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Virus-Induced Gene Silencing of Endogenous Genes and Promotion of Flowering in Soybean by *Apple latent spherical virus*-Based Vectors

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1. Introduction

Soybean, which has been traditional food in Asian countries, is one of the most important crops due to its high quality protein and oil. Various functional components derived from secondary metabolite also have received significant attention in terms of human health. Elucidation of the gene function associated with the biosynthesis of various seed components at molecular level will provide valuable information for improvement of soybean. A great deal of information at the molecular level of soybean, including expression sequence tag (EST) libraries, microarray data, genome sequences, genetic linkage map, comparative genomics, and DNA markers has been reported (Alkharouf et al., 2004; Cheng et al., 2008; Choi et al., 2007; Grant et al., 2010; Haerizadeh et al., 2009; Hecht et al., 2005; Hisano et al., 2007; Nelson and shoemaker., 2006; Schmutz et al., 2010; Shoemaker et al., 2004; Wong et al., 2009; Zhu et al., 2005). This information can provide the candidate gene sequences controlling important traits of soybean, and the functional gene analysis tool for soybean is very important to identify the genes associated with important traits, soybean management, and efficient genetic improvements of soybean.

Agrobacterium- and biolistic-mediated technologies have been developed for the introduction of foreign genes into plants (Klein et al., 1987; Zambryski et al., 1983). As another approach, a virus vector-mediated gene delivery system has been developed for several plants. Plant virus vectors could be used for both the expression of the foreign genes and the suppression of the target genes by virus-induced gene silencing (VIGS) in infected plants (Gleba et al., 2004; Purkayastha and Dasgupta., 2009). A virus-mediated gene delivery system is quick and does not require transformation and regeneration techniques, and is widely used for functional gene analysis in plants. Especially, it is an attractive tool for major crop plants including soybean, in which efficient transformation is not established for various cultivars.

In soybean, five virus vectors were constructed from *Clover yellow vein virus* (ClYVV), *Soybean mosaic virus* (SMV), *Bean pod mottle virus* (BPMV), *Cucumber mosaic virus* (CMV), and *Apple latent spherical virus* (ALSV) (Igarashi et al., 2009; Masuta et al., 2000; Nagamatsu et al., 2007; Wang et al., 2006; Zhang and Ghabiral., 2006). ClYVV and SMV have been developed for foreign gene expression by a fusion polyprotein expression strategy, and BPMV- and ALSV-based vectors have been developed for both the expression of foreign genes and VIGS

of endogenous genes. CMV-based vector has also been used for the induction of VIGS of endogenous genes in soybean.
In this chapter, we describe the use of ALSV vector for VIGS of endogenous genes at all growth stages of soybean plants and seeds. We also report the promotion of flowering in soybean, irrespective of their stem-termination type, maturity group, and production area.

2. *Apple latent spherical virus* (ALSV) and ALSV-based vector

ALSV, classified into a genus Cheravirus (Le Gall et al. 2007), has isometric virus particles ca. 25 nm in diameter, and contains two ssRNA species (RNA1 and RNA2) and three capsid proteins (Vp25, Vp20, and Vp24) (Koganezawa et al. 1985; Le Gall et al. 2007; Li et al. 2000) (Fig.1a and b). The virus was originally isolated from an apple tree in Japan (Koganezawa et al. 1985), and has been shown to infect apple latently (Ito and Yoshida 1997). ALSV-RNA 1 is 6813 nt in length excluding the 3' poly(A) tail and has a single open reading frame (ORF) encoding a replication-associated protein of 243 K (Li et al. 2000). RNA 2 is 3385 nt in length excluding the 3' poly (A) tail, and also has a single ORF encoding a 42 K movement protein (MP) on the N-terminal side and three capsid proteins in the C-terminal region (Li et al. 2000; Yoshikawa et al. 2006). The genes of both RNA1 and RNA2 are expressed as single polyprotein precursors, and matured viral gene products are yielded by proteolysis of the polyproteins. The MP and three capsid proteins are all indispensable for the cell-to-cell movement of ALSV (Yoshikawa e al., 2006). Vp20, one of the three capsid proteins of ALSV, is a silencing suppressor which interferes with systemic silencing (Yaegashi et al. 2007). Horizontal transmission of ALSV does not occur easily in the field because the natural spread of ALSV from infected trees to neighboring trees has not been observed in the apple orchard since the virus was first detected in 1984 (Nakamura et al., 2010). On the other hand, vertical transmission of ALSV could occur via seed in apple, although the transmission rate is low (Nakamura et al., 2010).
ALSV-based vector was constructed from the infectious cDNA clone of RNA2 (pEALSR2) by duplication of protease cleavage site between the C terminus of MP and the N terminus of Vp25 (Li et al., 2004) (Fig.1c). The resulting vector (pEALSR2L5R5) could be used for the expression of foreign genes and for a reliable and effective VIGS among a broad range of plants by co-inoculation with infectious cDNA clone of RNA1 (pEALSR1) (Fig.1c) (Igarashi et al., 2009; Li et al., 2004; Yaegashi et al., 2007; Yamagishi & Yoshikawa, 2009).

