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# Virus-induced gene silencing in various *Prunus* species with the *Apple latent spherical virus* vector

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ABSTRACT

Virus-induced gene silencing (VIGS) has been used as a rapid and effective tool for functional analysis of genes in various plants, including woody fruit tree species. We previously reported the successful induction of VIGS of the endogenous *PHYTOENE DESATURASE* (*PDS*) gene in apricot (*Prunus armeniaca* L.) using *Apple latent spherical virus* (ALSV) vectors. In contrast, our attempts to infect Japanese apricot (*Prunus mume* Siebold & Zucc.) with ALSV vectors was unsuccessful, suggesting that species- and/or cultivar-dependent differences of ALSV susceptibility may exist in *Prunus*. In this study, we investigated whether this VIGS-based gene evaluation system using ALSV vectors was applicable to seven *Prunus* species, including apricot, sweet cherry (*Prunus avium* L.), almond [*Prunus dulcis* (Mill.) D. A. Webb.], peach (*Prunus persica* Batsch), Japanese apricot, Japanese plum (*Prunus salicina* Lindl.), and European plum (*Prunus domestica* L.). ALSV vectors carrying part of the apricot *PDS* gene sequence were amplified in *Nicotiana benthamiana*, and inoculated into the cotyledons of *Prunus* seedlings by particle bombardment. Typical *PDS*-silenced phenotypes, characterized by uniform discoloration of the upper leaves, were observed in sweet cherry and some cultivars of apricot and almond several weeks after inoculation. The amounts of *PDS* mRNA in the infected leaves were significantly reduced, while a 21 nt antisense small RNA, which was assumed to play a central role as a guide RNA in *PDS* mRNA degradation, was highly accumulated. However, ALSV infection of Japanese apricot, Japanese plum, European plum, and the other cultivars of apricot and almond was unsuccessful. Furthermore, although the infection rate of ALSV in peach was high, severe pale spots (a viral infection symptom) were observed in the infected leaves. These results collectively suggested that the efficiency of ALSV infection and VIGS could vary depending on species and/or cultivar in *Prunus*. The possible use of the ALSV-mediated VIGS system for functional analysis of genes in *Prunus* is discussed.



## 1. Introduction

Virus-induced gene silencing (VIGS) is a useful reverse genetics tool for functional analysis of plant genes. This approach is based on plant endogenous defense responses against invading foreign agents such as viruses or viroids, and used to induce the knock-down of target gene expression through a post-transcriptional gene silencing (PTGS) mechanism. VIGS is triggered by the infection of recombinant virus vectors carrying partial sequences of the target genes to be silenced. When the virus vectors infect the plants, double-stranded (ds) RNA and/or higher-order structures of single-stranded (ss) RNA are formed during virus replication. Antiviral responses in plants are triggered by the presence of such aberrant dsRNA structures, and cleave them into small interfering RNAs (siRNAs) through Dicer-like (DCL) enzymes. The siRNAs produced from the viral RNA and inserted target gene fragment are integrated into the endonuclease-containing component called the RNA-induced silencing complex (RISC). Of the sense and antisense strands of the siRNA duplex, the one incorporated into the RISC is called the guide strand, while the degraded one is called the passenger strand. When the antisense strand of the siRNA duplex is incorporated into the RISC as a guide strand, it subsequently serves as a guide to induce the degradation of complementary viral RNA or the mRNA of target genes in a homology dependent manner (Axtell, 2013; Liu et al.,

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2002; Lu et al., 2003; Mine and Okuno, 2008; Robertson, 2004; Voinnet, 2005).

So far, more than 35 different plant viruses have been developed as VIGS-vectors, and are increasingly used for functional analysis of genes in various plant species because they can induce rapid knock-down phenotypes of target genes without the lengthy transformation step using *Agrobacterium* (Purkayastha and Dasgupta, 2009; Senthil-Kumar and Mysore, 2011; Unver and Budak, 2009). Although most virus vectors were initially established for herbaceous model plants, such as *Nicotiana benthamiana*, recent studies have shown the successful application of some virus vectors for the silencing of endogenous genes in woody fruit tree species, such as Tobacco rattle virus (TRV)-based vectors for apple (*Malus* x *domestica* Borkh.) and peach (*Prunus persica* Batsch) (Bai et al., 2015; Li et al., 2012; Zhou et al., 2015), and *Citrus leaf blotch virus* (CLBV)-based vectors for *Citrus* species (Agüero et al., 2014). Among the various virus vectors reported, *Apple latent spherical virus* (ALSV) vectors are particularly promising because they have been used to effectively induce VIGS in a broad range of plant species including rosaceous fruit tree species, such as apple, pear (*Pyrus communis* L.), and Japanese pear (*Pyrus pyrifolia* Nakai) (Igarashi et al., 2009; Ito et al., 2012; Sasaki et al., 2011b; Takahashi et al., 2013; Yamagishi and Yoshikawa, 2009). ALSV consists of isometric virus particles ca. 25 nm in diameter, and contains two ssRNA species (RNA1 and RNA2) and three capsid proteins (Vp25, Vp20, and Vp24) (Koganezawa et al., 1985; Li et al., 2000). It can induce systemic VIGS including the meristematic region without causing any viral symptoms in most host plants (Igarashi et al., 2009; Sasaki et al., 2011b). Moreover, ALSV is not transmitted from infected plants to neighboring plants horizontally even in orchards, which enables safe management of the recombinant virus in terms of biological contamination (Nakamura et al., 2011). Because of these advantages, ALSV vectors have been used not only for VIGS-based functional analyses of plant genes but also for various practical studies, such as the induction of transcriptional gene silencing (TGS) of a transgene and endogenous gene through DNA methylation (Kon and Yoshikawa, 2014), development of virus-vaccines against pathogenic viruses (Satoh et al., 2014; Taki et al., 2013; Tamura et al., 2013), and promotion of flowering in rosaceous fruit or legume species (Sasaki et al., 2011b; Yamagishi et al., 2011, 2014; Yamagishi and Yoshikawa, 2011). Although the ALSV vector system has been applied to a limited number of fruit tree species at present, it has the potential to be used for the evaluation of gene functions or genetic improvement in a broader range of fruit tree species considering the wide host range of ALSV.

