Virus-induced gene silencing in *Prunus* fruit and nut tree species by *Apple latent spherical virus* vector

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General Introduction

Fruit production of Prunus

The genus *Prunus*, which belongs to the tribe Amygdaleae of the subfamily Amygdaloideae in the family Rosaceae, includes many economically important fruit and nut tree species, such as apricot (*P. armeniaca* L.), Japanese apricot (*P. mume* Siebold & Zucc.), sweet cherry (*P. avium* L.), sour cherry (*P. cerasus* L.), peach and nectarine [*P. persica* (L.) Batsch], European plum (*P. domestica* L.), Japanese plum (*P. salicina* Lindl.), and almond [*P. dulcis* (Mill.) D. A. Webb.] (McNeill et al., 2012; Potter et al., 2007). As fruit trees of *Prunus* bear characteristic fruits that have a stone (hardened, lignified endocarp) enclosing a seed, this tribe is commonly referred to as ‘‘stone fruit’’. The edible fresh is a juicy mesocarp in most species, while the nut rather than fresh is consumed in almond. According to FAOSTAT (http://faostat3.fao.org/home/E.), 4,111,076 tons of apricots, 2,294,455 tons of cherries, 1,348,628 tons of sour cherries, 21,638,953 tons of peaches and nectarines, 11,528,337 tons of plums, and 2,917,894 tons of almonds were produced annually in the world in 2013. Some of them are also cultivated commercially in Japan with the annual production in 2013 as follows; 123,700 tons of apricots, 18,100 tons of cherries, 124,700 tons of peaches and nectarines, and 21,800 tons of plums.

Recent progress in genetics and genomics in Prunus

Some *Prunus* species are valuable not only for commercial fruit production, but also as experimental models of genetics and genomics in other fruit and nut crops. Peach has become one of the most important reference species for them due to the following reasons; 1) its high economic value, 2) self-compatibility allowing for development of self-crossed progeny, 3) compact genome size estimated to be 265 Mbp (Verde et al., 2013), approximately twice that of *Arabidopsis thaliana*, 4) relatively short juvenile phase of 1 to 3 years. Over the past few years, the international Rosaceae research community has collaboratively collected a large amount of genetic and genomic resources for peach and other representative crops in the Rosaceae, such as apple (*Malus* × *domestica* Borkh.) and strawberry (*Fragaria* species) (Shulaev et al., 2008). These resources include expressed sequence tags (ESTs), bacterial artificial chromosomes (BAC) libraries, physical and genetic maps, molecular markers, bioinformatics tools, and recently-released whole genome sequences, which are available in the Genome Database for Rosaceae (GDR; https://www.rosaceae.org/; Yamamoto et al., 2016). Among *Prunus* species, whole genome sequences have been reported for peach (Verde et al., 2013) and Japanese apricot (Zhang et al., 2012), with apricot, sweet cherry, and plum currently being sequenced (Jung and Main, 2014). For peach, an unbiased, high-quality draft genome sequence could be obtained using doubled haploid cultivar ‘Lovell’ with completely homozygous genotype (Verde et al., 2013). With incorporating and upgrading gene prediction and annotation with transcript

assemblies obtained from RNA-Seq reads from different peach tissues and organs, a total of 26,873 protein-coding genes were predicted in the latest version of the peach genome, Peach v2.0.a1. (GDR; https://www.rosaceae.org/species/prunus_persica/genome_v2.0.a1; Verde et al., 2013).

The availability of these increasing genetic and genomic resources has enabled sequence-based functional and comparative genomics to obtain the transfer knowledge of genes or other genetic elements as well as evolutional insights between different *Prunus* species (Jung and Main, 2014). Owing to the information, combined with the already existing wealth of biological and morphological data in various *Prunus* species, it has become easier to identify molecular markers or candidate 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). Rapid advances in the high-throughput sequencing technology and bioinformatics tools would further facilitate these studies. This trend provides us good opportunities for bridging the gap between basic studies and breeding or production of *Prunus*, because identification of genetic determinants for desirable/undesirable traits or elucidation of molecular mechanisms regulating them could lead to develop highly accurate molecular markers or artificial regulation methods of agriculturally important traits, which are applicable in *Prunus* production and breeding programs.

Functional evaluation of plant genes

In addition to the accumulation of various genetic and genomic resources, the advent of high-throughput sequencing technologies has been accelerating identification of drafted genes of which functions were not yet defined well in various plant species (Gilchrist and Haughn, 2010) including *Prunus*. Although the functions of these genes can be predicted based on the patterns of nucleotide or putative amino acid sequences or on their expression related to specific traits, functional verification *in vivo* would be much preferable for better understanding of the actual roles of the genes.

Over the last 20 years, a large number of techniques have been developed for gene function evaluation, some of which are applicable to many species, and each of which has advantages and limitations (Gilchrist and Haughn, 2010). They are commonly divided into gain-of-function and loss-of-function studies. Both approaches are represented by *Agrobacterium*-mediated stable transformation to generate plants constitutively expressing foreign genes, or antisense RNA or hairpin RNA to produce small interfering RNAs (siRNAs) which trigger the degradation of complimentary mRNA of target genes through the post-transcriptional gene silencing (PTGS) mechanism, respectively. A modified version of these transgenic techniques includes expression control of the transgenes under regulation of organs or developmental stages specific promoters, or promoters that can be induced by external environmental factors (Estornell et

al., 2008). In most transgenic approaches, tissue-culture steps for selecting transformed cells and regenerating an entire plant from them are needed after transformation. The mutagenesis-based approaches have been also used for both gain- and loss-of-gene function studies in plants. These approaches involve screening of insertion, deletion, or point mutation in the populations mutagenized by means of T-DNA or transposon insertion, or chemical mutagenesis (Gilchrist and Haughn, 2010). One of the disadvantages of these approaches is the fact that large populations are required for detecting insertion, deletion, and mutation on target genes of interest because these modifications are induced randomly in the genome (Gilchrist and Haughn, 2010).

Challenges of functional evaluation of genes in Prunus

Although extensive efforts have been made to develop various types of tools for functional evaluation of genes in a broad range of plant species, successful utilization of the newly-developed tools are still largely restricted to model plants such as *A. thaliana* or rice (*Oryza sativa* L.). Especially, it is difficult in many cases to apply mutagenesis-based approaches for perennial fruit crops considering the space, cost, and labor necessary for maintaining large mutant populations. Transgenic approaches have been thus mainly utilized for functional evaluation of genes in fruit tree species.

Within the genus *Prunus*, many studies to establish and optimize *Agrobacterium*-mediated transformation systems using regeneration from leaf explants or seed derived tissues have been reported in apricot (Laimer da Câmara Machado et al., 1992; Petri et al., 2004, 2008a; Pratesi et al., 2004), Japanese apricot (Gao et al., 2010; Gao-Takai and Tao, 2014), sweet cherry (Druart et al., 1998; Pratesi et al., 2004; Sgamma et al., 2015), sour cherry (Dolgov and Firsov, 1999; Song and Sink, 2005, 2006), peach (Hammerschlag and Smigocki, 1998; Padilla et al., 2006; Pérez-Clemente et al., 2004; Scorza et al., 1995b), European plum (Petri et al., 2008b; Mante et al., 1991; Scorza et al., 1994, 1995a, b; Tian et al., 2009), Japanese plum (Urtubia et al., 2008), and almond (Ainsley et al., 2001; Archilletti et al., 1995; Miguel and Oliveira, 1999; Ramesh et al., 2006). However, most of the studies have not gone beyond basic development of transformation system using reporter genes, and functional verification of agriculturally important genes has still lagged behind, except for a few species such as European plum. Technical difficulties in transformation systems in *Prunus* are as follows. First, the efficiencies of both *Agrobacterium*-mediated transformation and following regeneration are generally low and highly dependent on genotypes in *Prunus* (Petri and Burgos, 2005). Second, even if transgenic plants are successfully obtained, it generally takes several years to characterize the traits in reproductive organs because of a long juvenile phase of perennial tree species, although this period can be shortened by co-introducing flowering related genes such as *FLOWERING LOCUS T* (*FT*) with the target genes as shown in European plum and other fruit species such as apple, pear (*Pyrus communis* L.) and *Citrus* species (Endo et al., 2005; Matsuda et al.,

2009; Srinivasan et al., 2012; Tanaka et al., 2014). Third, the genetic backgrounds of transformed plants from seed derived tissues are different from those of the heterozygous original cultivars or lines in clonally propagated fruit trees. Although transformation experiments to investigate the functions of *Prunus* genes have been alternatively performed using other model species, such as *A. thaliana* or *Populus* species (Chen et al., 2013; Esumi et al., 2009, 2010; Sasaki et al., 2011a; Wang et al., 2013), it is often difficult to precisely evaluate gene functions associated with some fruit tree-specific traits in heterologous plant systems. The development of an efficient gene evaluation system in *Prunus* is therefore very important for rapid progress in functional studies of genes in *Prunus*.

Genetic improvement of Prunus

Transformation approaches could be utilized not only for basic studies to characterize gene functions, but also for genetic engineering of agriculturally important traits in fruit tree species, because only desirable traits can be introduced into existing genetic resources more rapidly and less costly than conventional breeding programs. In the last two decades, genetic transformation of fruit crops has mainly focused on enhancing disease resistance, increasing tolerance of abiotic stresses, improving fruit quality, reducing generation time, and modifying other plant growth habits (Rai and Shekhawat, 2014). The most notable instances of such genetic improvement in fruit crops are transgenic papaya (*Carica papaya* L.) and European plum which exhibit high levels of resistances to *Papaya ringspot virus* (PRSV) and *Plum pox virus* (PPV), respectively, thorough PTGS of the viral coat protein (CP) genes (Kumari et al., 2015; Scorza et al., 2013; Ilardi and Nicola-Negri, 2011; Tripathi et al., 2008). In the latter case, after extensive testing and risk assessment in laboratory, greenhouse, and in the field over 20 years, PPV-resistant plum cultivar, 'HoneySweet', was successfully authorized for cultivation in the USA in 2011 (Scorza et al., 2013). Genetic engineering was also used for European plum to enhance the tolerance to salt and water stresses by overexpression of transgenes encoding cytosolic antioxidant enzymes (Diaz-Vivancos et al., 2013, 2016). In another study, European plum transformed with *PtFT1* gene from poplar (*P. trichocarpa*) exhibited early, continuous flowering and produced normal fruits in the greenhouse within 1 year to 10 months (Srinivasan et al., 2012), which is utilized to shorten the breeding cycle of European plum in a breeding system named 'FasTrack' (http://ucanr.edu/sites/fastrack/). In addition to European plum, successful development of *Prunus necrotic ringspot virus* (PNRSV) resistance was reported in transgenic cherry rootstocks, and even in the non-transgenic sweet cherry scion grafted on them through siRNA-mediated gene silencing (Song et al., 2013; Zhao and Song, 2014). Although deployment of genetic engineering for improvement of *Prunus* is, to date, limited to only a few species described above, development of alternative methods to stable genetic transformation could lead to promote genetic improvement of various *Prunus* species, as well as functional evaluation of

genes in them.

Virus-induced gene silencing

Virus-induced gene silencing (VIGS) is a useful reverse genetics tool that could be an alternative to stable genetic transformation or other approaches 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 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 with the RNase III-type dsRNA endonuclease activity. The siRNAs thus 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., 2002; Lu et al., 2003; Mine and Okuno, 2008; Robertson, 2004; Voinnet, 2005). Both RNA and DNA viruses are recognized by the plant defense mechanisms and can induce the siRNA-mediated VIGS in infected plants (Baulcombe, 1999).

Advantages of VIGS and development of various VIGS vectors

The VIGS technique has several advantages over other reverse genetics approaches as described below (Lu et al., 2003; Purkayastha and Dasgupta, 2009; Senthil-Kumar and Mysore, 2011; Unver and Budak, 2009). First, knock-down phenotypes of VIGS target genes can be observed in a relatively short period, several weeks after inoculation of viral vectors, without the lengthy transformation and regeneration steps usually necessary for *Agrobacterium*-mediated stable transformation system. Second, partial sequence information of target genes, such as ESTs, is usually sufficient to construct the VIGS vectors. Third, gene functions are able to be analyzed even if loss-of-function of the targeted genes can cause plant or embryo lethality through mutagenesis or stable transformation approaches. Fourth, this method has the potential to silence multicopy genes or genes of different plant species with similar genetic backgrounds.

Because of these advantages, several types of plant viruses have been modified into VIGS vectors and increasingly used for functional studies of plant genes in the last two decades. The VIGS

technique was initially inspired through the observation of highly specific antiviral state in transgenic tobacco plants expressing CP gene of *Tobacco etch virus* (TEV) (Lindbo et. al., 1993). Then, the first successful application of VIGS was reported in 1995, in which knock-down of endogenous *PYTOENE DESATURASE* (*PDS*) gene was demonstrated in *Nicotiana benthamiana* using *Tobacco mosaic virus* (TMV) vectors (Kumagai et al., 1995). Although most virus vectors had been initially established for herbaceous model plants, such as *N. benthamiana*, specific viral vectors have been increasingly developed and optimized for a broad range of desired plant species, such as *A. thaliana*, *Solanum* species, legume species, rice, wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), maize (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), cassava (*Manihot esculenta* Crantz), and strawberry (*F.* × *ananassa*) (Bennypaul et al., 2012; Brigneti et al., 2004; Chai et al., 2011; Chung et al., 2004; Constantin et al., 2004; Ding et al., 2006; Faivre-Rampant et al., 2004; Fofana et al., 2004; Fu et al., 2005; Holzberg et al., 2002; Turnage et al., 2002; Tuttle et al., 2008; Yan et al., 2012; Zhang and Ghabrial, 2006). So far, more than 37 VIGS vectors have been developed for even only dicotyledonous plants (Ramanna et al., 2013), and used to characterize genes involved in various traits such as disease resistance (van der Linde et al., 2011), abiotic stress tolerance (George et al., 2010; Senthil-Kumar et al., 2008), nutrient acquisition or nutrient stress (Pacak et al., 2010; Xia et al., 2012), and other developmental processes (Yang et al., 2010; Zhu et al., 2010).