3. Efficient inoculation of ALSV vectors to soybean

Efficient inoculation of virus vector to plant is one of the important steps for successful use of virus vector. In CIYVV-, SMV-, and BPMV-based vectors, rub-inoculation of DNA clones has been successfully conducted in soybean (Masuta et al., 2000; Seo et al., 2009; Zhang et al., 2010). Unfortunately, direct inoculations of an infectious cDNA clones of ALSV by mechanical or particle bombardment were less efficient in soybean plants. However, infection efficiency of ALSV-based vector has been improved using the biolistic inoculation of total RNAs from ALSV-infected *Chenopodium quinoa* leaves. By this method, almost 100% of the infection can be achieved consistently in soybean plants (Yamagishi & Yoshikawa, 2009).
One of the requirements for plant virus vectors is that the virus does not induce symptoms on plants. In BPMV-based vector, mild strains have been used for vector construction (Zhang et al., 2010). When wild-type (wt)ALSV was inoculated into two cotyledons at the

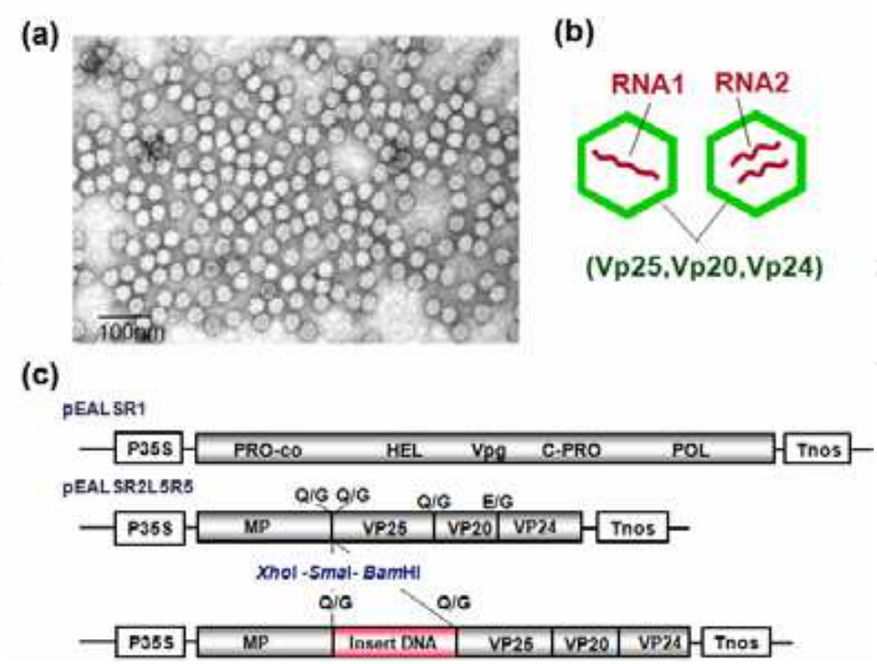

Fig. 1. Electron microscopic image (a), diagrammatic representation of ALSV particles (b), and schematic representation of the infectious cDNA clones of RNA1 (pEALSR1) and RNA2–based vector (pEALSR2L5R5) which were constructed by creating artificial protease processing site by duplicating the Q/ G protease cleavage site between MP and Vp25 (c). P35S, enhanced CaMV 35S promoter; Tnos, nopaline synthase terminater; PRO-co, protease cofactor; HEL, NTP-binding helicase; C-PRO, cysteine protease, POL, RNA polymerase; MP, 42K movement protein; Vp25, Vp20, and Vp24, capsid proteins.

