Virology 492 (2016) 166–178

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Development of tobacco ringspot virus-based vectors for foreign gene expression and virus-induced gene silencing in a variety of plants

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ARTICLE INFO

Article history:
Received 1 February 2016
Returned to author for revisions
23 February 2016
Accepted 27 February 2016

Keywords:
Tobacco ringspot virus
Foreign gene expression
Virus-induced gene silencing
Functional genomics
Phytoene desaturase
Nicotiana benthamiana
Arabidopsis
Legumes
Cucurbits

ABSTRACT

We report here the development of tobacco ringspot virus (TRSV)-based vectors for the transient expression of foreign genes and for the analysis of endogenous gene function in plants using virus-induced gene silencing. The jellyfish green fluorescent protein (GFP) gene was inserted between the TRSV movement protein (MP) and coat protein (CP) regions, resulting in high in-frame expression of the RNA2-encoded viral polyprotein. GFP was released from the polyprotein via an N-terminal homologous MP-CP cleavage site and a C-terminal foot-and-mouth disease virus (FMDV) 2 A catalytic peptide in *Nicotiana benthamiana*. The VIGS target gene was introduced in the sense and antisense orientations into a *Sna*BI site, which was created by mutating the sequence following the CP stop codon. VIGS of *phytoene desaturase* (*PDS*) in *N. benthamiana, Arabidopsis* ecotype Col-0, cucurbits and legumes led to obvious photo-bleaching phenotypes. A significant reduction in *PDS* mRNA levels in silenced plants was confirmed by semi-quantitative RT-PCR.

1. Introduction

In recent years, the use of plant viruses as vectors for delivering genes into plants has been increasingly reported. Many RNA and DNA plant viruses with various viral genomes and host ranges have been and are still being engineered for foreign protein production. Such plant viruses are also being used to investigate plant genomic functions (Aguero et al., 2012; Hefferon, 2014; Lim et al., 2015; Wang et al., 2014). Impressive advances have also been made in the development of strategies for constructing viral vectors (Gleba et al., 2014), as well as viral vector delivery systems (Liu et al., 2002; Ryu et al., 2004; Seo et al., 2009; Tuttle et al., 2012; Yan et al., 2012). Plant virus-based vectors are capable of yielding high levels of proteins in plants at a low cost and in a short period of time. To date, the β-glucuronidase (GUS) and jellyfish green fluorescent (GFP) marker proteins as well as important pharmaceutical proteins such as commercial antibodies, antigens and vaccines, have been successfully produced in plants via plant virus-based expression systems (Gleba et al., 2007; Zhang et al., 2013). Virus-induced gene silencing (VIGS) is a fast and efficient reverse genetic tool for plant functional genomics, as the phenotypes induced by the down-regulation of an endogenous plant gene can be noticed in a short time. Consequently, VIGS has been widely used to characterize the plant genes involved in plant development, disease resistance, cellular signaling and metabolic regulation (Purkayastha and Dasgupta, 2009). In the past few years, improvements in VIGS systems have extended both the range of plants suitable for VIGS and the persistence of gene silencing in some plants (Hsieh et al., 2013; Purkayastha et al., 2010; Senthil-Kumar and Mysore, 2011; Yamagishi and Yoshikawa, 2009). Additionally, two-component gene-silencing systems, such as the satellite virus-induced silencing system (SVISS), have also been efficiently applied in some plants (Gossele et al., 2002; Liou et al., 2014; Zhou and Huang, 2012).

Recently, the genomic resources of many important crop species have become publicly available due to the rapid progress of next-generation sequencing technologies. Knowledge of genomic sequences provides a wealth of information for discovering genes involved in critical traits such as crop evolution as well as improving breeding strategies. Cucurbits such as melon, watermelon, cucumber, pumpkin and squash are economically important fruit and vegetable crops. The accumulation of valuable cucurbit gene sequence resources has encouraged the

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http://dx.doi.org/10.1016/j.virol.2016.02.025
0042-6822/

development of technologies to elucidate gene functions. VIGS could become a useful platform for functional genomics in cucurbits, as these crops are infected by an ever-growing number of plant viruses. An apple latent spherical virus (ALSV)-based VIGS system has been reported to work efficiently in cucurbits (Igarashi et al., 2009). However, the number of VIGS vectors currently available for cucurbits are rather limited. This limitation may be due in part to the characteristics of viral genomes or the strategies used to construct VIGS vectors. The exploitation of new VIGS vectors for cucurbits will therefore be of great value in facilitating an understanding of the functionality characteristics of cucurbit genes.

Legumes belonging to the family *Leguminosae* are important crops; these legumes are capable of symbiotic nitrogen fixation with rhizobia. Because legumes are recalcitrant to genetic transformation, VIGS has become an attractive alternative tool for functional genomics studies in legumes. To date, VIGS systems in legumes have been developed based on the bean pod mottle virus (BPMV) (Zhang et al., 2009, 2010; Diaz-Camino et al., 2011), pea early browning virus (PEBV) (Constantin et al., 2004), ALSV (Igarashi et al., 2009; Yamagishi and Yoshikawa, 2009), cucumber mosaic virus (CMV) (Nagamatsu et al., 2007), tobacco streak virus (TSV) (Jossey, 2012), *Sunnhemp mosaic virus* (SHMV) (Varallyay et al., 2010), white clover mosaic virus (WCIMV) (Ido et al., 2012) and soybean yellow common virus (SYCMV) (Lim et al., 2015). Soybean (*Glycine mix*) is a well-known and widely studied legume species, as its seeds are rich in oils and proteins. Wild soybean (*Glycine soja*) is the closest wild relative of the domesticated soybean and contains valuable genetic resources that have been lost in soybeans following its long history of domestication. Thus, the VIGS system for wild soybean may greatly facilitate the exploitation of novel gene functions as well as the domestication and improvement of soybeans.