High-throughput forward genetics screening with VIGS

Although VIGS has been mainly used as a reverse genetics tool to study target gene functions in plants, it can be also applied to large scale forward genetics screening by cloning cDNA libraries into VIGS vectors and observation of loss-of-function phenotypes in infected plants. Lu et al. (2003) assessed the function of 4992 *N. benthamiana* cDNAs in disease resistance against bacterial phytopathogen, *Pseudomonas syringae*, using *Potato virus X* (PVX) vectors, and identified clones corresponding to heat shock protein 90 (HSP90) as a cofactor of disease resistance whose knock-down resulted in the suppression of hypersensitive response (HR) cell death against the pathogen. In another study, *Tobacco rattle virus* (TRV) vectors were used to screen 400 ESTs of tomato (*S. lycopersicum* L.), leading to the identification of important roles for *SlMADS1* and its *N. benthamiana* homologs, *NbMADS4-1* and *-2*, in floral development (Dong et al., 2007).

Viral vectors for expression of foreign genes in plants

Viral vectors can be used for expression of foreign genes in plants as well as for induction of VIGS. Recombinant viruses expressing reporter genes have been routinely used to monitor and elucidate many different aspects of virus infection in intact plants, such as replication, infectivity to plants of different genotypes, gene function, cross protection, cell-to-cell and long-distance

movement, spatial separation, localization within the host cell, and insect transmission (Andrade et al., 2007; Baulcombe et al., 1995; Dawson and Folimonova, 2013; Dawson et al., 1988; Dietrich and Maiss, 2003; Dolja et al., 1992; Folimonova, 2012; Folimonova et al., 2008, 2010; Hagiwara et al., 1999; Ion-Nagy et al., 2006; Liu et al., 2009; Padgett et al., 1996; Ratcliff et al., 1999; Roberts et al., 1997; Tatineni and Dawson, 2012; Tatineni et al., 2008, 2011; Verver et al., 1998). Along with these studies, considerable efforts have been made to use the expression viral vectors for rapid and high-yield production of many pharmaceutical proteins such as vaccine antigens and antibodies in infected plants (Cañizares et al., 2005; Gleba et al., 2004, 2005, 2007; Lico et al., 2008; Rybicki, 2010). Besides the protein production, several application studies using viral expression vectors have been reported in recent years. McGarry and Ayre (2012) demonstrated an emerging technique to induce precocious flowering in cotton by expressing *FT* gene with *Cotton leaf crumple virus* (CLCrV) vectors. In other studies, viral vectors carrying sequence-specific nucleases such as ZFN or TALEN, or that can express guide RNA for CRISPR/Cas9 system, have been successfully used for targeted gene editing (Marton et al., 2010; Vainstein et al., 2011; Yin et al., 2015).

Application of viral vectors for fruit tree species

In contrast to the herbaceous plants, only a few studies on virus vectors in fruit tree species had been reported until recently in terms of both VIGS and foreign gene expression, mainly because of the lack of efficient viral vectors (Robertson, 2004). Furthermore, woody plants are usually more resistant to virus infections and the mechanical inoculation methods routinely used for herbaceous plants are not readily applicable to fruit tree species (Yamagishi et al., 2010; Yanase et al., 1979). However, recent studies have shown the successful application of some virus vectors for the silencing of endogenous genes and/or expression of foreign genes in woody fruit tree species, such as TRV vectors for apple and *Malus* crabapple (Li et al., 2012; Tian et al., 2015), *Citrus tristeza virus* (CTV) vectors and *Citrus leaf blotch virus* (CLBV) vectors for *Citrus* species (Agüero et al., 2014; Dawson and Folimonova, 2013), and *Grapevine virus A* (GVA) vectors and *Grapevine leafroll-associated virus-2* (GLRaV-2) vectors for grapevine (*Vitis vinifera* L.) (Kurth et al., 2012; Muruganantham et al., 2009). For recent intriguing study, Velázquez et al. (2016) reported the successful induction of precocious flowering and normal fruiting in juvenile citrus by expressing *FT* genes with CLBV vectors, and proposed a possible use of this system as a new tool for genetics and breeding of citrus.

In *Prunus* species, TRV-based VIGS vectors were used for functional assessment of genes associated with fresh color of peach fruits (Bai et al., 2016; Zhou et al., 2015). TRV-mediated VIGS was also applied for sweet cherry to identify and characterize genes involved in fruit color, ripening, and drought tolerance (Li et al., 2015; Shen et al., 2014). However, successful viral infection and resultant phenotypic changes were limited to only a small part of inoculated organs (mostly fruits) in

these studies, and VIGS vectors which are able to spread systemically in infected plants are still not available in *Prunus*. Although green fluorescent protein (GFP)-tagged PPV vectors were successfully used for *in vitro* maintained peach and apricot plants to study PPV/host-*Prunus* interactions (Lansac et al., 2005), it is still unknown whether PPV can be practically used as effective viral vectors for VIGS or expression of other foreign genes. Therefore, development and evaluation of new viral vectors applicable for *Prunus* species are needed to promote gene function analyses and other application studies in *Prunus*.

Apple latent spherical virus vector

Among the various virus vectors reported, *Apple latent spherical virus* (ALSV) vectors are particularly promising because they have been successfully used for both VIGS of endogenous genes and expression of foreign genes in a broad range of plant species including not only herbaceous plant species but also rosaceous fruit tree species such as apple, pear, and Japanese pear (*Pyrus pyrifolia* Nakai) (Igarashi et al., 2009; Ito et al., 2012; Li et al., 2004; Sasaki et al., 2011b; Takahashi et al., 2007, 2013; Yaegashi et al., 2007; Yamagishi and Yoshikawa, 2009; Yamagishi et al., 2011, 2016b).

ALSV, which is classified into the genus *Cheravirus*, consists of isometric virus particles (ca. 25 nm in diameter) with a hexagonal outline, and contains two ssRNA species (RNA1 and RNA2) and three capsid proteins (Vp25, Vp20, and Vp24) (Li et al., 2000). The virus is composed of two components, M and B, which are thought to contain two molecules of RNA2 and a single molecule of RNA1, respectively (Li et al., 2000). RNA1 (6813 nt excluding the 3' poly (A) tail) has a single open reading frame (ORF) encoding a 243K polypeptide which contains the consensus motifs of the protease cofactor, the NTP-binding helicase, the cysteine protease and the RNA polymerase from the N-terminus (Li et al., 2000). RNA2 (3385 nt excluding the 3' poly (A) tail) also has a single ORF encoding a 108K polypeptide which contains 42K movement protein (MP) on the N-terminal side and three capsid proteins in the C-terminal region (Li et al., 2000). Translated polyproteins undergo further processing to produce functional proteins. It was found that one of the three capsid proteins, Vp20, acts as a silencing suppressor which interferes with systemic silencing (Yaegashi et al., 2007).

ALSV was originally isolated during an investigation to identify the causal agent of apple russet ring disease from an apple tree 'Indo' which had been grafted with 'Fuji' showing fruit russet ring symptoms (Koganezawa et al., 1985; Li et al., 2000). Since it appeared that ALSV is not a causal agent of the disease and infects apple latently (Ito and Yoshida, 1997), ALSV has been utilized as the viral vector. Infectious cDNA clones of ALSV RNAs were initially modified into viral vectors for expression of foreign genes in plants (Li et al., 2004), and those carrying fluorescent proteins were used to analyze cell-to-cell movement of ALSV in infected plant tissues (Yoshikawa et al., 2006). Spatial distribution of identical and two distinct virus populations labeled with different

fluorescent proteins in co-infected plants was also tested with ALSV (Takahashi et al., 2007). ALSV vectors carrying GFP were used for evaluating VIGS of transgene in transgenic tobacco plants expressing GFP (Yaegashi et al., 2007). ALSV vectors were then evaluated as VIGS vectors for silencing of endogenous genes, and shown to effectively induce reliable VIGS of endogenous genes in a broad range of herbaceous plants including *Nicotiana* species, *A. thaliana*, tomato, cucubit species, legume species, wild rose (*Rosa rugose* Tunb. ex Murray), and gentian (*Gentiana triflora*) (Igarashi et al., 2009; Ito et al., 2012; Takahashi et al., 2013; Yamagishi and Yoshikawa, 2009; Yamagishi et al., 2016b), as well as rosaceous fruit tree species such as apple, pear, and Japanese pear (Sasaki et al., 2011b). ALSV can spread throughout the whole inoculated plant and 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 multiple pathogenic viruses through VIGS of viral genome (Satoh et al., 2014; Taki et al., 2013;Tamura et al., 2013), and promotion of flowering in various plants including rosaceous fruit species, legume species, *Nicotiana* species, *A. thaliana*, gentian, and lisianthus (*Eustoma grandiflorum*) by expression and/or VIGS of flowering related genes (Fekih et al., 2016; Sasaki et al., 2011b; Yamagishi and Yoshikawa, 2011; Yamagishi et al., 2011, 2014, 2016a). Some of these techniques have attracted considerable attention as a novel type of genetics and breeding tools which can introduce new traits into infected plants without altering host genome sequence unlike the conventional genetic transformation systems (Gleba et al., 2004; Purkayastha and Dasgupta, 2009). 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.

The objective of this study

As it is currently difficult to evaluate gene functions in *Prunus*, an ALSV-mediated gene evaluation system might provide an alternative tool to enable such studies in *Prunus*. This system also could be used as a tool for breeding and genetic improvement in *Prunus*. The main objective of this study is to develop VIGS-based gene evaluation system with ALSV vectors in a wide range of *Prunus* species and cultivars. Furthermore, the amount and distribution of small RNA in the infected *Prunus* species were also analyzed to obtain basic insights into successful induction of VIGS. The

possible use of the ALSV-mediated VIGS system for gene function analysis in *Prunus* species is discussed.

Chapter 1.

Virus-induced gene silencing in apricot (*Prunus armeniaca* L.) and Japanese apricot (*P. mume* Siebold & Zucc.) with the *Apple latent spherical virus* vector.

1.1. Introduction

ALSV vectors have been shown to effectively induce stable VIGS in a wide range of plant species, including rosaceous fruit tree species such as apple, pear, and Japanese pear. As a first step toward the establishment of an efficient gene evaluation system in *Prunus*, a VIGS-based gene evaluation system for two *Prunus* fruit tree species, apricot and Japanese apricot, was tested using ALSV vectors.

1.2. Materials and Methods

Plant materials and RNA extraction

Seedlings of apricot 'Heiwa' and Japanese apricot 'Nanko' were grown in a growth chamber under LD conditions (16/8 h light/dark photoperiod) at 25°C. Young leaves of the apricot and Japanese apricot seedlings were collected, immediately frozen in liquid nitrogen, and stored at -80°C until use. Total RNA was extracted from the collected leaves using the hexadecyl-trimethyl-ammonium bromide (CTAB) method and cDNA was synthesized as described in Yamane et al. (2011). Seeds of apricot and Japanese apricot were rinsed under running tap water overnight, placed on a layer of filter paper moistened with distilled water in petri dishes, and germinated under 4°C, dark conditions. Just after germination, the seedlings were used for viral inoculation as described below.

Construction of ALSV vectors

We selected the *PDS* gene as a target for VIGS because it was one of the most widely used markers of VIGS in previous studies (Kumagai et al., 1995). Based on nucleotide sequence alignment of *PDS* genes of apricot (DDBJ/EMBL/GenBank Acc. No. AY822065) and other woody plant species, a conserved region of the apricot *PDS* gene (*ParPDS*; 108 bp; Fig. 1-1A) was amplified from cDNA from apricot leaves using the primer pair ParPDS-F and ParPDS-R (Table 1-1). The RT-PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and their DNA sequences were determined using a CEQ8000 and DTCS Quick Start Master Mix (Beckman Coulter, Fullerton, CA, USA).

To construct pBICAL1 (Fig. 1-2), an 8-kb fragment containing ALSV RNA1 expression cassette was amplified from pEALSR1 (Li et al., 2004) using primers SalI/pUC18-R and

ClaI/nosT-L, which included *Sal*I and *Cla*I sites, respectively (Table 1-1). The generated PCR product was digested with *Sal*I and *Cla*I and inserted into the same sites of pBICP35 (Mori et al., 1991), producing pBICAL1. For pBICAL2 construction (Fig. 1-2), a 4.5-kb fragment containing ALSV RNA2 expression cassette was amplified from pEALSR2L5R5 (Li et al., 2004) using primers SalI/pUC18-R and ClaI/nosT-L (Table 1-1). The generated PCR product was digested with *Sal*I and *Cla*I and inserted into the same sites of pBICP35, producing pBICAL2.