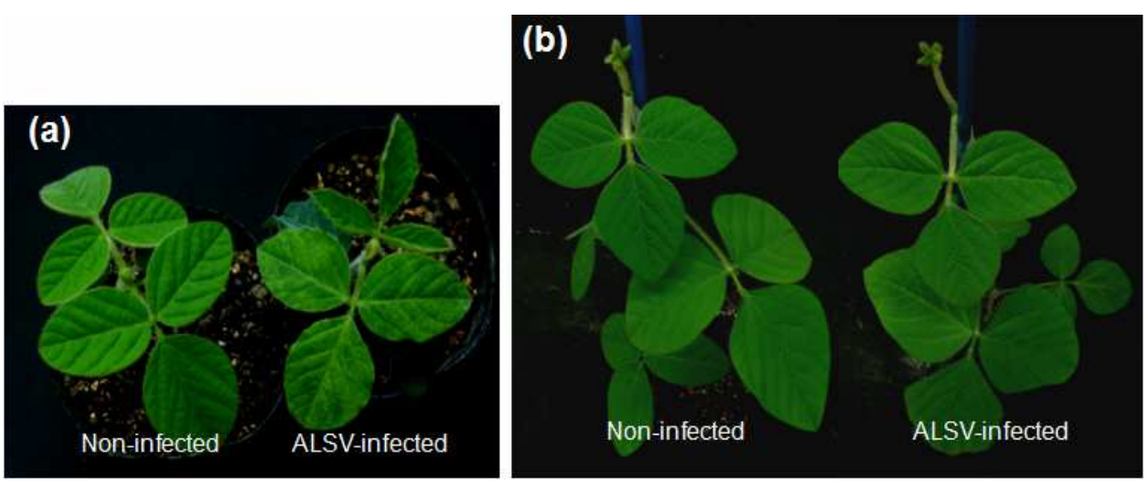

Fig. 2. Modulating symptoms of ALSV-infected soybean during development (cv. Jack). (a) Right, first and second trifoliolate leaves with mosaic symptom in soybean infected with ALSV at three nodes stage. Left, a non-infected soybean. (b) Right, symptomless third and forth trifoliolate leaves at the later growth stage of infection shown in (a). Left is a non-infected soybean seedling shown in (a).

emergence stage of soybean, mosaic symptom appeared on unifoliate, first, and second trifoliate leaves (Fig. 2a). However, mosaic symptom is no longer observed above the third trifoliate leaves (Fig. 2b), though the virus is systemically infected in all upper leaves (Yamagishi & Yoshikawa, 2009).

We also confirmed that ALSV could infect systemically all eight soybean cultivars ('Chamame', 'Enrei', 'Suzukari', 'Hatayutaka', 'Nemasirazu', 'Tanbaguro', 'Dewamusume', and 'Jack') with different stem-termination type, maturity group, and production area. The infection of ALSV vectors does not affect the vegetative and reproductive growth of these soybean cultivars even cvs. 'Tanbaguro' and 'Jack' which are susceptible to all Japanese SMV strains, and cv. 'Dewamusume' which is resistant to all Japanese SMV strains and CMV.

4. VIGS in a vegetative stage of soybean plants

Phytoene desaturase (PDS) is an enzyme required for biosynthesis of carotenoid which protects chlorophyll from photo-bleaching in plants. When cotyledons of soybean at the emergence stage were inoculated with soyPDS-ALSV containing a 300 bp fragment of the soybean *PDS* (*soyPDS*) (Igarashi et al., 2009; Yamagishi & Yoshikawa, 2009), systemic infection was established 1–2 weeks post inoculation (wpi), and all soybean plants infected with soyPDS-ALSV showed an uniform photo-bleached phenotype typical of PDS inhibition on upper, uninoculated leaves, petioles and stems from about 3 wpi (Fig. 3a and Fig.3c

