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A co-silencing system for functional analysis of genes without visible phenotype in tomato plant development and fruit ripening using tobacco rattle virus

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ABSTRACT

Virus-induced gene silencing (VIGS) is known as a powerful tool for identifying gene functions during plant growth and development. Tobacco rattle virus (TRV) based VIGS has been successfully employed in many plants. However, there have been few reports that VIGS can be used to study the function of gene at fruit level, especially the genes without visible silencing phenotype. Here we used miniature tomato cultivar Micro-Tom (*Solanum lycopersicum* L.) as material to silence *phytoene desaturase* (*SlPDS*) and *SlARG2*, an important gene encoding arginase in arginine metabolism, separately or simultaneously with TRV agroinoculated by sprout vacuum-infiltration to test the utility of concurrently silencing two genes. When *SlPDS* and *SlARG2* were co-silenced in the same tissue at the same time, there are no compromises in silencing efficiency and silencing efficacy compared with *SlPDS* or *SlARG2* silenced separately. Moreover, the silence of *SlPDS* and *SlARG2* with TRV agroinoculated by sprout vacuum-infiltration occurred at the early stage of tomato plant, and the silence can extend to full red stage of fruit. Therefore, our results provide a means for functional analysis of genes, particularly the genes with no obvious visible phenotype after silencing, involved in all of the metabolic networks during fruit development and ripening.

1. Introduction

Virus-induced gene silencing (VIGS) is an RNA-mediated antiviral defense mechanism of plant, which has been recently developed as an attractive reverse genetics tool, wherein the transcript of the target gene is inhibited by a viral vector in a sequence homology-dependent manner (Liu et al., 2002a,b; Kandoth et al., 2013). Compared to stable genetic manipulation methods, such as transfer DNA (T-DNA)-based transformation and CRISPR (clustered regularly interspaced short palindromic repeats) genome editing systems, this method enables rapid, easy, efficient and high throughput analysis of gene functions (Burch-Smith et al., 2004). Despite the advantages, VIGS also has several inherent limitations, such as inconsistency and transiency of silencing, and lack of a visually discernable silencing phenotype for many genes (Burch-Smith et al., 2004). These limitations underscore the importance of determining the temporal and spatial characteristics of targeted gene silencing for efficient downstream functional analysis. Usually, a reporter gene that shows visible silencing phenotype is used as a positive control to circumvent this limitation. *Phytoene desaturase* (*PDS*) encodes an enzyme that catalyzes phytoene dehydration to form ζ-carotene, which is an important step in the carotenoid biosynthesis pathway (Cunningham and Gantt, 1998; Fu et al., 2005). Inhibition of *PDS* expression results chlorophyll degradation in the presence of light, which is easily detectable as a photobleaching phenotype in chlorophyll-rich tissues. Therefore, the phenotypic changes caused by *PDS* silencing are most frequently used as a positive control in experiments on VIGS analysis (Fu et al., 2005; Velásquez et al., 2009).

The VIGS vectors available for silencing studies in plants are mainly derived from RNA viruses, such as tobacco mosaic virus (Kumagai et al., 1995), tobacco rattle virus (TRV) (Liu et al., 2002a; Ratcliff et al.,

Abbreviations: VIGS, virus-induced gene silencing; TRV, tobacco rattle virus; PDS, phytoene desaturase; CRISPR, clustered regularly interspaced short palindromic repeats; LB, left border; RB, right border; RdRp, RNA-dependant RNA polymerase; MP, movement protein; 16K, 16 Kd protein; R, self- cleaving ribozyme; N, NOS terminator; CP, coat protein; MCS, multiple cloning sites; MES, 2-(N-morpholino)-ethanesulfonic acid; sRT- PCR, semi-quantitative RT-PCR; qPCR, quantitative real-time PCR; Ct, threshold cycle

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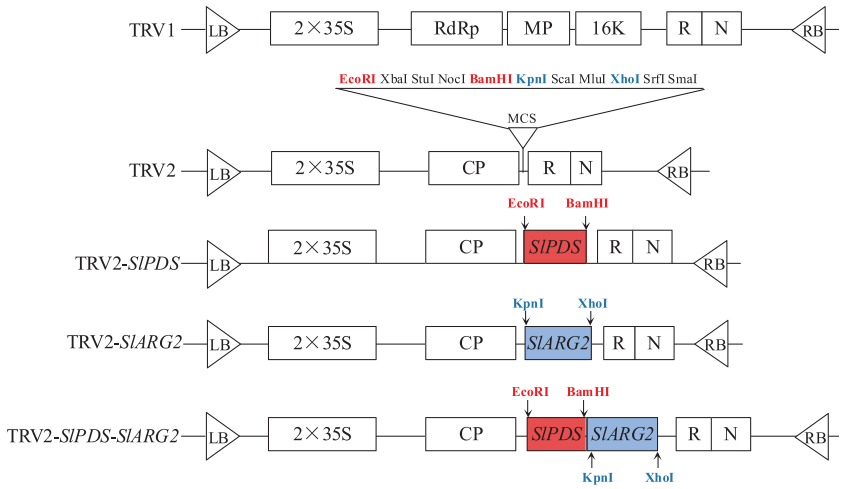

Fig. 1. Schematic of TRV1, TRV2 and its derivative constructs. LB: left border; RB: right border; RdRp: RNA- dependant RNA polymerase; MP: movement protein; 16K: 16 Kd protein; R: self- cleaving ribozyme; N: NOS terminator; CP: coat protein; MCS: multiple cloning site.