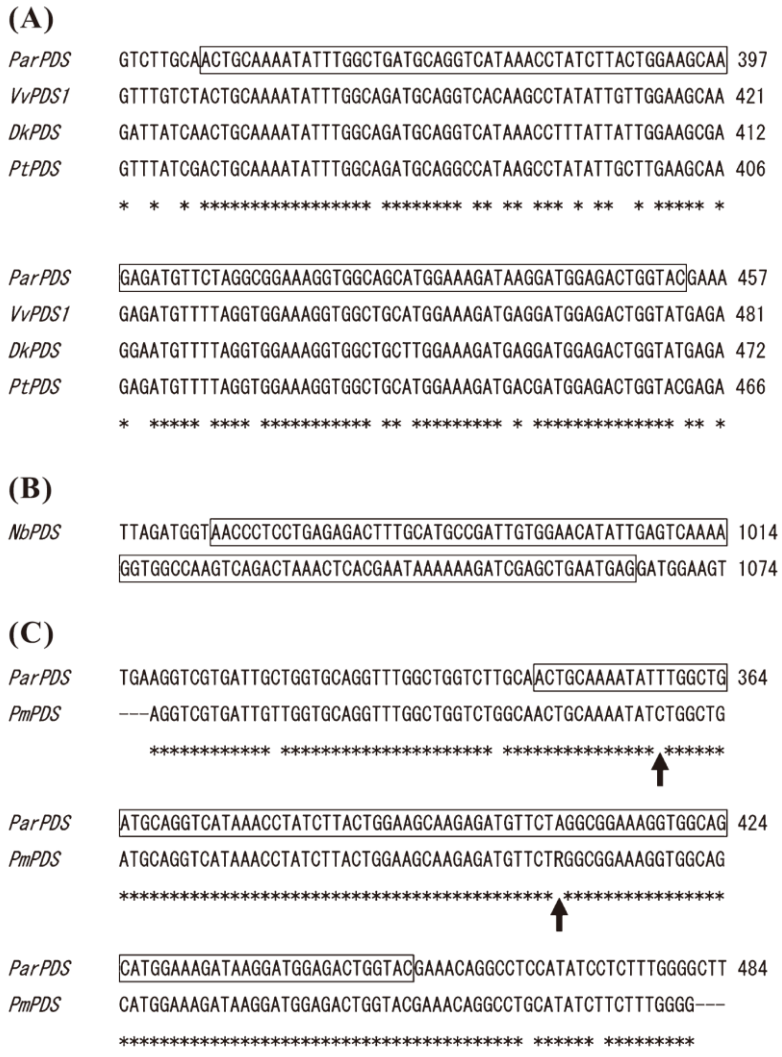

Fig. 1-1. Nucleotide sequences of the plant *PDS* genes used in this study. Numbers on the right side represent the nucleotide position from the start codon in the CDS of each gene. Asterisks indicate identical nucleotides. (A) Multiple alignment of nucleotide sequences of *PDS* orthologs of several woody plant species. The *Prunus armeniaca PDS* (*ParPDS*) region used for construction of pBICAL2-ParPDS is boxed. The sequence data used were *ParPDS* (AY822065), *Vitis vinifera PDS1* (*VvPDS1*; JQ319635), persimmon (*Diospyros kaki*) 'Xiao Fang Shi' *PDS* (*DkPDS*; GU112527) from the GenBank/EMBL/DDBJ database, and poplar (*Populus trichocarpa*) predicted *PDS* (*PtPDS*; XM_002321068) from a NCBI reference sequence. (B) The nucleotide sequence of *Nicotiana benthamiana PDS* (*NbPDS*; EU165355). The *NbPDS* region used for construction of pBICAL2gsPDS is boxed. (C) Alignment of nucleotide sequences of *ParPDS* and *Prunus mume PDS* (*PmPDS*). The *ParPDS* region used for construction of pBICAL2-ParPDS is boxed. Arrows indicate the SNPs between *ParPDS* and *PmPDS*. R indicates A or G.

Table 1-1. Primer sequences used in this study.

Primer name	Sequence (5′-3′)[z]	Usage
ParPDS-F	ACTGCAAAATATTTGGCTGATG	Cloning of partial sequence of *ParPDS*
ParPDS-R	GTACCAGTCTCCATCCTTATCTTTC	Cloning of partial sequence of *ParPDS*
SalI/pUC18-R	GGGGGTCGACGGTTTTCCCAGTCACGACGTTG	Construction of pBICAL1, pBICAL2 and pBICAL2gsPDS
ClaI/nosT-L	GGGATCGATCTAGTAACATAGATGAC	Construction of pBICAL1, pBICAL2 and pBICAL2gsPDS
ParPDS-XhoI-F	ATATACTCGAGACTGCAAAATATTTGGCTGATG	Construction of pBICAL2-ParPDS
ParPDS-BamHI-R	TATATGGATCCGTACCAGTCTCCATCCTTATCTTTC	Construction of pBICAL2-ParPDS
NtPDS110XhoI/R	AGCTCGAGAACCCTCCTGAGAGAGACTT	Construction of pEALSR2gsPDS
NtPDS211BamHI/L	GAGGATCCCTCATTCAGCTCGATCTT	Construction of pEALSR2gsPDS
PrunusPDS-insert-F	CTTCCACTTTCCGAGCCTCT	Comparison of *PDS* sequences of apricot and Japanese apricot
PrunusPDS-insert-R	ACCAAGCTCACCAAACAGGT	Comparison of *PDS* sequences of apricot and Japanese apricot
ALSV-F	CTTCTAGTTTGCATAGATCTGACCCA	RT-PCR for ALSV detection
ALSV-R	TTTCAAGAGTTCTCCCCCATAAGATT	RT-PCR for ALSV detection
ParPDS-real-F	GGATGAGGAAACAGGGCATA	RT-PCR for expression analysis of *ParPDS*
ParPDS-real-R	CCTGAAGGAATCGGTTCAAA	RT-PCR for expression analysis of *ParPDS*
PavUbi-F	CGAACCCTAGCCGATTACAA	Reference of RT-PCR analysis
PavUbi-R	AGTGGTTCGCCATGAAAGTC	Reference of RT-PCR analysis

[z] Restriction sites for *Sal*I, *Cla*I, *Xho*I, and *Bam*HI are underlined.

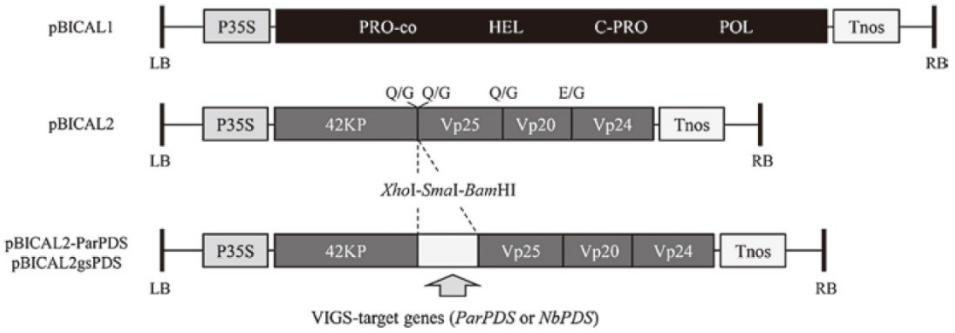

Fig. 1-2. Schematic representations of the T-DNA regions of ALSV constructs used in this study. pBICAL1 and pBICAL2 were designed for the expression of ALSV RNA1 and RNA2, respectively, under the control of CaMV35S promoter sequence. Partial sequences of the VIGS target genes (*ParPDS* and *NbPDS*) were inserted between the coding sequences of 42KP and Vp25 using *Xho*I and *Bam*HI restriction sites. LB, left border; P35S, Cauliflower mosaic virus 35S promoter; PRO-co, protease cofactor; HEL, NTP-binding helicase; C-PRO, cysteine protease; POL, RNA polymerase; Tnos, nopaline synthase terminator; RB, right border; 42KP, 42K movement protein; Vp25, Vp20, and Vp24, capsid proteins.

To construct ALSV vectors carrying the partial sequence of *ParPDS*, the cloned DNA sequence was amplified by PCR with PrimeSTAR GXL DNA polymerase (Takara Bio, Shiga, Japan) using the primer pair ParPDS-XhoI-F and ParPDS-BamHI-R, which included *Xho*I and *Bam*HI sites, respectively (Table 1-1). The PCR products were double-digested with *Xho*I and *Bam*HI and ligated in frame with the coding sequences for the movement and Vp25 capsid proteins flanking the cloning site of pBICAL2 digested with the same enzymes. The resultant construct, designated pBICAL2-ParPDS (Fig. 1-2), was introduced into a disarmed *Agrobacterium* strain, EHA105, by electroporation using electro competent EHA105 cells produced by a standard technique using 10%

(w/v) glycerol and a MicroPulser electroporator (BIO-RAD, Hercules, CA, USA). pBICAL1 and pBICAL2 were also introduced separately into EHA105.

To construct ALSV vectors carrying the partial sequence of the *N. benthamiana PDS* (*NbPDS*) gene, 102-nucleotide partial fragment of *NbPDS* (Fig. 1-1B) was amplified from cDNA derived from *N. benthamiana* RNA using primers NtPDS110XhoI/R and NtPDS211BamHI/L, which included *Xho*I and *Bam*HI sites, respectively (Table 1-1). The fragment was digested with *Xho*I and *Bam*HI and inserted into the same sites of pEAL2SR2L5R5, producing pEALSR2gsPDS. A 4.6 kb-fragment containing ALSV RNA2 expression cassette was amplified from pEALSR2gsPDS using primers SalI/pUC18-R and ClaI/nosT-L (Table 1-1). The generated PCR product was digested with *Sal*I and *Cla*I and inserted into the same sites of pBICP35. The resultant construct, designated pBICAL2gsPDS (Fig. 1-2), was introduced into EHA105.

Comparison of PDS sequences of apricot and Japanese apricot

It has been reported that more than 23 successive nucleotides with 100% sequence identity to the target gene are necessary for VIGS (Purkayastha and Dasgupta, 2009). As apricot and Japanese apricot have similar genetic backgrounds, ALSV vectors for VIGS of *ParPDS* could be applicable for the silencing of Japanese apricot *PDS* (*PmPDS*). To confirm this, we determined the nucleotide sequence of the *PmPDS* region corresponding to the partial sequence of *ParPDS* used for the construction of pBICAL2-ParPDS. RT-PCR was performed with cDNA from Japanese apricot leaves using the primer pair PrunusPDS-insert-F and PrunusPDS-insert-R (Table 1-1). PCR amplification was performed with a program of 95°C for 3 min followed by 35 cycles of 95°C for 20 sec, 60°C for 15 sec, and 72°C for 45 sec. After alkaline phosphatase and exonuclease treatment, direct sequencing of the PCR products was performed using a CEQ8000 and DTCS Quick Start Master Mix (Beckman Coulter).

Amplification of ALSV vectors in N. benthamiana

N. benthamiana plants were used to produce and amplify wild-type (wt) and recombinant ALSV particles. They were grown under 16 h LD conditions at 25°C for about 10 days after sowing, and then grown under 16 h LD conditions at 23°C. Young seedlings 17–21 days after germination (3–4 leaf stage; Fig. 1-3A) were used for *Agrobacterium*-mediated viral inoculation. Leaves of *N. benthamiana* were simultaneously inoculated with pBICAL1/EHA105 and pBICAL2/EHA105, or pBICAL1/EHA105 and pBICAL2-ParPDS/EHA105, using toothpick. Frozen stocks of EHA105 harboring each plasmid were added separately to about 200 μL LB liquid medium. The suspensions were incubated for 2–3 h at 28°C and spread on LB agar plates with an appropriate antibiotic. After incubation for 1–2 days at 28°C, fresh colonies of pBICAL1/EHA105 and pBICAL2/EHA105, or pBICAL1/EHA105 and pBICAL2-ParPDS/EHA105, were suspended together in 100 μL of 10 mM

$MgCl_2$ containing acetosyringone (100 μg·mL^{-1}). The suspensions were kept at 23°C for 3–4 h. The tip of a sterilized toothpick was dipped in the suspension and inserted into *N. benthamiana* leaves. Four to six inoculations were made at different points on each leaf of 17–21 days old young *N. benthamiana* plants (Fig. 1-3A). The inoculated plants were grown under 16 h LD conditions at 23°C. pBICAL1/EHA105 and pBICAL2gsPDS/EHA105 were also infected into *N. benthamiana* because chlorosis of upper leaves, a phenotype of *PDS* gene silencing caused by the amplification of NbPDS-ALSV, could be used as a good indicator to estimate the movement and amplification of wtALSV and ParPDS-ALSV.

Successful viral infection was determined by the microtissue direct RT-PCR method 2–3 weeks post inoculation (wpi) according to Hosokawa et al. (2006) with slight modifications. PCR amplification was performed with a program of 32 cycles of 95°C for 20 sec, 58°C for 15 sec, and 72°C for 45 sec with an initial heating at 95°C for 3 min using the primer pair ALSV-F and ALSV-R (Table 1-1) corresponding to nucleotide positions 1396–1421 and 1573–1598 of ALSV RNA2 (AB030941), respectively. This primer pair was designed to amplify the region containing the cloning site to confirm the existence of inserted sequences in ALSV RNA2. Young upper leaves and stipules of infected *N. benthamiana* were collected, stored at -80°C, and used for the extraction of total RNA to be used as an inoculum.