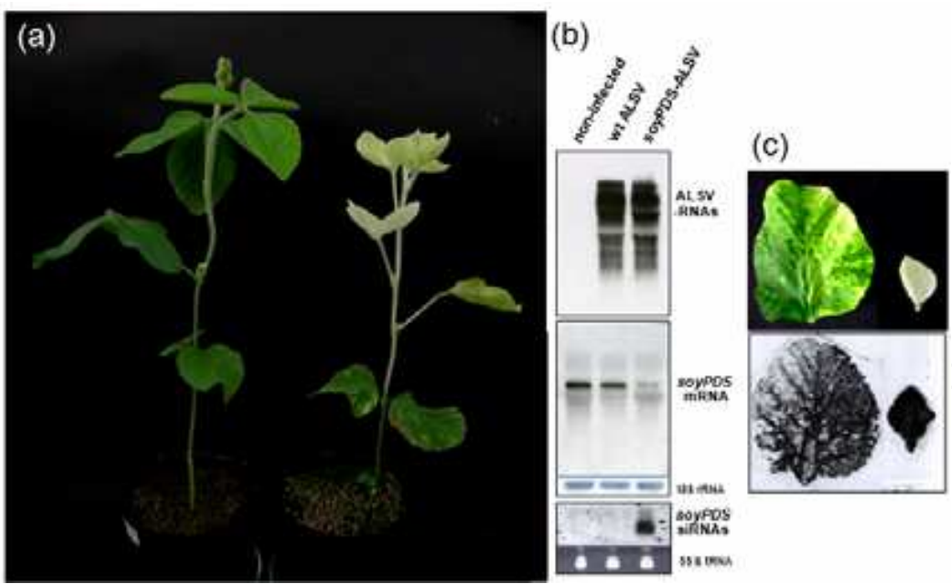

Fig. 3. Phenotype and the accumulation pattern of ALSV RNAs in the leaves of soybean plants infected with *soyPDS*-ALSV. (a) Right, photo-bleaching of a soybean plant (cv.'Jack') infected with *soyPDS*-ALSV 3 weeks post inoculation. Left, non-infected plant. (b) Northern blot hybridization analysis of soybean plants infected with soyPDS-ALSV. Detection of ALSV RNAs (upper panel), *soyPDS*-mRNA (middle panel), and *soyPDS*-siRNAs (lower panel) in a non-infected, wtALSV infected, and soyPDS-ALSV infected soybean plants. (c) Detection of the ALSV RNA distribution pattern in the soybean leaves (cv. 'Tanbaguro').

Left, partly photobleached unifoliolate leaf; right, uniformly photobleached third trifoliolate leaf. ALSV signal is strongly detected from the region showing photobleaching. (b) is reprinted with kind permission from Springer Science+Business Media: <Plant Molecular Biology, Virus-induced gene silencing in soybean seeds and the emergence stage of soybean plants with *Apple latent spherical virus* vectors., vol. 71, 2009, page 20, Yamagishi, N. & Yoshikawa, N., figure 3, and any original (first) copyright notice displayed with material>.

upper panel). Northern blot analysis showed that *soyPDS* mRNA clearly decreased in the photo-bleached leaves infected with soyPDS-ALSV, in contrast with wtALSV-infected leaves which was comparable to non-infected leaves (Fig. 3b middle panel). *SoyPDS* specific siRNAs, a hallmark of RNA silencing (Hamilton and Baulcombe 1999), also accumulated in the photo-bleached leaves infected with soyPDS-ALSV (Fig. 3b lower panel), indicating that the photo-bleaching of soybean leaves infected with soyPDS-ALSV was due to VIGS of *soyPDS*. Tissue-blot hybridization showed that soyPDS-ALSV was detected from the photo-bleached area in infected leaves, indicating that RNA silencing of *soyPDS* mRNA was induced in the tissues infected with soyPDS-ALSV (Fig. 3c).

One of the advantages of ALSV vector for VIGS in plants is that virus was distributed uniformly throughout infected plants and induced a highly uniform RNA silencing phenotype as shown in Fig. 3a. This is due to the fact that ALSV can replicate in the shoot apical meristem and leaf primordia (Fig. 4) and induce VIGS as soon as target gene expresses occurs in newly developed vegetative tissues. In contrast, SMV could not invade into the shoot apical meristem and leaf primordia (Fig. 4).

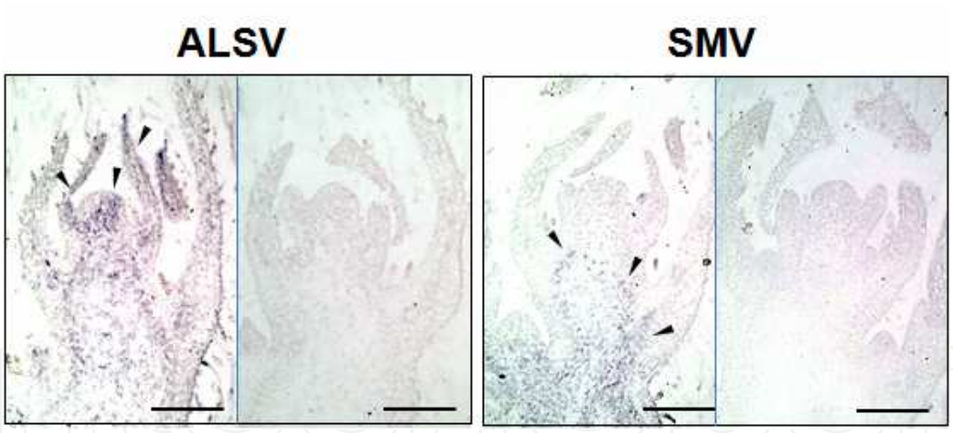

Fig. 4. *In situ* hybridization analysis of the distribution of ALSV-RNAs and SMV-RNAs in shoot apices of soybean seedlings (cv. 'Tanbaguro'). Left, infected; Right, non-infected. ALSV-RNAs could be detected from all tissues including the meristematic tissue and leaf primordia (arrowheads). In contrast, SMV-RNAs were detected in main stem tissues downward of stem apex (arrowheads), but not in the meristematic tissue and leaf primordia. Purple staining indicates the presence of viral RNAs. Scale bars: 200 μm. *In situ* hybridization analysis of the distribution of ALSV-RNAs is reprinted with kind permission from Springer Science+Business Media: <Planta, Expression of *FLOWERING LOCUS T* from *Arabidopsis thaliana* induces precocious flowering in soybean irrespective of maturity group and stem growth habit., doi: 10.1007/ s00425-010-1318-3, 2011, Yamagishi, N. & Yoshikawa, N., figure 2, and any original (first) copyright notice displayed with material>.

