acting sequences that regulate the expression of the 3′ genes generally are located immediately upstream of their ORFs. We refer to these sequences as 'controller elements' (CE) instead of 'promoters' because we have not been able to determine whether the mode of production of the 3′ sgRNAs is by promotion from an internal sequence of the minus strand or termination during minus strand synthesis followed by amplification of positive-stranded sg mRNAs (Gowda et al., 2001). The 3′ CEs generally consist of one or two stem-loop structures with a downstream (plus sense) +1 site corresponding to the 5′ terminal adenosine of the mRNA (Gowda et al., 2001; Ayllón et al., 2003, 2004, 2005). Mutational analysis and characterization of the context of the +1 site of the sg mRNAs showed opportunities for manipulation of levels of expression several fold up or down (Ayllón et al., 2003).

The present CTV vector has an extra gene inserted between the minor and major coat proteins, at position 6 from the 3′ terminus (Folimonov et al., 2007). Although this prototype vector has worked well, this position was chosen arbitrarily. With CTV having 10 3′-genes, there are numerous other positions that could have been chosen for expression of a foreign gene. Since genes placed closer to the 3′ terminus tend to be expressed in greater amounts, there are opportunities for higher levels of expression of the foreign gene if CTV could tolerate such insertions. In this work, we attempted to explore the limits of manipulation to construct vectors that CTV will tolerate. We found that the virus tolerated insertions of an extra gene in most positions within the 3′ region of the genome and that the more 3′ positions resulted in greater expression of the foreign gene. Yet, there were some limits. Large inserts depressed production of sgRNAs of the more 5′-positioned genes and reduced replication and spread within citrus trees. The larger insertions were better accommodated in more 5′ positions where there was interference with fewer sgRNAs resulting in more replication. We also examined strategies to produce foreign proteins without adding additional sgRNAs based on IRESs or protease processing strategies. The overall result is that CTV is amazingly tolerant to manipulation at several positions within the genome giving a multitude of different vector strategies that are viable.

Results

Addition of an extra gene at different locations within the CTV genome – Insertions at the p13 gene site

The CTV vector developed previously (Folimonov et al., 2007) has an additional gene cassette (an ORF to express a specific

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