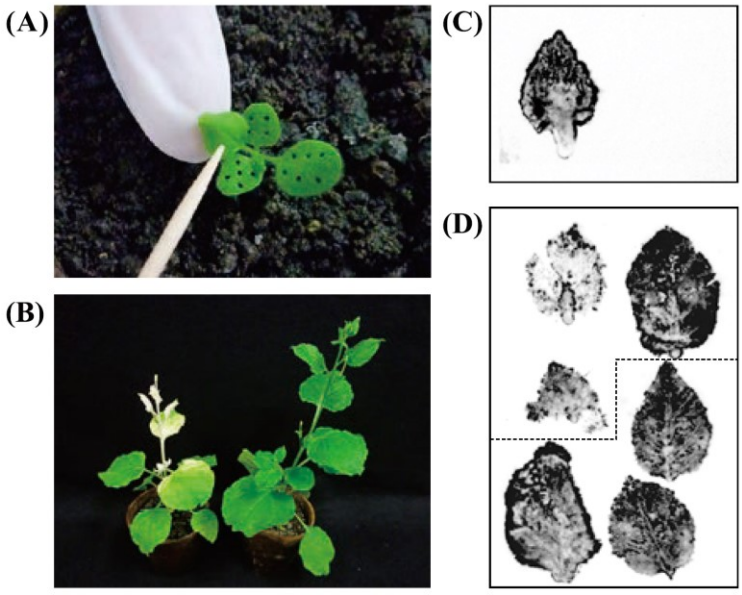

Fig. 1-3. Production and amplification of ALSV vectors in *N. benthamiana*. (A) *Agrobacterium*-mediated viral inoculation into *N. benthamiana* using a toothpick. (B) *N. benthamiana* infected with NbPDS-ALSV (left) and a healthy control plant with no viral inoculation (right) (30 dpi). (C) Tissue blot analysis of a silenced white leaf infected with NbPDS-ALSV (left) and a healthy leaf with no viral inoculation (right). (D) Tissue blot analysis of leaves infected with ParPDS-ALSV (above the broken line) and wtALSV (below the broken line).

Viral inoculation to apricot and Japanese apricot seedlings

Total RNA containing amplified wtALSV or ParPDS-ALSV was isolated from infected *N. benthamiana* using TRIzol reagent (Life Technologies Japan, Tokyo, Japan), purified by phenol/chloroform extraction, and used for inoculation of apricot and Japanese apricot seedlings by particle bombardment, essentially as described in Yamagishi et al. (2010). Briefly, 8 mg gold particles (0.6 mm in diameter; BIO-RAD) were sonicated in 50 µL RNase-free water for at least 1 min using Ultrasonic Cleaner SU-27TH (Sibata Scientific Technology, Tokyo, Japan). Then, 50–100 µL of RNase-free water containing 200 µg total RNA extracted from infected *N. benthamiana* was added to the gold particle suspension with continuous vortex mixing. After mixing, a 1/10 volume of 5 M ammonium acetate and twice the volume of isopropyl alcohol was added to precipitate the RNA. The mixture was kept at -20°C for at least 1 h. After removing the supernatant, the gold particles coated with total RNA were washed with 1 mL dehydrated 99.5% ethanol three times to remove water, and resuspended in 2.4 mL dehydrated 99.5% ethanol. The suspension was used to coat the inside of a plastic tube (50 cm) with gold particles according to the manufacturer's instructions. The gold-coated tubing was cut into pieces (1.25 cm), each of which contained 5 µg total RNA, and used for biolistic inoculation.

The seed coats were gently removed from apricot and Japanese apricot seedlings just after germination and the cotyledons were bombarded (Fig. 1-4A, B) with gold particles coated with total RNA at a pressure of 280–300 psi using a Helios Gene Gun system (BIO-RAD). Bombardments were performed four and three times on each side of the cotyledons of apricot and Japanese apricot, respectively. After acclimatization in the dark at 4°C and 100% relative humidity, the inoculated seedlings were planted in soil and grown in a growth chamber under 16 h LD conditions at 25°C.

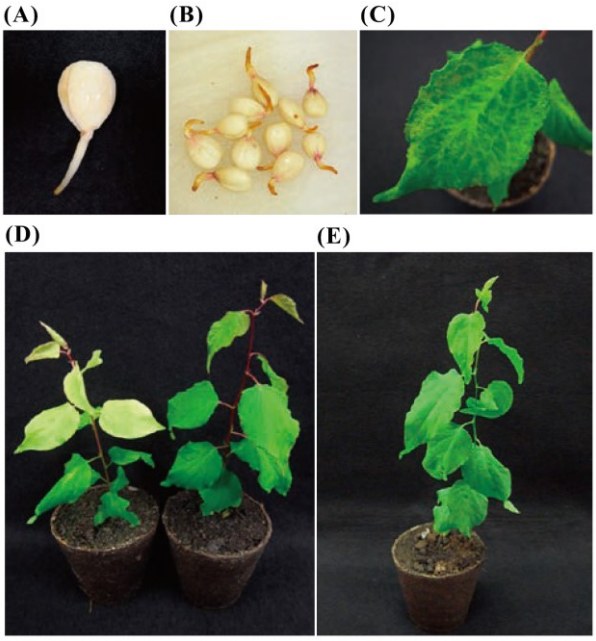

Fig. 1-4. Apricot and Japanese apricot seedlings infected with ALSV vectors. (A) An apricot seedling and (B) Japanese apricot seedlings just after germination used for viral inoculation by particle bombardment. (C) Leaf discoloration along the veins of the upper leaves of apricot seedlings infected with ParPDS-ALSV (19 dpi). (D) An apricot seedling infected with ParPDS-ALSV (left) and a healthy plant with no viral inoculation (right) (30 dpi). (E) An apricot seedling infected with wtALSV (36 dpi).

RT-PCR analysis

Total RNA was extracted from the upper leaves of inoculated apricot and Japanese apricot seedlings by a small-scale CTAB method according to Sasaki et al. (2011b) with slight modifications. cDNA was synthesized using 500 ng total RNA with ReverTra Ace qPCR RT Master Mix with gDNA Remover and a mixture of oligo (dT) and random primers (TOYOBO, Osaka, Japan). cDNA equivalent to the amount synthesized from 5 ng total RNA was used as a template for RT-PCR analysis. RT-PCR for detection of ALSV was carried out using the same primer pair and PCR program that were used for virus detection in *N. benthamiana*. Semi-quantitative RT-PCR analysis of mRNA levels of the *PDS* gene was performed using the primer pair ParPDS-real-F and ParPDS-real-R (Table 1-1) and a PCR program consisting of 32 cycles of 95°C for 20 sec, 60°C for 15 sec, and 72°C for 45 sec with initial heating at 95°C for 3 min. The PCR products were electrophoresed on a 1% (w/v) agarose gel, stained with ethidium bromide, and detected using a UV illuminator. In addition, real-time RT-PCR with the same primer pair was conducted using SYBR Green Master Mix and a LightCycler 480 (Roche Diagnostics, Mannheim, Germany). This PCR was performed with a program of 95°C for 5 min followed by 45 cycles of 95°C for 10 sec, 60°C for 20 sec, and 72°C for 1 sec. Gene specific amplification was confirmed by melting curve analysis. Three technical replicates were performed. As a reference, the *UBIQUITIN* gene-specific primer pair PavUbi-F and PavUbi-R (Yooyongwech et al., 2008; AF298826; Table 1-1) was used for RT-PCR and real-time RT-PCR analyses.

Tissue blot analysis

The distribution of ALSV in the leaves of inoculated *N. benthamiana* and apricot seedlings was confirmed by tissue blot analysis as described previously (Yamagishi and Yoshikawa, 2009) using a DIG-labeled RNA probe complementary to ALSV RNA2.

1.3. Results and Discussion

Efficient amplification of ALSV vectors in N. benthamiana

In the original method (Li et al., 2004), ALSV vectors for particle bombardment were produced and amplified in *Chenopodium quinoa* by mechanically inoculating infectious cDNA clones designed from ALSV RNA1 and RNA2. In this study, we used *N. benthamiana* to amplify ALSV vectors more efficiently. The use of *N. benthamiana* made it possible to avoid the time consuming inoculation step that was necessary for the original viral amplification method with *C. quinoa*. Using modified binary Ti-plasmids with CaMV35S promoter sequence (Fig. 1-2), agroinfection became possible. When *Agrobacterium tumefaciens* strains pBICAL1/EHA105 and pBICAL2gsPDS/EHA105 were inoculated using a toothpick, a uniform photo-bleached phenotype,

which is a typical symptom of VIGS of the *NbPDS* gene, was observed in the upper leaves of almost all inoculated plants 2–3 wpi, as described previously (Igarashi et al., 2009; Fig. 1-3A, B). Using this phenotypic change as an indicator of viral amplification, direct microtissue RT-PCR and tissue blot analyses were performed to confirm the systemic infection of wtALSV or ParPDS-ALSV in *N. benthamiana* inoculated with pBICAL1/EHA105 and pBICAL2/EHA105, or pBICAL1/EHA105 and pBICAL2-ParPDS/EHA105. When RT-PCR of the upper leaves was conducted using ALSV RNA2-specific primers, almost all inoculated plants produced 251-bp and 353-bp PCR fragments corresponding to wtALSV and ParPDS-ALSV, respectively (data not shown). Although Igarashi et al. (2009) indicated that ALSV vectors carrying a partial sequence of the *PDS* gene longer than 300 bp lost their insert sequences 30–40 days post inoculation (dpi) in tobacco plants, such loss of inserted sequences was not observed in our experiments for *N. benthamiana* plants infected with NbPDS-ALSV and ParPDS-ALSV. The shorter length of partial *PDS* gene sequences used in this study (102 bp of *NbPDS* and 108 bp of *ParPDS*) could explain the different results obtained in our study and by Igarashi et al. (2009). Tissue blot analysis of inoculated plants using a DIG-labeled RNA probe complementary to ALSV RNA2 indicated that ALSV was accumulated uniformly in the upper leaves (Fig. 1-3C, D). These results suggested that *N. benthamiana* was successfully infected with ALSV vectors by *Agrobacterium*-mediated viral inoculation using a toothpick, which could offer an easy and fast way to produce and amplify ALSV vectors for particle bombardment.

Infection of apricot and Japanese apricot seedlings with ALSV vectors

The infection rates of ALSV for apple, pear, Japanese pear, and soybean [*Glycine max* (L.) Merr.] were reported to be almost 100% when ALSV RNAs were introduced into the cotyledons of seedlings just after germination by particle bombardment (Sasaki et al., 2011b; Yamagishi and Yoshikawa, 2009; Yamagishi et al., 2010). As very high infection efficiencies were obtained with apple, pear, and Japanese pear, we followed these methods to inoculate apricot and Japanese apricot. Total RNA isolated from *N. benthamiana* infected with wtALSV and ParPDS-ALSV was used to coat gold particles, and the cotyledons of apricot and Japanese apricot seedlings just after germination were bombarded with these gold particles using a helium gun (Fig. 1-4A, B). RT-PCR with ALSV RNA2-specific primers produced PCR fragments corresponding to wtALSV (251 bp) and ParPDS-ALSV (353 bp) for the upper leaves 2–3 wpi in 2 of 12 seedlings infected with wtALSV (16.7%) and in 5 of 45 seedlings infected with ParPDS-ALSV (11.1%), respectively (Table 1-2; Fig. 1-5). Apricot seedlings infected with wtALSV exhibited no obvious virus infection symptoms and showed normal growth comparable to healthy plants with no viral infection (Fig. 1-4D, E). These results suggested that ALSV vectors could be effectively used to evaluate gene functions in apricot because viral symptoms often hinder the correct evaluation of phenotypic changes caused by silencing of the VIGS-target gene. Conversely, we detected neither wtALSV nor

ParPDS-ALSV from the leaves of 10 and 25 Japanese apricot seedlings infected with these virus vectors, respectively (Table 1-2). This result may indicate that species- or cultivar-dependent differences of ALSV susceptibility exist in *Prunus*. As we only used seedlings from a single cultivar each of apricot and Japanese apricot, it is unclear whether the differential susceptibility was dependent on species or cultivar. In either case, we need to consider genotype-dependent variation in ALSV susceptibility when the ALSV vector system is used to evaluate gene functions in *Prunus*.

Table 1-2. Infection rates of ALSV vectors into apricot and Japanese apricot seedlings.

Plant species	Virus vector	Number of infected/ inoculated seedlings	Infection rate (%)
Apricot (*P. armeniaca*)	wtALSV	2/12	16.7
	ParPDS-ALSV	5/45	11.1
Japanese apricot (*P. mume*)	wtALSV	0/10	0
	ParPDS-ALSV	0/25	0

VIGS of the PDS gene in apricot

The *PDS* gene encodes a key enzyme in the carotenoid biosynthesis pathway and carotenoid pigments are essential for the protection of chlorophyll from photo oxidation (Bartley and Scolnik, 1995). Because inhibition of the *PDS* gene leads to rapid degradation of chlorophyll and results in a photo-bleached phenotype in plants, it is widely used as a visible marker of VIGS (Kumagai et al., 1995).

Sequence analysis revealed that the partial sequence of *ParPDS* inserted into the ALSV construct contained more than 23 successive nucleotides with 100% identity to *PmPDS* (Fig. 1-1C). Thus, we targeted the *PDS* gene for VIGS in apricot and Japanese apricot using the same ALSV vector, ParPDS-ALSV.