Tobacco ringspot virus (TRSV, genus *Nepovirus*, subgroup A, family *Secoviridae*) is a viral disease that attacks a wide range of herbaceous and woody plants. For example, TRSV causes bud blight disease in soybeans (Demski et al., 1989) and mottled leaves in cucurbits (Abdalla et al., 2012). Further research has demonstrated that the host range of TRSV includes not only plants but also insects as a result of host shifting (Li et al., 2014). Vectors for the transmission of TRSV between host species generally include nematodes, insects and seeds. The TRSV contains two positive-sense and single-stranded polyadenylated RNAs, each of which is translated into a large polyprotein. Although TRSV has been detected in several host plants, the number of complete TRSV genome sequences available in GenBank remains limited. In a previous study, we reported a complete genome sequence of TRSV from a soybean isolate. Based on this sequence, we subsequently generated biologically active infectious cDNA clones (Zhao et al., 2015). In this work, we describe for the first time the development of viral vectors based on TRSV for both foreign gene expression and VIGS in plants. TRSV-based vectors have the potential for VIGS in *N. benthamiana*, *Arabidopsis* ecotype Col-0, cucurbits and legumes. This VIGS system is not limited to vegetative tissues but is also suitable for reproductive organs such as flowers and fruits.

2. Results

2.1. Transient expression of GFP in TRSV-based expression vectors

GFP was inserted into vector pT2C2A (see "Section 4") between the MP and CP genes, generating vector pT2C2AGFP as an RNA2 component for use in a TRSV-based foreign gene expression system (Fig. 1B). The MP-CP cleavage site in RNA2 was previously identified as Cys/Ala (C/A) (Buckley et al., 1993). GFP was released from the polyprotein via processing of the MP-CP cleavage site and a FMDV 2A catalytic peptide.

The use of viral FMDV 2A catalytic peptide prevents homologous recombination, improving the stability of the construct (Gopinath et al., 2000). Self-processing cleavage of FMDV 2A occurs between the 2A glycine and 2B proline in the FMDV polyprotein (Ryan et al., 1991). In pT2C2AGFP, we fused the sequence encoding the FMDV 2A catalytic peptide (APAKQLLNFDLLKLAGDVESNPGP) to the 5′ end of

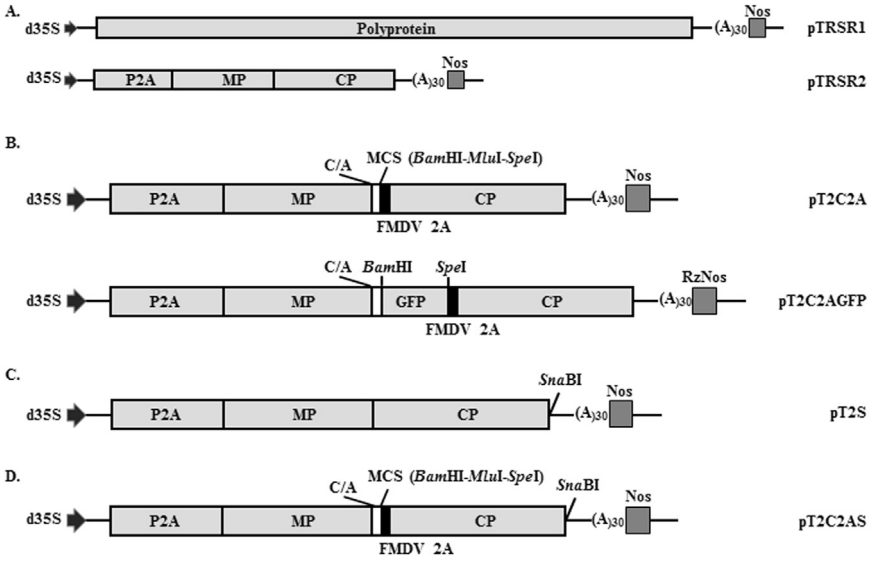

Fig. 1. Schematic representation of TRSV cDNA infectious clones and genomic modifications. A. Schematic representation of TRSV cDNA infectious clones including pTRSR1 and pTRSR2. B. Schematic representation of pT2C2AGFP for use in TRSV-based GFP expression vectors. The GFP was located between the MP-CP cleavage site (C/A) and the additional FMDV 2A catalytic peptide in vector pT2C2A using *Bam*HI and *Spe*I restriction enzymes sites. The FMDV 2A catalytic peptide was fused to the N terminus of the CP. C. Schematic representation of pT2S for use in TRSV-based VIGS vectors. pT2S was generated by creating a *Sna*BI site downstream of the stop codon for the CP coding region in pTRSR2. D. Schematic representation of pT2C2AS for use in TRSV-based gene expression or silencing vectors. The MCS was used for the expressed gene cloning and the *Sna*BI site was used for the endogenous gene insertion. Light gray boxes in each plasmid represent open reading frames. Black block arrow represents a duplicated CaMV 35S promoter (d35S). Dark gray box in each plasmid represents the nopaline synthase (Nos) terminator. White box indicates the duplicated 19 amino acids from the CP N terminus. Solid black box indicates the FMDV 2A catalytic peptide.