5. VIGS in a reproductive stage of soybean plants infected with soyPDS-ALSV and soyIFS2-ALSV

The VIGS by ALSV vector could be maintained in reproductive growth of soybean, and ALSV could infect about 20-30% of the embryos of seeds on ALSV-infected soybean plants (Yamagishi & Yoshikawa, 2009). Fig. 5a showed the full pod stage of soyPDS-ALSV infected soybean cv. 'Enrei', on which photo-bleached full pods were set. All pods and seed coats on soybean plants infected with soyPDS-ALSV showed a highly uniform photo-bleached phenotype similar to leaves of infected plants (Fig. 5b). On the other hand, some embryos showed photo-bleached phenotype and others normal green phenotype (Fig. 5b). Northern blot analysis of infected plants indicated that soyPDS-ALSV was detected in all pods, seed coats, and embryos showing white color phenotype, but not in green embryos (Fig. 5c). *SoyPDS* siRNAs were also detected in white embryos, but not in green embryos (Fig. 5d), confirming that soyPDS-ALSV could induce VIGS of soyPDS gene in the embryos of seeds on infected plants.

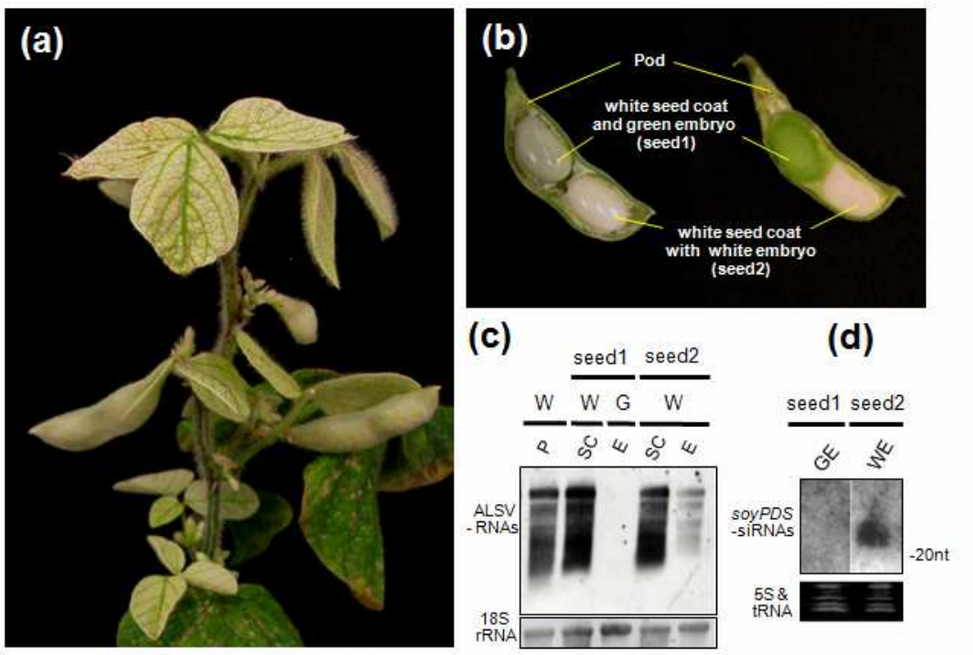

Fig. 5. VIGS in soybean plants at reproductive stage by soyPDS-ALSV. (a) VIGS of *soyPDS* gene in a soybean plant (cv. Enrei) infected with soyPDS-ALSV at the full pod stage. (b) The seeds in a soybean plant infected with soyPDS-ALSV. Left, colors of both pod and seed coats are white; Right, Longitudinal sections of a pod and seeds. An embryo of one seed shows white color and that of another seed shows green color. (c) Detection of ALSV-RNAs from pods (P), seed coats (SC), and embryos (E) from a soyPDS-ALSV infected soybean plants (cv. Enrei) shown in (b) as seed 1 and seed 2. W and G indicate white and green color, respectively. (d) Detection of *soyPDS* -siRNAs in the green embryo (GE) and white embryo (WE) shown in (b) as seed 1 and seed 2. Reprinted with kind permission from Springer Science+Business Media: <Plant Molecular Biology, Virus-induced gene silencing in soybean seeds and the emergence stage of soybean plants with *Apple latent spherical virus* vectors., vol. 71, 2009, pages 19 and 20, Yamagishi, N. & Yoshikawa, N., figures 2 and 3, and any original (first) copyright notice displayed with material>.