Of the 45 apricot and 25 Japanese apricot seedlings bombarded with ParPDS-ALSV, 5 apricot and 0 Japanese apricot seedlings were successfully infected with ParPDS-ALSV (Table 1-2). Uniform discoloration of the upper leaves, a typical phenotype of *PDS* gene knockdown, was observed in all infected apricot seedlings 2–3 wpi (5–6 leaf stage; Fig. 1-4D). The discoloration first appeared along the veins of the upper leaves (Fig. 1-4C) and then spread to the whole area of newly generated leaves (Fig. 1-4D). Tissue blot analysis was performed to confirm the systemic infection of ALSV in these photo-bleached leaves because RT-PCR analysis could check virus infection of only a small part of leaves. The result showed that ALSV was accumulated uniformly in silenced upper leaves (Fig. 1-6).

The infected seedlings exhibited slightly stunted growth, which could have been caused by the inhibition of photosynthesis, and stopped growing within 3 months post inoculation (16–18 leaf

stage). The silenced phenotype persisted throughout plant growth. However, photo-bleached unfolded upper leaves occasionally turned yellow or light green later in their development (data not shown). Similar results were observed in wild rose, in which *PDS*-silenced photo-bleached leaves turned green, and then newly developed leaves showed a photo-bleached phenotype (Ito et al., 2012). Although ALSV vectors containing the insert sequence were detected by RT-PCR from leaves showing this "recovery phenotype" (data not shown), the efficiency of VIGS may have declined because of reduced virus concentration or other environmental factors. In fact, it has been reported in previous studies that some growth conditions such as temperature or humidity affect the efficiency or stability of silencing (Chellappan et al., 2005; Fu et al., 2006; Tuttle et al., 2008; Wang et al., 2013).

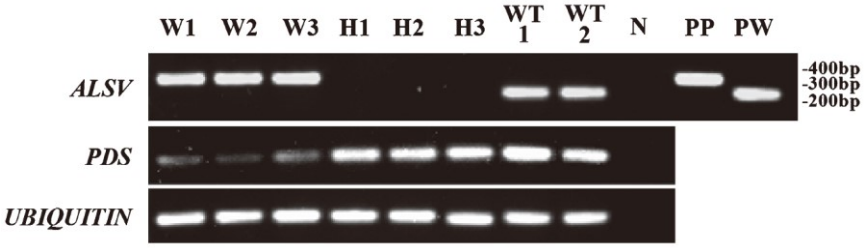

Fig. 1-5. Semi-quantitative RT-PCR analysis of ALSV RNA2, *PDS*, and *UBIQUITIN* genes in the leaves of apricot seedlings infected with ALSV vectors. The infection of wtALSV and ParPDS-ALSV was confirmed by PCR fragments corresponding to 251 and 353 bp, respectively. The *UBIQUITIN* gene was used as a reference. W1–3, silenced white leaves infected with ParPDS-ALSV; H1–3, healthy leaves with no viral inoculation; WT1–2, leaves infected with wtALSV; N, distilled water (negative control); PP, pBICAL2-ParPDS (positive control for ParPDS-ALSV); PW, pBICAL2 (positive control for wtALSV). Each number represents leaves from independent seedlings.

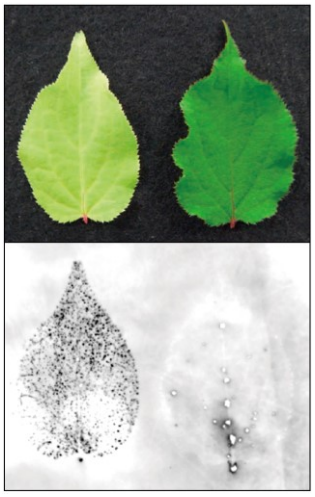

Fig. 1-6. Tissue blot analysis of a leaf from an apricot seedling infected with ALSV vectors. Discoloration of a leaf infected with ParPDS-ALSV (left) and a healthy leaf with no viral inoculation (right). Upper and lower pictures are of the same leaves.

We further investigated the amount of *PDS*-mRNA in the leaves of apricot seedlings infected with ParPDS-ALSV by semi-quantitative RT-PCR and real-time RT-PCR analysis to confirm that the discoloration of leaves was caused by silencing of the *PDS* gene. Semi-quantitative RT-PCR indicated that the amount of *PDS* transcripts in the photo-bleached leaves of apricot seedlings infected with ParPDS-ALSV was considerably decreased compared with the seedlings infected with wtALSV or healthy, uninoculated seedlings (Fig. 1-5). Real-time RT-PCR also indicated that the amount of *PDS*-mRNA was distinctly reduced in silenced white leaves (Fig. 1-7). These results collectively suggested that ALSV vectors could successfully induce VIGS of the *PDS* gene. ALSV-mediated VIGS should therefore be a useful tool for the evaluation of endogenous gene functions in apricot.

The results obtained in this study, however, raise several issues that need to be addressed before gene functions in apricot and other *Prunus* fruit tree species can be effectively assessed using ALSV-mediated VIGS. First, the infectivity of ALSV could vary with species or cultivar in *Prunus*. We need to select the right species or genotypes for ALSV-mediated gene functional analysis. Second, the efficiency of ALSV infection into apricot using particle bombardment was low compared with ALSV infection into apples and pears (Sasaki et al., 2011b; Yamagishi et al., 2010). Although this could be also related to genotype differences, we need to optimize infection methods and plant growth conditions after inoculation. Third, the stability of the virus or VIGS has yet to be determined fully. Finally and most importantly, we also need to develop an ALSV infection system for adult plant materials so that we can test gene functions in reproductive organs. Further studies are needed before we can fully utilize the VIGS-based gene evaluation system for *Prunus* using ALSV vectors.

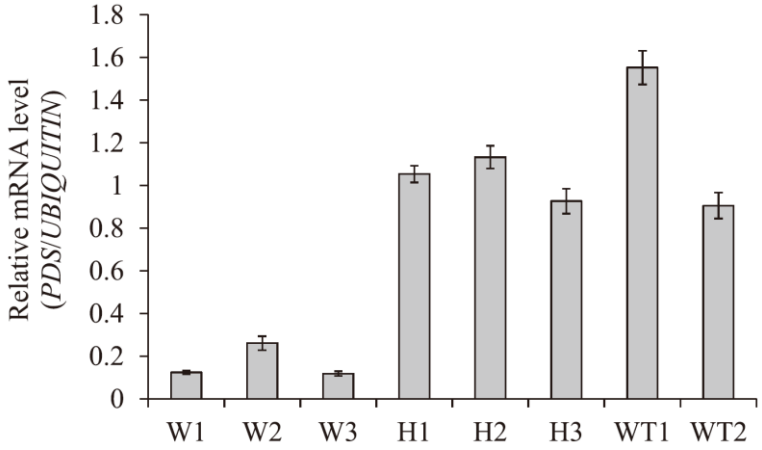

Fig. 1-7. Real-time RT-PCR analysis for mRNA levels of the *PDS* gene in the leaves of apricot seedlings infected with ALSV vectors. mRNA levels are shown as relative values against those of the *UBIQUITIN* gene. For the abbreviations used in each lane, see the legend of Figure 5. Vertical bars represent ± SD (n = 3).

1.4 Summary

In chapter 1, we investigated whether a VIGS-based gene evaluation system using ALSV vectors could be applied to two *Prunus* fruit tree species, apricot and Japanese apricot. The ALSV vector carrying a partial sequence of the *ParPDS* gene was amplified in *N. benthamiana*, and inoculated into the cotyledons of apricot and Japanese apricot seedlings by particle bombardment. Uniform discoloration of the upper leaves, a typical phenotype of *PDS* knock down, was observed several weeks after inoculation in apricot seedlings. The amount of *PDS*-mRNA was distinctly reduced in silenced white leaves, suggesting that ALSV vectors could successfully induce VIGS of the *PDS* gene. ALSV-mediated VIGS should therefore be utilized as a powerful tool for functional assessment of endogenous genes in apricot. On the other hand, our attempts to infect wild and recombinant ALSVs into Japanese apricot seedlings were unsuccessful, suggesting that ALSV susceptibility could vary depending on species and/or cultivar in *Prunus*. We discussed the possible use of this VIGS-based gene evaluation system in *Prunus*.

Chapter 2.

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

2.1. Introductiom

VIGS has been used as a rapid and effective tool for functional analysis of genes in various plants, including woody fruit tree species. In chapter 1, VIGS of the endogenous *PDS* gene was successfully induced in apricot using ALSV vectors, while infection of Japanese apricot with ALSV vectors was unsuccessful, suggesting that species- and/or cultivar-dependent differences of ALSV susceptibility may exist in *Prunus*. In chapter 2, the efficacy of VIGS-based gene evaluation system using ALSV vectors is tested with seven *Prunus* species, including apricot, Japanese apricot, sweet cherry, peach, European plum, Japanese plum, and almond. In addition, the amount and distribution of small RNA in the *Prunus* species infected with ALSV vectors is characterized.

2.2. Materials and Methods

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 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.

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 in chapter 1 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 *Agrobacterium* strain, EHA105, and inoculated into *N. benthamiana* as described below. The resultant recombinant virus was designated ParPDS-ALSV.

In general, *Prunus* species have a similar genetic background, and the nucleotide sequences of orthologous genes are highly conserved among different *Prunus* species. Indeed, the cDNA sequences of the *PDS* regions corresponding to the partial sequence of *ParPDS* cloned into pBICAL2-ParPDS were 98–100% identical among apricot 'Heiwa', sweet cherry 'Satonishiki', almond 'Nonpareil' and 'Carmel', peach 'Ohatsumomo', Japanese apricot 'Nanko', Japanese plum 'Sordum', and European plum 'Sanctus Hubertus' (data not shown; Fig. 1-1C in chapter 1). Thus, we were able to use the same constructs, pBICAL1 and pBICAL2-ParPDS, for VIGS of the *PDS* genes in all of the *Prunus* species and cultivars.

Viral amplification in N. benthamiana

pBICAL1/EHA105 and pBICAL2-ParPDS/EHA105 were simultaneously inoculated into young leaves of *N. benthamiana* using a toothpick as described in chapter 1. To confirm the successful infection and amplification of ParPDS-ALSV, microtissue direct RT-PCR was performed 2–3 wpi according to the procedure described in chapter 1.

Viral inoculation of Prunus seedlings

Total RNA containing ParPDS-ALSV was extracted from infected *N. benthamiana* using TRIzol reagent (Life Technologies), purified by phenol/chloroform extraction, and used for viral inoculation by particle bombardment essentially as described in chapter 1. The seed coats were gently removed from *Prunus* seedlings just after germination, and their cotyledons were bombarded with gold particles coated with total RNA at a pressure of 280–320 psi using a Helios Gene Gun system (BIO-RAD). Each side of the cotyledons was bombarded 3–4 times. After acclimatization in the dark at 4°C and 100% relative humidity for 2 days, the inoculated seedlings were planted in soil and grown in a growth chamber under 16/8 h LD conditions at 22°C.

RT-PCR analysis

Total RNA was extracted from the upper leaves of inoculated *Prunus* seedlings about 1 month post inoculation (mpi) by a small-scale CTAB method according to Sasaki et al. (2011b) with slight modifications. After cDNA synthesis, RT-PCR for detection of ALSV and real-time RT-PCR analysis of the mRNA levels of the *PDS* genes were carried out as described in chapter 1. As a reference, the *UBIQUITIN* gene-specific primer pair PavUbi-F and PavUbi-R (Yooyongwech et al., 2008; AF298826) was used for both analyses. For real-time RT-PCR analysis, we used two or three biological replicates of each cultivar, and three technical replications were performed. Only two biological replications were conducted for the apricot cultivars 'Shinyo' and 'Shinshuomi' because only two infected plants were obtained, while three biological replications were conducted for the other cultivars. Mean values of *PDS* mRNA levels were compared between infected and healthy plants using Student's *t*-test.

For the inoculated almond 'Nonpareil' and 'Carmel' seedlings, lower and upper leaves, stems, and roots were collected about 1.5–2 mpi, and used for RT-PCR and real-time RT-PCR analyses. We used one biological replicate for each cultivar, and the *PDS* mRNA levels of each sample were shown as means of three technical replicates. For inoculated peach seedlings, lower and upper leaves were collected about 2.5 mpi and used for RT-PCR and tissue blot analyses as described below.

Tissue blot analysis

The distribution of ALSV in the lower and upper leaves of inoculated peach seedlings was confirmed by tissue blot analysis as described previously (Yamagishi and Yoshikawa, 2009) using a DIG-labeled RNA probe complementary to ALSV RNA2.

Small RNA sequencing analysis

To investigate the population and distribution of small RNA associated with the ALSV RNAs and *PDS* mRNA in *Prunus* seedlings infected with ALSV vectors, small RNA sequencing analysis was performed essentially as described in Akagi et al. (2014) with slight modifications. Briefly, total RNA was extracted from infected leaves of apricot 'Shinyo', sweet cherry 'Satonishiki', almond 'Nonpareil', and peach 'Ohatsumomo', and healthy leaves of 'Nonpareil' and 'Ohatsumomo' by a small-scale CTAB method about 1 mpi. We used a single biological replicate for each cultivar. The fraction of <200 nt RNA was concentrated from total RNA using a mirVana miRNA Isolation kit (Life Technologies). About 200 ng of concentrated <200 nt RNA was used for small RNA library construction with a NEBNext Small RNA Library Prep Set (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol. PCR enrichment was performed using 15 PCR amplification cycles. The enriched libraries were purified firstly using AMPure XP (Beckman Coulter) with a 1.1:1 (v/v) AMPure: reaction volume to remove >ca 50 bp insertions. Next, the

supernatant was purified using AMPure XP with a 3:1 (v/v) AMPure: reaction volume to filter self-ligated adapter dimers. Library quality and quantity were assessed using an Agilent BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) and Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). The constructed libraries were sequenced on an Illumina HiSeq 2500 sequencer (50 bp single-end reads).