2001), tomato golden mosaic virus (Peele et al., 2001), potato virus X (Ruiz et al., 1998), apple latent spherical virus (Igarashi et al., 2009), bean pod mottle virus (Zhang and Ghabrial, 2006), and cabbage leaf curl virus (Turnage et al., 2002). Among these viral VIGS vectors, TRV-based VIGS vectors had been widely used to silence gene in *Solanaceous* plant species due to the wide host range and mild virus infection symptoms (Ratcliff et al., 2001; Ramegowda et al., 2014). TRV is a single-stranded RNA virus with bipartite genomes. In the TRV vector, TRV RNA1 (TRV1) encodes RNA polymerase, movement protein and cysteine-rich protein; TRV RNA2 (TRV2) consists of a capsid protein gene and multiple cloning sites (MCS) for target gene insertion (Senthilkumar and Mysore, 2014; Liu et al., 2002b). The modified TRV promotes the insertion of viral sequences and subsequent infection, and directly targets host plant growth. Tomato is a typical species of *Solanaceae*, and its genome is currently being sequenced. Therefore, tomato has become an excellent model system for the analysis of gene function, physiological metabolism and fruit development and ripening (Gerszberg et al., 2015; Luengwilai et al., 2012). The functions of genes related to ethylene biosynthesis, perception, and cell wall degradation during fruit ripening in tomato have been investigated using TRV-VIGS (Xie et al., 2006; Li et al., 2011). However, for most genes not related to pigment synthesis, silencing does not produce a readily visible phenotype. Usually, a co-silencing system including the target gene and *PDS* reporter gene is used to analyze the function of genes that lack of a visually discernable silencing phenotype using VIGS technology. However, information regarding the silencing efficiency and efficacy of co-silencing systems in plants, especially in fruit tissue, is limited. In addition, VIGS-induced gene silencing in fruit ripening stage of many plants is challenging. Therefore, it is vital to select suitable materials for studying the function of fruit development and ripening-related genes.

SlARG2, a second gene encoding arginase, is a key gene in L-arginine metabolism, the silence of which lacks readily visible phenotype (Zhang et al., 2012). In this study, we constructed a co-silencing TRV-VIGS system with the *SlPDS* reporter gene and *SlARG2* target gene in the tomato plant. The silencing efficiency and efficacy of *SlPDS* or *SlARG2* in different tissues of the tomato plant were analyzed; furthermore, the durability of *SlPDS* or *SlARG2* silencing with fruit ripening was also detected, which might provide technical reference for functional analysis of genes lacking a visually discernible silencing phenotype in plants, especially in fruits.

2. Materials and methods

2.1. Plant materials

Tomato (*Solanum lycopersicum* L. cv. Micro-Tom) was used for the experiments. Seeds were germinated at 25 °C in Petri dishes containing Whatman No. 1 filter paper moistened with distilled water. After the sprouts reaching 0.5–1 cm in length, the germinating seeds were subject to sprout vacuum-infiltration. Treated sprouts were sown in pots in a growth chamber, which was kept at 23 ± 2 °C and 60–70% relative humidity with 16/8 h light/dark photoperiod.

Leaves, sepals, petals, and fruits at mature green (I, approximately 28d after flowering), breaker (II, approximately 34 d after flowering), pink (III, approximately 38 d after flowering), light red (IV, approximately 42 d after flowering) and full red (V, approximately 45 d after flowering) stages were separately picked and used for further analysis.

2.2. RNA extraction and first-strand cDNA synthesis

Total RNA were extracted from tissues of wild-type, silenced and non-silenced (infiltrated with empty vector TRV1 and TRV2) plants using trizol reagent according to Zhang et al. (2010), and then treated with RNase-free DNase I (Promega, Madison, WI, USA) to remove any remaining genomic DNA. Reverse transcription was performed using M-MLV reverse transcriptase (Promega, Madison, WI, USA), oligo(dT)$_{18}$ primer and 2 μg of total RNA DNA-free.

2.3. Plasmid construction

The VIGS vectors TRV1 and TRV2 have been described in detail (Liu et al., 2002a, 2002b). And, all the constructs were assembled into the TRV2 vector.

TRV2-*SlPDS* construction. To construct TRV2-*SlPDS*, a 445-bp fragment of *SlPDS* (GenBank accession number X59948) was PCR-amplified from tomato cDNA using primers (forward: 5'-GAC CCG GAA TTC AGT CGG AGT ACC TGT GAT-3' with an engineered EcoRI restriction site and reverse: 5'- GCA CGC GGA TCC CAA GTA TTT CTG TTT CGT GT-3' with an engineered BamHI restriction site). The resulting product was cloned into TRV2 to form TRV2-*SlPDS* (Fig. 1).

TRV2-*SlARG2* construction. To construct TRV-*SlARG2*, a 408-bp fragment of *SlARG2* (GenBank accession number AY656838) was PCR-amplified from tomato cDNA using primers (forward: 5'-CGG GGT ACC AAA GGG CGT GTA TCT ATC CGT GG-3' with an engineered KpnI restriction site and reverse: 5'- CCG TTC CTC GAG TTT GGG AGG CAA GTT TAC AG-3' with an engineered XhoI restriction site). The resulting