Isoflavone in soybean seeds is one of the functional components which plays diverse roles in plant-microbe interactions (Dixon and Sumner 2003; Subramanian et al. 2005), and it functions in many ways for human health (Chiechi and Micheli. 2005; Cornwell et al. 2004). Isoflavone synthase (IFS) encoded by two genes, *IFS-1* and *IFS-2*, which share 93% nucleotide identities of the coding region, is the key enzyme in the formation of isoflavones in soybean (Subramanian et al. 2005). Inoculation of soyIFS2-ALSV containing 237 bp fragment of the soybean *IFS-2* to soybean resulted in the embryo of about 30% of mature seeds being harvested from soybean plants (cv. Enrei) which were infected with soyIFS2-ALSV (Yamagishi & Yoshikawa, 2009). RT-PCR analysis showed that the transcripts of *soyIFS-2* and *soyIFS-1* decreased in the cotyledons of seeds infected with soyIFS2-ALSV. *SoyIFS-2*-siRNAs were detected in the cotyledons of seeds infected with soyIFS2-ALSV.The isoflavone contents in the cotyledons of mature seeds infected with soyIFS2-ALSV were lower than those in non-infected cotyledons.

VIGS of the *soyIFS-1* was less efficient than that of the soyIFS-2 gene by soyIFS2-ALSV infection (Yamagishi & Yoshikawa, 2009). Although soyIFS-1 and soyIFS-2 share 92% sequence identity over the 237 nucleotide stretch in soyIFS2-ALSV, the insert has only three identical sequence stretches of more than 23 nucleotides, which is the minimum required for VIGS (Lacomme et al. 2003; Thomas et al. 2001). The sequence identity and the identical sequences stretch may be important for clear VIGS of endogenous gene in soybean by ALSV vectors (Yamagishi & Yoshikawa, 2009).

6. VIGS in an early-growth stage of soybean plants infected with soyPDS-ALSV through seed transmission

Growth in the early developmental stages could affect the later growth and final yield in soybean. The VIGS system in early growth stages in soybean will be a valuable tool for functional gene analysis, management, and efficient breeding of soybean. However, it is not easy to establish the systemic infection of virus in very young seedlings like this case just after germination. When the mature seeds from soybean cv.'Enrei' infected with soyPDS-ALSV were sown on the soil, about 30% of seedlings showed a highly uniform photo-bleached phenotype at the emergence stage (Yamagishi & Yoshikawa, 2009). The photobleaching was maintained in their cotyledons, hypocotyls, and unifoliate leaves after emergence as shown in Fig. 6a. Both soyPDS-ALSV and *soyPDS*-siRNAs were detected from the photo-bleached seedlings, but not from the green seedlings (Fig. 6b and c). The other seedlings (about 70%) from infected soybean cv.'Enrei' grew as normal green plants and were not infected with soyPDS-ALSV.

7. The promotion of flowering in soybean plants by ALSV vector

Soybean, which originated in China, is a short days plant and has been adapted to various climates and environments during cultivation. It has resulted in many variations in flowering traits, for example, different maturity groups by differences of photoperiod sensitivity (Fukui and Arai, 1951; Fukui 1963; Shanmugasundaram, 1981), two types of stem growth habit, one is determinate which loses vegetative activity on the stem apex when reproductive growth starts, the other is indeterminate which can maintain vegetative activity on the stem apex through reproductive growth (Bernard, 1972; Tian et al., 2010), and the long juvenile period characteristic under short-day condition (Neumaier and James,1993;

Carpentieri-Pípolo et al., 2002). The variation of requirements in flowering might have been an obstacle to efficient soybean breeding. For example, soybean varieties adapted to low latitudes are difficult to induce flowering in a high latitude climate despite the importance of genetic resources.

The flowering integrator gene *FLOWERING LOCUS T (FT)* in *Arabidopsis thaliana* is conserved between diverse plant species, and FT protein is thought to be the flowering signal ''florigen'', a universal long-distance mobile signal. FT protein interacts with the bZIP transcription factor, FD, and activates floral identity genes in the meristematic tissue of the shoot apex (Abe et al., 2005; Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Wigge et al., 2005). In soybean, Kong et al. Identified ten *FT* homologs. Ectopic expression in *A. thaliana* confirmed that two *FT* homologs, *GmFT2a* and *GmFT5a*, had the same function as *A. thaliana FT* (Kong et al., 2010). On the other hand, the expression of FT gene from *A. thaliana* promoted precocious flowering in heterologous plant species, such as cucurbits and *Solanaceae* (Lin et al., 2007; Li et al., 2009; Yamagishi et al., 2011).