The sequenced reads were demultiplexed according to the index sequences, and then were trimmed for quality (phred score >20) and adapter sequence contamination with custom Python scripts developed in the Comai laboratory and available online (http://comailab.genomecenter.ucdavis.edu/index.php/Barcoded data preparation tools). Reads shorter than 19 bp and longer than 25 bp were discarded. The 19–25 bp reads obtained were mapped to the ALSV RNA1 (AB030940), ALSV RNA2 (AB030941), and *ParPDS* mRNA (AY822065) sequences. Alignment of the reads to these sequences was conducted using Burrows-Wheeler Aligner (BWA) with default parameters (Li and Durbin, 2009). The number of reads mapped to each sequence was obtained from the alignment file produced by the Sequence Alignment/Map (SAM) tool (Li et al., 2009), and expressed as reads per kilobase of sequence per million reads (RPKM). The coverage and distribution of reads matching the ALSV RNAs were visualized as the sum of the sense and antisense strand reads using Integrative Genomics Viewer (IGV) ver. 2.3 (Robinson et al., 2011), while the sense and antisense strand reads that matched the cDNA sequence of *ParPDS* were shown separately.

2.3. Results and Discussion

Infection of Prunus seedlings with ALSV vectors

ParPDS-ALSV was detected in the inoculated seedlings of apricot ‘Shinyo’ (11.1%), ‘Shingetsu’ (12.5%), and ‘Shinshuomi’ (9.5%), sweet cherry ‘Satonishiki’ (20%), almond ‘Nonpareil’ (30%) and ‘Carmel’ (29.4%), and peach ‘Ohatsumomo’ (85.7%) at about 1 mpi (Table 2-1). The infection rates of ParPDS-ALSV in apricot cultivars were consistent with that in apricot ‘Heiwa’ (11.1%) as reported in chapter 1. Apricot, sweet cherry, and almond seedlings infected with ALSV exhibited no obvious viral symptoms. This indicates that ALSV can be used effectively for gene functional evaluation in these species because viral symptoms often disturb the correct observation of knock-down phenotypes of VIGS-target genes. Although the infection rate of ParPDS-ALSV in ‘Ohatsumomo’ peach was highest among the inoculated *Prunus* species and cultivars, the infected seedlings showed severe viral symptoms (pale spots) in the upper leaves (Fig. 2-5A, B; see details later). We could not detect ParPDS-ALSV from the inoculated seedlings of apricot ‘Nanbuhachisuke’ or ‘Niigataomi’, almond ‘Marcona’, Japanese apricot ‘Ryukyokoume’, ‘Benisashi’, ‘Koshinoume’, or ‘Hachiro’, Japanese plum ‘Sordum’, or European plum ‘Sanctus Hubertus’ (Table 2-1). As no

ALSV infection was observed with 'Nanko' Japanese apricot in chapter 1, Japanese apricot may have resistance against ALSV. These results indicated that ALSV susceptibility could vary depending on species and cultivar in *Prunus*.

Note that the efficiencies of ALSV infection in *Prunus* were lower than in results obtained with apple, pear, Japanese pear, and soybean (Sasaki et al., 2011b; Yamagishi and Yoshikawa, 2009; Yamagishi et al., 2010). In these species, about 100% ALSV infection efficiencies were obtained using almost the same inoculation method. However, adjustment and optimization of each viral inoculation step may result in improvement of the ALSV infection efficiency in *Prunus* species and cultivars.

Table 2-1. Infection rates of ParPDS-ALSV in *Prunus* species one month post inoculation.

Plant species	Cultivars	Number of infected/inoculated seedlings	Infection rate (%)
Apricot (*P. armeniaca*)	'Shinyo'	2/18	11.1
	'Shingetsu'	3/24	12.5
	'Shinshuomi'	2/21	9.5
	'Nanbuhachisuke'	0/22	0
	'Niigataomi'	0/15	0
Sweet cherry (*P. avium*)	'Satonishiki'	6/30	20
Almond (*P. dulsis*)	'Nonpareil'	12/40	30
	'Carmel'	5/17	29.4
	'Marcona'	0/10	0
Peach (*P. persica*)	'Ohatsumomo'	12/14	85.7
Japanese apricot (*P. mume*)	'Ryukyokoume'	0/9	0
	'Benisashi'	0/19	0
	'Koshinoume'	0/17	0
	'Hachiro'	0/31	0
Japanese plum (*P. salicina*)	'Sordum'	0/20	0
European plum (*P. domestica*)	'Sanctus Hubertus'	0/27	0

VIGS of PDS genes in apricot, sweet cherry, and almond

In this study, we used the *PDS* gene as a target for VIGS because knock-down of this gene results in a rapid photo-bleached phenotype in plants (Bartley and Scolnik, 1995; Kumagai et al., 1995). Based on the high sequence homologies of *PDS* genes among *Prunus* species, we were able to target the *PDS* gene of each *Prunus* species for VIGS with the same ALSV vector, ParPDS-ALSV, containing a partial sequence of the apricot *PDS* gene.

In apricot, 2 of the 18 inoculated seedlings of 'Shinyo', 3 of the 24 inoculated seedlings of 'Shingetsu', and 2 of the 21 inoculated seedlings of 'Shinshuomi' were successfully infected with ParPDS-ALSV (Table 2-1). In contrast, ALSV was not detected from the 22 and 15 inoculated seedlings of 'Nanbuhachisuke' and 'Niigataomi', respectively. As mentioned above, whether ALSV can infect these two cultivars needs to be carefully determined because the ALSV infection rates in the other apricot cultivars tested were low (about 10%). Uniform discoloration of the upper leaves, a characteristic phenotype of *PDS* downregulation, was observed in all infected seedlings 3–5 wpi (5–14 leaf stage; Fig. 2-1A–C). Real-time RT-PCR indicated that the amounts of *PDS* mRNA in the

photo-bleached leaves were significantly decreased compared with those in healthy leaves with no viral inoculation (Fig. 2-1D). These results suggested that ALSV could successfully induce VIGS of *PDS* genes in these apricot cultivars. Most of the infected seedlings were slightly smaller and grew more slowly than seedlings with no viral inoculation. Some of them stopped growing within 3 months, probably because of insufficient photosynthesis resulting from *PDS* knock-down. Discoloration of the upper leaves remained in these plants, while new yellow or light green upper leaves were generated in the other infected seedlings as they grew further. In these plants, the VIGS efficiency seemed to decrease, probably because of a lower virus concentration or environmental factors. A similar "recovery phenotype" of leaf color was also reported in apricot 'Heiwa' and wild rose infected with ALSV vectors targeting *PDS* genes (Ito et al., 2012; see chapter 1).

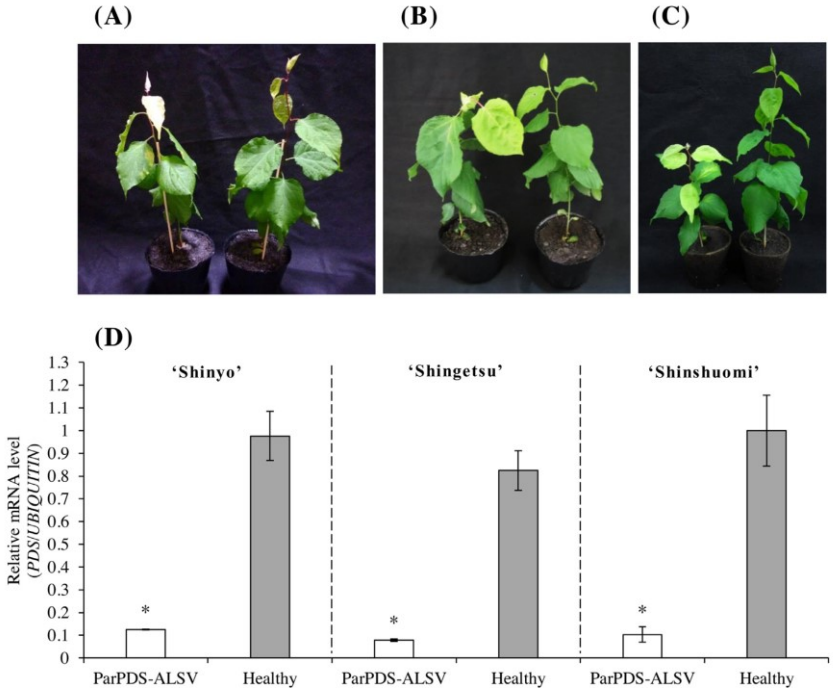

Fig. 2-1. VIGS of the *PDS* gene in apricot seedlings infected with ALSV vectors. (A) Seedlings of 'Shinyo', (B) 'Shingetsu', and (C) 'Shinshuomi' infected with ParPDS-ALSV (left) and healthy plants with no viral inoculation (right) (42, 41, 36 dpi, respectively). (D) Real-time RT-PCR analysis of *PDS* mRNA levels in the leaves of apricot seedlings infected with ALSV vectors. *PDS* mRNA levels are shown as mean values ± SE (n = 2–3; biological replicates) normalized against the *UBIQUITIN* gene. ParPDS-ALSV, photo-bleached leaves infected with ParPDS-ALSV; Healthy, healthy leaves with no viral inoculation. A single asterisk indicates significant difference ($P < 0.05$) using Student's *t*-test.

In sweet cherry, 6 of the 30 inoculated seedlings of 'Satonishiki' were successfully infected with ParPDS-ALSV (Table 2-1). The upper leaves of all infected seedlings exhibited uniform photo-bleached phenotypes 2–3 wpi (3–5 leaf stage; Fig. 2-2A). Real-time RT-PCR showed that the amounts of *PDS* transcript in the photo-bleached leaves were significantly reduced

compared with those in uninoculated leaves (Fig. 2-2B). These results indicated that VIGS of *PDS* genes was effectively induced by ALSV vectors in sweet cherry 'Satonishiki'. Although stunted growth was observed in the infected seedlings, almost all of them continued to grow for more than 3 months. Unlike apricot, the *PDS*-silenced phenotype persisted, and a "recovery phenotype" of leaf color was not observed in sweet cherry.

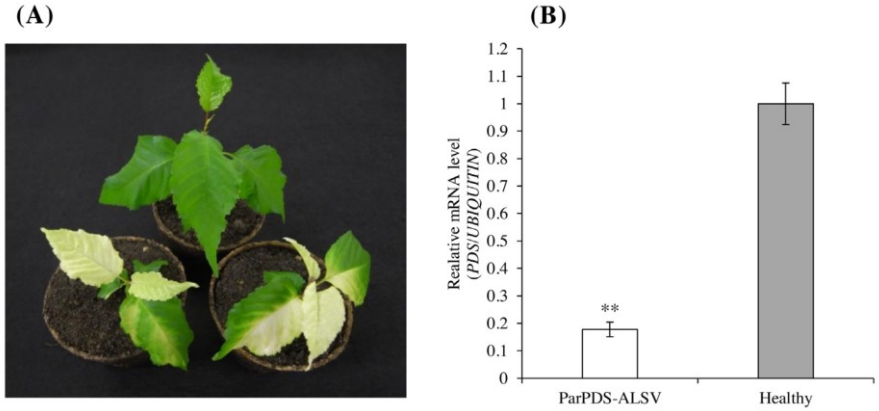

Fig. 2-2. VIGS of the *PDS* gene in sweet cherry seedlings infected with ALSV vectors. (A) Seedlings of 'Satonishiki' infected with ParPDS-ALSV (below) and a healthy plant with no viral inoculation (above) (36 dpi). (B) Real-time RT-PCR analysis of *PDS* mRNA levels in the leaves of sweet cherry seedlings infected with ALSV vectors. For details of real-time RT-PCR analysis, see the legend of Fig. 2-1. Double asterisks indicate significant difference ($P < 0.01$) using Student's *t*-test.

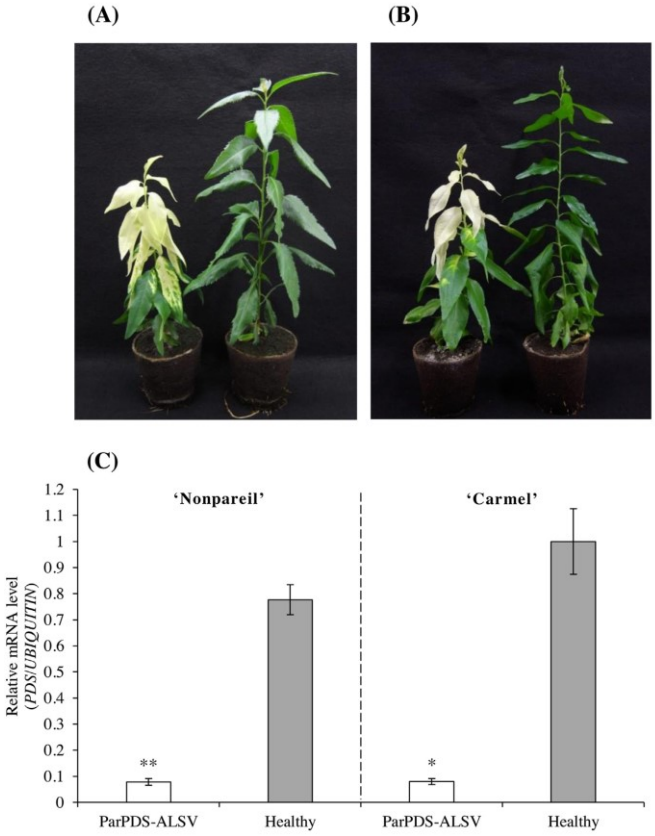

Fig. 2-3. VIGS of the *PDS* gene in almond seedlings infected with ALSV vectors. (A) Seedlings of 'Nonpareil' and (B) 'Carmel' infected with ParPDS-ALSV (left) and healthy plants with no viral inoculation (right) (44 and 43 dpi, respectively). (C) Real-time RT-PCR analysis of *PDS* mRNA levels in the leaves of almond seedlings infected with ALSV vectors. For details of real-time RT-PCR analysis, see the legend of Fig. 2-1. Single and double asterisks indicate significant difference ($P < 0.05$ and 0.01, respectively) using Student's *t*-test.