*Prunus*, which belongs to the family Rosaceae, includes many economically important fruit and nut tree species such as apricot (*Prunus armeniaca* L.), sweet cherry (*Prunus avium* L.), almond [*Prunus dulcis* (Mill.) D. A. Webb.], peach, Japanese apricot (*Prunus mume* Siebold & Zucc.), Japanese plum (*Prunus salicina* Lindl.), and European plum (*Prunus domestica* L.). Because of their relatively small genome size and short juvenile phase, some *Prunus* species have been used as genetic and morphological experimental models of woody fruit and nut plants to identify genes associated with agriculturally important traits, such as flowering, dormancy, self-incompatibility, fruit quality, virus resistance, and other growth habits (Dardick et al., 2013; Falchi et al., 2013; Tao and Iezzoni, 2010; Wells et al., 2015; Yamane, 2014; Zuriaga et al., 2013). The recent whole genome sequencing of peach has further facilitated these studies (Verde et al., 2013). So far, many candidate genes that could be used as potential molecular markers in breeding programs have been identified. However, functional verification of these genes has lagged behind because of the lack of an efficient transformation system in *Prunus*. Although transformation experiments to investigate the functions of *Prunus* genes have been performed using other model species, such as *Arabidopsis thaliana* or *Populus* species (Sasaki et al., 2011a; Chen et al., 2013), it is often difficult to precisely evaluate gene functions in heterologous plant systems. The development of an efficient gene evaluation system in *Prunus* is therefore very important for rapid progress in *Prunus* genetics and breeding.

As a first step toward the establishment of an efficient gene evaluation system in *Prunus*, we previously showed that ALSV vectors could successfully induce VIGS of the endogenous *PHYTOENE DESATURASE* (*PDS*) gene in apricot (Kawai et al., 2014). In this study, we assessed the VIGS efficiency of ALSV vectors in a wide range of *Prunus* species and cultivars. We found that ALSV vectors could successfully knock down mRNA from endogenous *PDS* genes in several cultivars of apricot, sweet cherry, and almond. Small RNA sequencing analysis revealed preferential accumulation of 21 nt antisense siRNAs in *PDS*-silenced leaves, suggesting their role as guide RNAs in VIGS of *PDS* genes. ALSV infection, however, was unsuccessful for Japanese apricot, Japanese plum, and European plum. Furthermore, peach seedlings infected with ALSV showed severe viral symptoms. These results strongly indicate that the efficiency of ALSV infection and VIGS varies with species and/or cultivar in *Prunus*. The possible use of the ALSV-mediated VIGS system for gene function analysis in *Prunus* species is discussed.

## 2. Materials and methods

### 2.1. Plant materials

Sixteen cultivars of 7 fruit tree species of *Prunus* were used in this study; apricot 'Shinyo', 'Shingetsu', 'Shinshuomi', 'Nanbuhachisuke', and 'Niigataomi', sweet cherry 'Satonishiki', almond 'Nonpareil', 'Carmel', and 'Marcona', peach 'Ohatsumomo', Japanese apricot 'Ryukyokoume', 'Benisashi', 'Koshinoume', and 'Hachiro', Japanese plum 'Sordum', and European plum 'Sanctus Hubertus'. Seeds of these cultivars were rinsed under running tap water overnight, placed on a filter paper soaked with distilled water in petri dishes, and germinated under 4 °C, dark conditions. Just after germination, the seedlings were used for viral inoculation by particle bombardment as described below.

*N. benthamiana* plants were used to produce and amplify recombinant ALSV particles for use as an inoculum in particle bombardment. They were grown under 16/8 h LD conditions (6000–8000 lux under cool-white fluorescent bulbs) at 25 °C for about 10 days after sowing, and then grown under 16/8 h LD conditions at 23 °C. Young plants at the 3–4 leaf stage (17–21 days after germination) were used for *Agrobacterium*-mediated viral inoculation as described below.

### 2.2. ALSV vectors and comparison of PDS sequences of Prunus

To assess the VIGS efficiency of ALSV vectors in various *Prunus* species, we selected the *PDS* gene, which encodes a key enzyme in carotenoid biosynthesis, as a target of silencing (Bartley and Scolnik, 1995; Kumagai et al., 1995). To express recombinant ALSV vectors targeting the *PDS* gene of each *Prunus* species, the binary plasmids pBICAL1 and pBICAL2-ParPDS described previously (Kawai et al., 2014) were used in this study. Briefly, pBICAL1 contains an expression cassette of ALSV RNA1 between CaMV35S promoter and nos terminator sequences. pBICAL2-ParPDS contains a 108 bp fragment of the apricot *PDS* (*ParPDS*) gene in-frame with an expression cassette of ALSV RNA2 located between CaMV35S promoter and nos terminator sequences. The partial sequence of *ParPDS* was amplified by RT-PCR using cDNA from apricot 'Heiwa' leaves, and ligated into the cloning site flanking the coding sequences of the movement and Vp25 capsid proteins in ALSV RNA2. These constructs were separately introduced into a disarmed