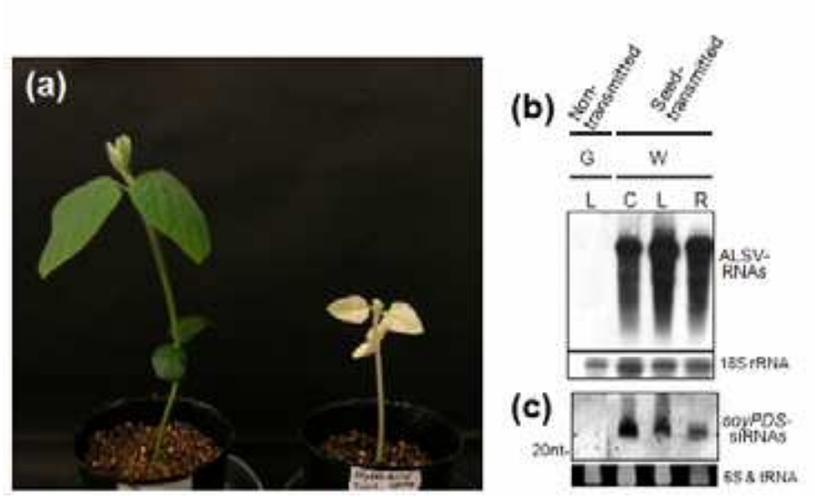

Fig. 6. VIGS of soyPDS gene in the next generation plant (cv. Enrei) from a plant infected with soyPDS-ALSV. (a) The next generation soybean seedlings. Left, a non-transmitted green seedling; Right, a *soyPDS*-silenced white seedling by seed transmission of soyPDS-ALSV. Both seedlings were germinated from seeds produced on a same soybean plant showing photobleaching. (b) and (c) Detection of ALSV-RNAs (b) and *soyPDS*-siRNAs (c) from cotyledon(C), leaf (L), and root (R) from a non-transmitted (green) and a soyPDS-ALSV seed transmitted (white) seedlings shown in (a). G and W indicate green and white color. (b) and (c) are reprinted with kind permission from Springer Science+Business Media: <Plant Molecular Biology, Virus-induced gene silencing in soybean seeds and the emergence stage of soybean plants with *Apple latent spherical virus* vectors., vol. 71, 2009, page 20, Yamagishi, N. & Yoshikawa, N., figure 3, and any original (first) copyright notice displayed with material>.

When FT-ALSV containing full-length sequence of *FT* was inoculated into the cotyledons of soybean 2-3 days after germination, soybean seedlings showed precocious flowering irrespective of the maturity groups in soybean (Yamagishi & Yoshikawa, 2011). For example, in Saitama Prefecture in Japan, it takes about 70 days and 90 days to blooming from germination in cvs. 'Suzukari' and 'Tanbaguro', respectively, (Fukui 1962). Both cultivars produced the flower buds on the main stem apex at 7 node stages at about 40dpi when FT-

ALSV was inoculated to the cotyledons 2-3 days after germination. Furthermore, indeterminate cultivars 'Dewamusume' and 'Jack' infected with FT-ALSV also produced the flower bud on the main stem apex under the long-day condition at about 40 dpi similar to that of determinate cultivars (Fig.7a) (Yamagishi & Yoshikawa, 2011). In the same conditions, non-infected soybean cvs. 'Dewamusume' and 'Jack' continued vegetative growth until at least 3 month-post inoculation (mpi) (Yamagishi & Yoshikawa, 2011). Taken together with these results, FT-ALSV infection could terminate the vegetative growth of soybean by production of flower buds on the main stem apex irrespective of their flowering traits.

Fig. 7b shows the phenotypes of FT-ALSV- infected and non-infected cv.'Dewamusume' at about 4 mpi. The pods and seeds on infected plant had matured after flowering (Fig. 7b left), in contrast with non-infected soybean which continues the vegetative growth under the same condition (Fig. 7b right). Seeds from these precocious flowering soybeans were normal