In almond, 12 of the 40 inoculated seedlings of ‘Nonpareil’ and 5 of the 17 inoculated seedlings of ‘Carmel’ were successfully infected with ParPDS-ALSV (Table 2-1). However, ALSV infection of 10 seedlings of ‘Marcona’ was unsuccessful. As a relatively stable infection efficiency (about 30%) was obtained with ‘Nonpareil’ and ‘Carmel’, ‘Marcona’ might be intrinsically resistant to ALSV infection. Uniform discoloration of newly generated leaves and stipules was observed in all infected almond seedlings 2–4 wpi (5–18 leaf stage; Fig. 2-3A, B). The *PDS* mRNA levels were distinctly decreased in the photo-bleached leaves compared with those in the control leaves (Fig. 2-3C). These results suggested that ALSV infection could trigger efficient knock-down of the *PDS* genes in these almond cultivars. Although almost all infected seedlings exhibited severely stunted growth, they continued growing for more than 3 months. However, as observed with apricot, some of the infected almond plants showed a “recovery phenotype” of leaf color later in their growth, suggesting that the VIGS efficiency might have also declined.

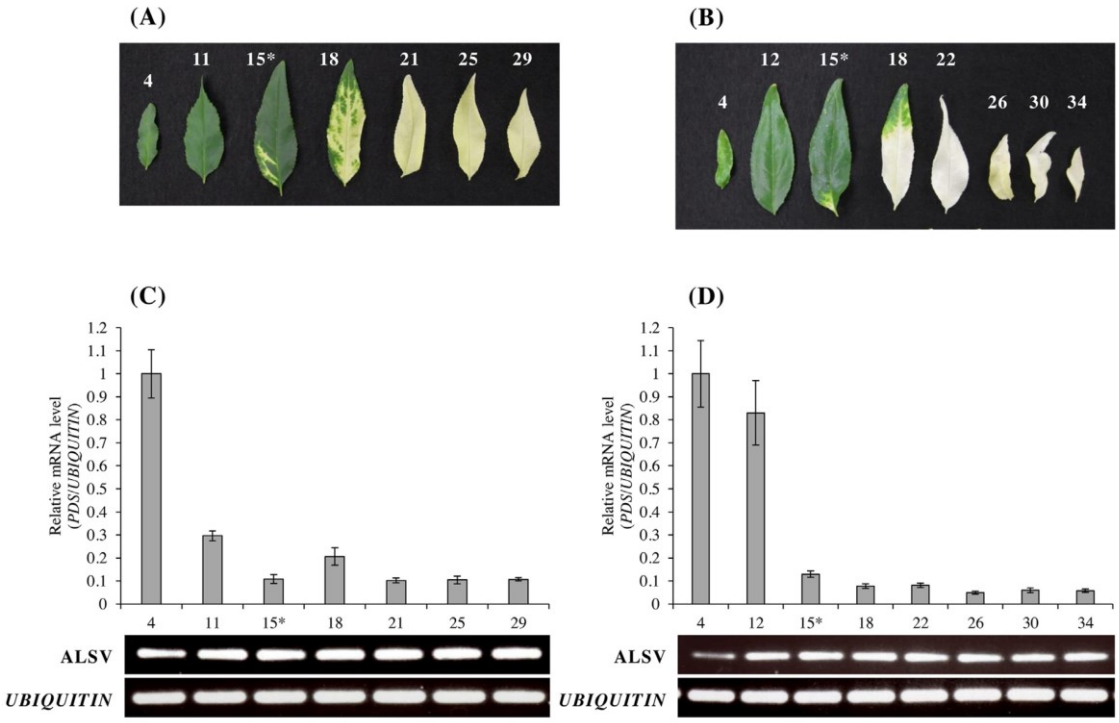

Fig. 2-4. Lower and upper leaves of (A) ‘Nonpareil’ and (B) ‘Carmel’ seedlings infected with ALSV vectors. Each number represents the leaf position in the seedling. Asterisks indicate the leaves in which discoloration was first observed. (C) Real-time RT-PCR analysis of *PDS* mRNA levels (above) and RT-PCR analysis of ALSV RNA2 (below) in lower and upper leaves of ‘Nonpareil’ and (D) ‘Carmel’ seedlings infected with ALSV vectors. The *UBIQUITIN* gene was used as a reference for both analyses, and *PDS* mRNA levels are shown as mean values ± SE (n = 3; technical replicates) normalized against the *UBIQUITIN* gene. Each number in (C) and (D) corresponds to the leaf samples shown in (A) and (B), respectively.

We further investigated the distribution of ParPDS-ALSV in the infected almond seedlings, including the root, stem, and lower and upper leaves. ParPDS-ALSV was detected from all organs investigated, indicating that the ALSV vectors spread systemically in the infected almond seedlings (Fig. 2-4A–D; data not shown). Interestingly, ParPDS-ALSV was detected not only from the photo-bleached upper leaves of 'Nonpareil' and 'Carmel' seedlings, but also from lower leaves with no visible leaf discoloration (Fig. 2-4A–D). Of these infected lower leaves, the amounts of *PDS* mRNA in 4th leaf of 'Nonpareil' and the 4th and 12th leaves of 'Carmel' were not reduced. This result might suggest that ALSV infection was limited to a small part of these leaves, and the virus concentration was insufficient to initiate VIGS of *PDS* genes. However, although the *PDS*-silenced phenotype was not observed, or was visible in only a limited area in the 11th and 15th leaves of 'Nonpareil' and the 15th leaf of 'Carmel', the *PDS* mRNA levels were reduced to the same levels as those observed in fully silenced upper leaves. This result suggests that ALSV amplification and subsequent degradation of *PDS* mRNA might have occurred after the accumulation of a sufficient amount of PDS protein to prevent chlorophyll photooxidation.

These results suggested that ALSV vectors could effectively induce VIGS of *PDS* genes in apricot, sweet cherry, and almond, if viral infection was successfully established. Systemic infection of ALSV was confirmed in almond seedlings, including the stem and root. Although the VIGS efficiencies of endogenous gene expression in these organs have yet to be investigated, it is possible that ALSV vectors can induce VIGS of endogenous genes in various organs.

This study highlighted several issues with regard to the stability of VIGS. These issues need to be addressed especially when we perform long-term evaluation of gene functions in fruit tree crops with long life cycles. The "recovery phenotype" observed in the infected seedlings of apricot and almond might be due to decreased VIGS efficiency, probably resulting from a lower virus concentration or environmental factors. In fact, it has been reported in herbaceous plants that the efficiency or stability of VIGS, or amounts of virus or virus-derived siRNAs, can vary depending on growth conditions such as temperature or humidity (Chellappan et al., 2005; Fu et al., 2006; Szittya et al., 2003; Tuttle et al., 2008; Wang et al., 2013). In the VIGS process, various factors, such as plant vigor, virus concentration, and the activities of the enzymes involved in PTGS, seem to coordinately determine the efficiency of gene silencing. To establish an efficient and stable VIGS system that can be used for functional evaluation of diverse target genes, we need to optimize growth conditions considering their integrative effects on VIGS efficiency in *Prunus* species.

Viral symptoms in infected peach seedlings

In peach, 12 of the 14 inoculated seedlings of 'Ohatsumomo' were infected with ParPDS-ALSV (Table 2-1). Although a high infection efficiency was obtained, leaves with severe pale spot symptoms were observed in infected peach seedlings about 2 wpi (Fig. 2-5A, B). Unlike

apricot, sweet cherry, and almond, neither discoloration of the upper leaves nor a significant reduction in the amount of *PDS* mRNA was observed in infected peach seedlings (Fig. 2-5A–D). This result suggested that ALSV vectors could not effectively induce VIGS of the *PDS* gene in peach 'Ohatsumomo'. About 50 days after bombardment, normal leaves formed on the branches from axillary buds, while the main branch stopped growing because of severe distortion of leaves and the shoot tip (Fig. 2-5C). We investigated whether ALSV was present in the newly generated upper leaves without symptoms by RT-PCR and tissue blot analyses. In both analyses, ALSV was detected only from lower leaves with symptoms, not from the upper leaves generated after the symptoms disappeared (Fig. 2-5E; data not shown). Strong ALSV signals were observed in the pale spots on the lower leaves by tissue blot analyses (Fig. 2-5E), suggesting that virus infection was limited to the area of viral symptoms. These results indicated that the application of ALSV-based VIGS for functional analysis of genes may be difficult in peach.

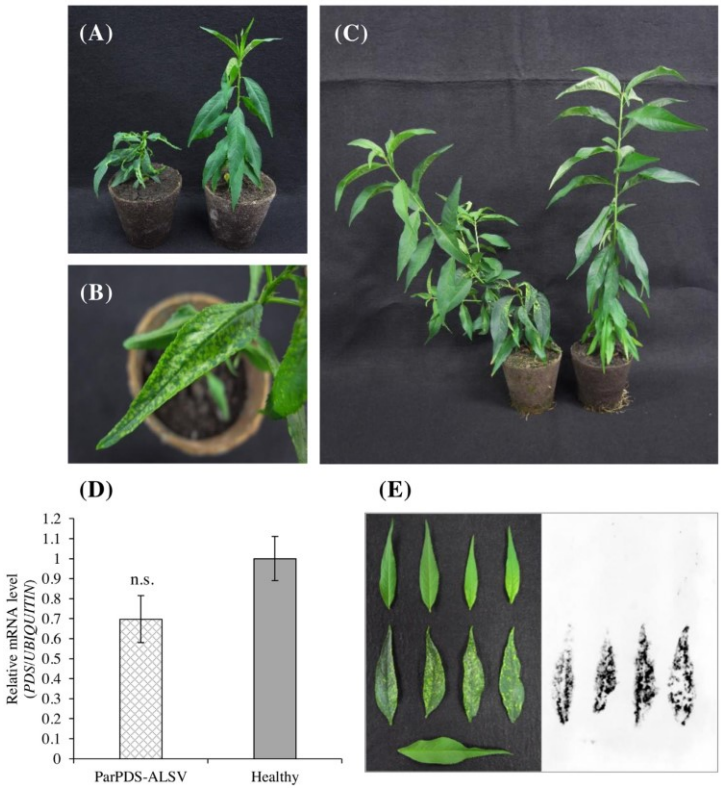

Fig. 2-5. Viral symptoms in peach seedlings infected with ALSV vectors. (A) A seedling of 'Ohatsumomo' infected with ParPDS-ALSV (left) and a healthy plant with no viral inoculation (right) at 30 dpi. (B) A leaf showing pale spot symptoms. (C) A peach seedling infected with ParPDS-ALSV (left) and a healthy plant (right) at 87 dpi. (D) Real-time RT-PCR analysis of *PDS* mRNA levels in the leaves of peach seedlings infected with ALSV vectors. For details of real-time RT-PCR analysis, see the legend of Fig. 2-1. ParPDS-ALSV, leaves showing symptoms after infection with ParPDS-ALSV; Healthy, healthy leaves with no viral inoculation. n.s. indicates non-significant ($P < 0.05$) using Student's *t*-test. (E) Tissue blot analysis of lower and upper leaves of 'Ohatsumomo' seedlings infected with ALSV vectors. Upper leaves with no symptoms (above), lower leaves with symptoms (middle), and a healthy leaf with no viral inoculation (below) are shown. The left and right pictures are of the same leaves.

It is still unclear whether other cultivars or genotypes of peach respond in the same way as ‘Ohatsumomo’. As peach is one of the most economically and experimentally important fruit trees in *Prunus*, it is worth studying ALSV infectivity and VIGS efficiency using various peach genotypes to assess whether a VIGS-based gene evaluation system can be used in peach.

Small RNA sequencing analysis in infected Prunus seedlings

In apricot, sweet cherry, and almond seedlings infected with ParPDS-ALSV, the entire population of small RNAs mapped to ALSV RNA1 and RNA2 was much larger than that in healthy almond and peach seedlings (Fig. 2-6A, B). Small RNA mapped to ALSV RNAs from uninfected seedlings, which were negative controls, was supposed to be generated from endogenous transcripts with a similar nucleotide sequence to the ALSV RNAs. In infected peach, the population of reads mapped to ALSV RNAs was also larger than that in uninfected plants, but obviously smaller than that in the other infected *Prunus* species (Fig. 2-6A, B). About 14–31 and 16–48 fold differences of RPKM for ALSV RNA1 and RNA2, respectively, were observed between peach and the other *Prunus* species. In a previous study on small RNA derived from virus vectors, Kishigami et al. (2014) performed deep sequencing analysis of siRNA (21–24 nt) to quantify ALSV in infected apple seedlings, and showed that the amount of siRNA originated from ALSV RNAs was consistent with that of viral RNA detected by real-time RT-PCR. Similarly, the small RNA population associated with ALSV RNAs in each *Prunus* species could be related to the amount of ALSV. In this case, the smaller read number observed in infected peach leaves might indicate lower accumulation of ParPDS-ALSV. Another possibility is that, although there was no significant difference in virus concentration among the infected *Prunus* species, the degree of viral RNA degradation and resultant small RNA production was different among them.