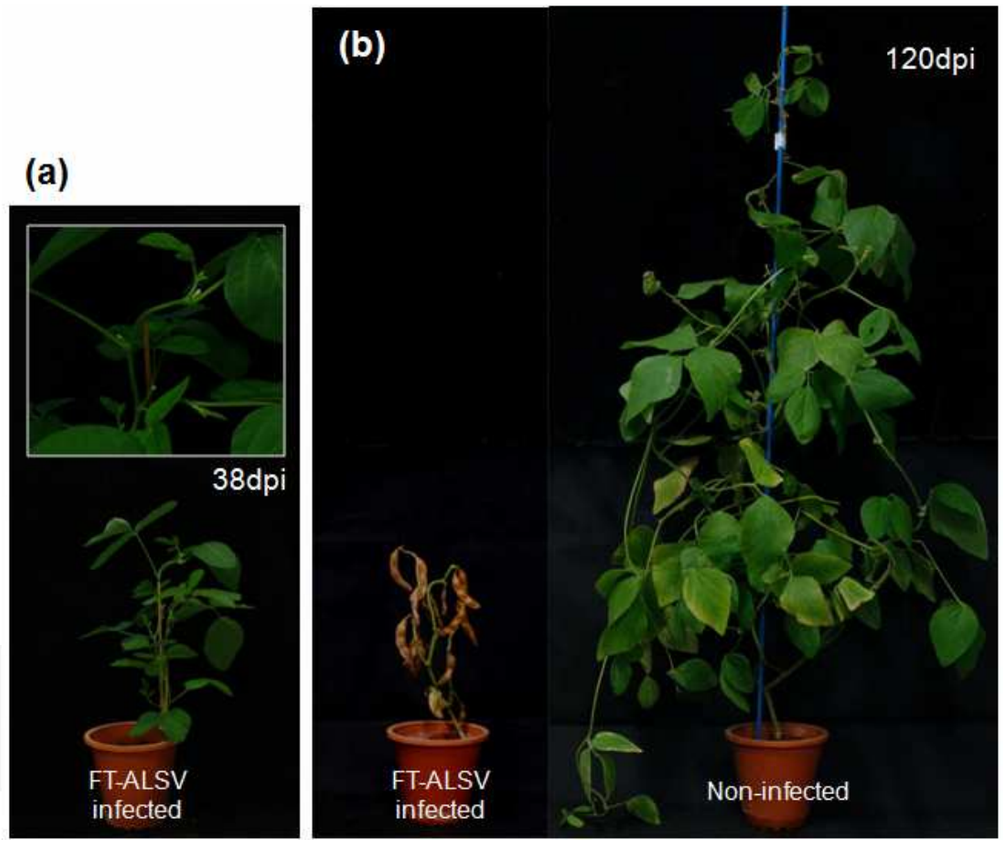

Fig. 7. Precocious flowering and termination of the vegetative growth of soybean by FT-ALSV infection. Plants were grown under a long-day condition. (a) The flower buds formation on the main stem apex of indeterminate cultivar 'Dewamusume' infected with FT-ALSV 38 dpi. (b) Comparison of an FT-ALSV- infected soybean cv. 'Dewamusume' shown in (a) about 120 dpi (left) and a non-infected control soybean cv. 'Dewamusume' (right). After the production of flower buds on the main stem apex, vegetative growth was terminated in FT-ALSV-infected soybean. Seeds matured on the soybean infected with FT-ALSV, in contrast with a non-infected plant which continues vegetative growth under the same condition.

in appearance and began germination when water was absorbed (Yamagishi & Yoshikawa, 2011). Thus, it is possible to reduce the generation time of soybean plants by FT-ALSV infection (Yamagishi & Yoshikawa, 2011). Although FT-ALSV transmitted through seeds to the next generation plants, virus-free plants also could be easily obtained. We think that precocious flowering by FT-ALSV in soybean would be a novel technology to obtain the gametophytoes for crossing and reducing the generation time in soybean breeding.

8. Consideration and prospects

As described above, ALSV vectors have several advantages for use as VIGS vectors in soybean. First, ALSV does not induce leaf symptoms above the third trifoliate leaves, and it does not affect the vegetative and reproductive growth of soybean plants. Second, ALSV can infect systemically various soybean cultivars with different stem-termination type, maturity group, and production area. Third, ALSV can infect systemically soybean plant including meristematic tissue and induce a highly uniform RNA silencing phenotype in upper leaves. Fourth, ALSV vector can induce VIGS of endogenous genes in all soybean growth stages including reproductive stage, seeds, and early growth stages of next generation.

On the other hand, ALSV vector cannot be applied to high-throughput analysis because of its gene expression strategy of viral genome (Igarashi et al., 2009). Recently, Zhang et al. have developed new BPMV-based vector, in which the cloning site for foreign sequences was introduced just after stop codon of a polyprotein in RNA2 (Zhang et al., 2010). This makes it possible to use BPMV-based vector for high-throughput analysis. This improvement can be applied to ALSV vector as well.

Infection of FT-ALSV induced precocious flowering in soybean, irrespective of the different stem growth habits and maturity types of the cultivars. This rapid flowering system using FT-ALSV will provide a powerful method for soybean breeding, in combination with molecular markers and genome sequence data.

9. Acknowledgements

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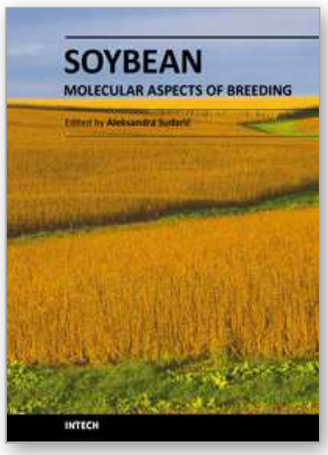

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