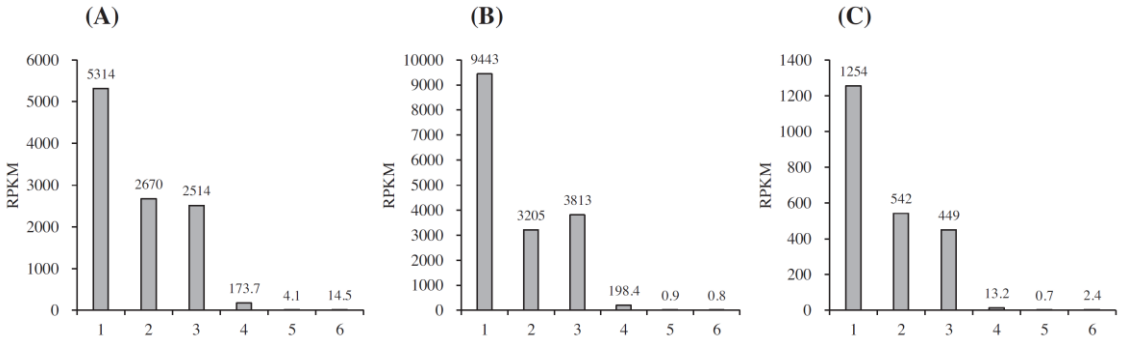

Fig. 2-6. Small RNA sequencing of the leaves of *Prunus* seedlings infected with ALSV vectors. The numbers of small RNA reads that match the sequences of (A) ALSV RNA1 (AB030940), (B) ALSV RNA2 (AB030941), and (C) the *ParPDS* mRNA (AY822065) are expressed as reads per kilobase of sequence per million reads (RPKM). Infected leaves of (1) apricot ‘Shinyo’; (2) sweet cherry ‘Satonishiki’; (3) almond ‘Nonpareil’; (4) peach ‘Ohatsumomo’; healthy leaves of (5) ‘Nonpareil’; (6) ‘Ohatsumomo’.

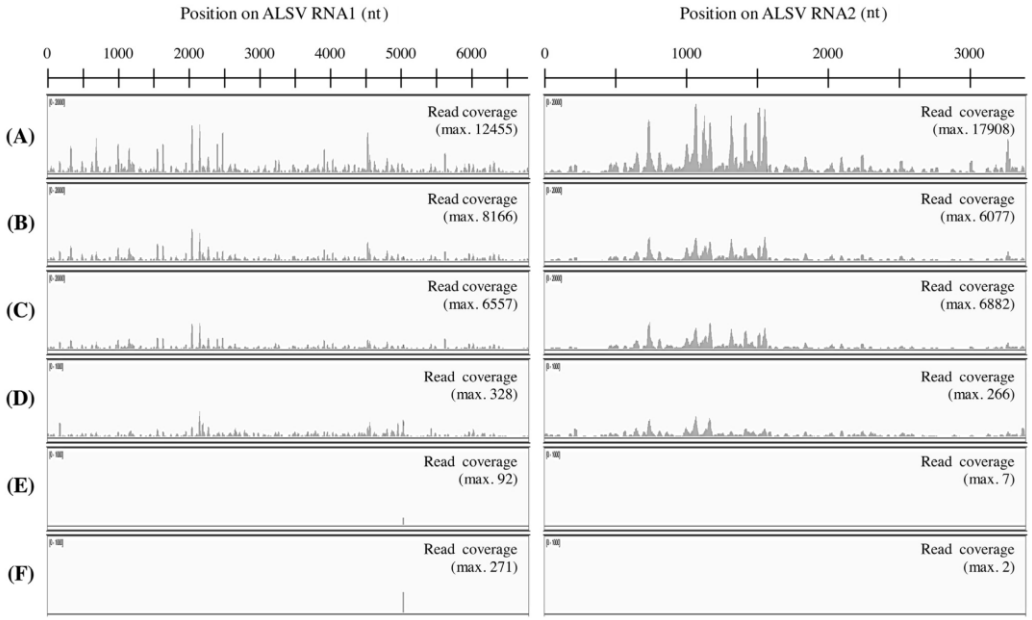

Fig. 2-7. Coverage and distribution of small RNA mapped to the ALSV RNAs. Small RNA reads derived from infected leaves of (A) apricot 'Shinyo', (B) sweet cherry 'Satonishiki', (C) almond 'Nonpareil', and (D) peach 'Ohatsumomo', and healthy leaves of (E) 'Nonpareil' and (F) 'Ohatsumomo' were mapped to the sequences of ALSV RNA1 (AB030940; left) and RNA2 (AB030941; right). The positions in ALSV RNA1 and RNA2 are shown on top. Read coverage is shown as the sum the sense and antisense strand reads. Note that the maximum data ranges of read coverage are set to 20,000 in (A)–(C), and 1000 in (D)–(F).

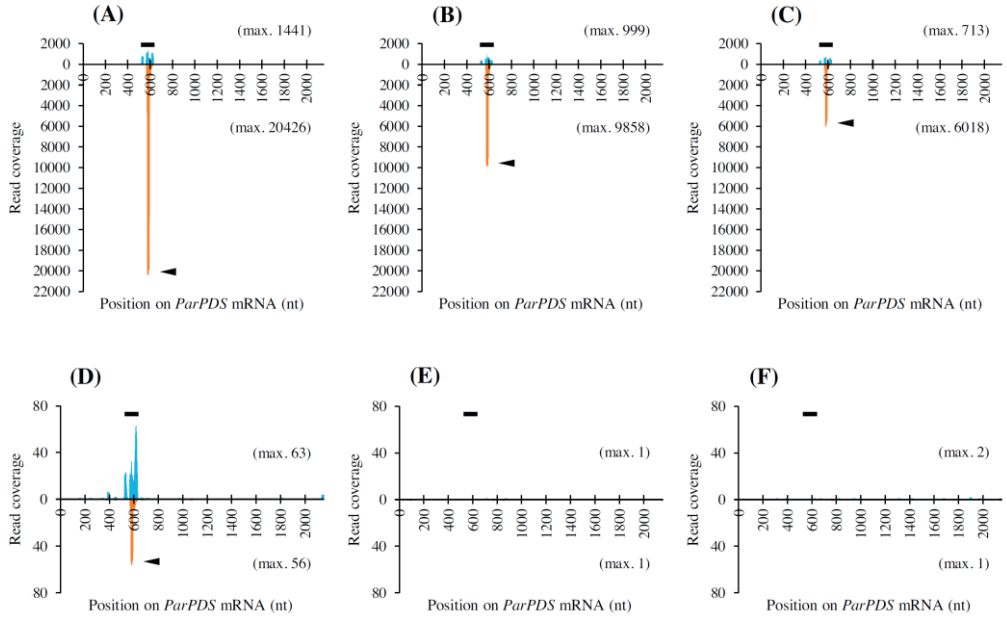

Fig. 2-8. Coverage and distribution of small RNA mapped to the *ParPDS* mRNA. Small RNA reads derived from infected leaves of (A) apricot 'Shinyo', (B) sweet cherry 'Satonishiki', (C) almond 'Nonpareil', and (D) peach 'Ohatsumomo', and healthy leaves of (E) 'Nonpareil' and (F) 'Ohatsumomo' were mapped to the *ParPDS* mRNA sequence (AY822065). Read coverages of the sense and antisense strands are shown separately above and below the *x*-axis, respectively. Note that the maximum data ranges of read coverage of (D)–(F) are lower than those of (A)–(C). The black bar represents the inserted partial sequence of the *ParPDS* gene (108 bp; 522–629 nt in the *ParPDS* mRNA). The arrowhead indicates the prominent coverage peak of antisense strand reads (21 nt; 573–593 nt in the *ParPDS* mRNA).

The small RNA in infected *Prunus* leaves was distributed entirely but unevenly across the ALSV RNA1 and RNA2 sequences, making some sharp peaks in the read coverage (Fig. 2-7). The positions of these coverage peaks were localized in the 5' regions of both ALSV RNA1 and RNA2, and consistent among *Prunus* species. A similar tendency in read coverage, to some extent, was also observed with apple seedlings infected with ALSV, in which abundant virus-derived siRNAs were associated with the regions near the 5' ends rather than the 3' ends of both ALSV RNA1 and RNA2 (Kishigamiet al., 2014). These results suggested that small RNA could be preferentially generated from ALSV genomes based on specific local nucleotide sequences or structural features, and that these sequence-based mechanisms of small RNA production were conserved in *Prunus*, and possibly, even among different plant species. In fact, such preferential generation of siRNA based on nucleotide sequences has been reported for RNAi or co-suppression in other plant and animal species (De Paoli et al., 2009; Ui-Tei et al., 2004).

The amount of small RNA mapped to the mRNA sequence of *ParPDS* showed a similar tendency to the small RNA that matched the ALSV RNAs in each sample; the RPKM values in *PDS*-silenced leaves of apricot, sweet cherry, and almond were much larger than those in healthy leaves of almond and peach, while the RPKM in infected peach leaves was considerably smaller than in other infected *Prunus* species (Fig. 2-6C). Interestingly, 21 nt antisense strand siRNAs (corresponding to 573–593 nt in the *ParPDS* mRNA sequence) were more abundant in the photo-bleached leaves of apricot, sweet cherry, and almond, making a single prominent peak in the read coverage within the inserted partial sequence of the *ParPDS* gene (corresponding to 522–629 nt in the *ParPDS* mRNA) (Fig. 2-8A–C). The amount of these antisense siRNAs in infected peach leaves, in which efficient VIGS of *PDS* gene was not observed, was much lower than in the other *Prunus* species (Fig. 2-8D). No prominent peak of small RNA was observed in healthy almond or peach seedlings (Fig. 8E, F). These results collectively suggested that these antisense siRNAs played a crucial role as guides in the cleavage of *PDS* mRNA.

Most of the small RNA, including the 21 nt antisense siRNAs, matched only a limited region of the inserted *ParPDS* fragment. The number of reads with hits outside this region of the inserted gene fragment was very low or negligible in all infected *Prunus* species (Fig. 2-8A–D). In some PTGS regulatory networks, dsRNA precursors can be newly generated by the recruitment of plant RNA-dependent RNA polymerase (RdRP) and small RNA produced by cleavage of primary transcripts. The newly synthesized dsRNA precursors are in turn processed into secondary siRNAs by the DCL enzyme. Some secondary siRNAs thus produced have the potential to induce degradation of the target mRNA, which leads to transitive and stable production of silencing signals (Axtell, 2013; Fei et al., 2013; Sijen et al., 2001; Voinnet, 2005). Such transitivity of small RNA has been also reported for VIGS of GFP transgenes in *N. benthamiana* and *A. thaliana*, in which secondary siRNAs produced by RDR6 were detected outside of the target region (Vaistij et al., 2002).

However, such amplification of silencing signals via secondary siRNAs did not appear to occur in this study, possibly because the endogenous *PDS* mRNA might be a poor template for RDR-dependent production of secondary siRNAs. It has been reported that PTGS of most plant endogenous genes by virus- or transgene-derived primary siRNAs does not trigger the production of secondary siRNAs, suggesting that the mRNA of endogenous genes is not a good template for RdRP (Aregger et al., 2012; Himber et al., 2003; Petersen and Albrechtsen, 2005; Vaistij et al., 2002). These results also indicate that the position and length of the inserted target sequence should be carefully selected when using ALSV vectors for functional evaluation of specific genes. In fact, Igarashi et al. (2009) showed that the position and length of the inserted partial sequence of the VIGS target gene greatly influenced the efficiency of VIGS. It should be noted, however, that the regulatory mechanisms of VIGS and siRNA production can vary with different kinds of host plants and target genes. The accumulation of data for various combinations of host plants/VIGS target genes in *Prunus* would enable us to design more efficient VIGS vectors for RNA silencing experiments.

2.4 Summary

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. In chapter 2, we investigated whether this VIGS-based gene evaluation system using ALSV vectors was applicable to seven *Prunus* species, including apricot, sweet cherry, almond, peach, Japanese apricot, Japanese plum, and European plum. ALSV vectors carrying part of the apricot *PDS* gene sequence were amplified in *N. 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.

Conclusion

ALSV-mediated VIGS could be a useful reverse genetics tool for the functional study of genes associated with agriculturally important traits in *Prunus*. The present study demonstrated that ALSV vectors could effectively induce VIGS of endogenous *PDS* genes in several cultivars of apricot, sweet cherry, and almond, although ALSV infectivity and VIGS efficiency seemed to vary depending on species and/or cultivar in *Prunus*. This study also showed the essential role of antisense siRNAs in inducing VIGS. Further optimization of viral inoculation procedures and growth conditions for infected plants will lead to the full use of ALSV vectors for evaluation of gene functions in various *Prunus* species. Nonetheless, the ALSV-mediated VIGS system reported here has the potential to enable high-throughput functional genomics that can facilitate the application of molecular biological and genetic information in *Prunus* production and breeding programs.

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