



# Virus-induced gene silencing in chili pepper by apple latent spherical virus vector



Chunjiang Li<sup>a,1</sup>, Hiroto Hirano<sup>b,1</sup>, Ichiro Kasajima<sup>c,1</sup>, Noriko Yamagishi<sup>c</sup>,  
Nobuyuki Yoshikawa<sup>a,c,\*</sup>

<sup>a</sup> Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan

<sup>b</sup> Frontier Research Laboratories, Institute for Innovation, Ajinomoto Co., Inc., Kawasaki, Kanagawa 210-8681, Japan

<sup>c</sup> Agri-Innovation Center, Iwate University, Morioka, Iwate 020-8550, Japan

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## ABSTRACT

Apple latent spherical virus (ALSV) can infect a variety of crops, usually without inducing symptoms. Partial gene sequences can be introduced into ALSV vectors for the induction of virus-induced gene silencing (VIGS). These features are beneficial for the estimation of gene functions in plants, with relatively concise experimental manipulations. Given that the infectability of chili peppers (*Capsicum* spp.) by ALSV was unknown, an ALSV infectivity test was performed on the highly pungent *Capsicum chinense* cultivar ‘Habanero’. The chili pepper plants were not infected after rub-inoculation with a crude homogenate of ALSV-infected *Chenopodium quinoa* leaves, whereas inoculating them with a concentrated ALSV virus preparation caused an infection. Inoculation with an ALSV RNA preparation by gold particle bombardment resulted in high infection rates (about 90%). The infection was systemic and the infected plants were symptomless. For the induction of VIGS, 201-nucleotide fragments of the *putative aminotransferase* (*pAMT*) gene were introduced into the ALSV vector. These ALSV vectors infected 80–90% of RNA-inoculated chili pepper seedlings. Expression of *pAMT*-mRNA was repressed in the placenta of immature fruit of infected plants. The silencing of *pAMT* in the infected plants caused a substantial decrease in capsaicin content and a concomitant moderate accumulation of the non-pungent bioactive metabolite capsiate in these plants. These results showed that ALSV could be used to study gene functions by VIGS and to enhance capsiate accumulation in chili pepper through genetic modification.

## 1. Introduction

Apple latent spherical virus (ALSV) is a member of the family Secoviridae (genus Cheravirus), and consists of two single-stranded RNA in its genome (RNA1 and RNA2) and three capsid proteins (VP25, VP20, and VP24) (Li et al., 2000). Foreign genetic sequences are introduced into cloning sites of the ALSV vector. This approach enables not only VIGS (virus-induced gene silencing) of the target gene of host plants, but also other genetic modifications, such as early flowering through expression of the *Flowering Locus T* (*FT*) gene and DNA methylation of the target sequence in the plant genome (Kasajima et al.,

2017a, 2017b). The ALSV vector has a favorable background as a genetics tool. ALSV can infect not only apple, the natural host, but also more than 30 other plant species, including the solanaceous plants tobacco, tomato, potato and petunia, and members of other plant families (Kasajima et al., 2017a; Li et al., 2019). As well as the wide host range, latent infection (without viral symptoms) is the advantage of ALSV vector, although preparation of ALSV vector is relatively laborious (takes time) due to the need to prepare high-concentration viral preparations for infection to crops. In the present study, ALSV vector was test used for VIGS in chili pepper.

Chili peppers comprise several species of the genus *Capsicum* in

**Abbreviations:** ALSV, apple latent spherical virus; bp, base pair(s); cDNA, complementary DNA; CMV, cucumber mosaic virus; CTAB, cetyltrimethylammonium bromide; dpi, day(s) post-inoculation; DW, dry weight; EDTA, ethylenediaminetetraacetic acid; kb, kilo-base(s); mpi, month(s) post-inoculation; nt, nucleotide(s); pAMT, putative aminotransferase; PHYVV, pepper huasteco yellow vein virus; RT-PCR, reverse transcription–polymerase chain reaction; TRV, tobacco rattle virus; Tris, tris(hydroxymethyl)aminomethane; VIGS, virus-induced gene silencing; VITGS, virus-induced transcriptional gene silencing; wtALSV, wild-type ALSV; XSB, XhoI–SmaI–BamHI

\* Corresponding author at: Laboratory of Plant Pathology, Faculty of Agriculture, Iwate University, Ueda 3-18-8, Morioka, Iwate 020-8550, Japan.

E-mail address: [yoshikawa@iwate-u.ac.jp](mailto:yoshikawa@iwate-u.ac.jp) (N. Yoshikawa).

<sup>1</sup> These authors equally contributed to this work.

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Solanaceae family. Chili peppers are originated in Central and South America (Kraft et al., 2014). Many cultivars are now grown around the world and are an economically important and popular crop used as a spice and in traditional medicine. Although the fruits of chili peppers are of medicinal value, the pungent taste is unfavorable to many people. The pungency is caused by capsaicinoids which consist mainly of capsaicin and dihydrocapsaicin (up to 90%), and the ratio of capsaicin to dihydrocapsaicin ranges from 1:1 to 2:1 according to species/cultivars (Iwai et al., 1979; Sanatombi and Sharma, 2008; Aza-González et al., 2011; Whiting et al., 2012, 2014). Capsaicin exerts multiple pharmacological and physiological effects in the human body, such as promotion of metabolism and suppression of fat accumulation (Ohnuki et al., 2001; Lejeune et al., 2003; Kawabata et al., 2006, 2009; Kang et al., 2010; Luo et al., 2011; Zheng et al., 2017). Capsaicin is also used as an analgesic in the form of topical ointments and nasal sprays (Fattori et al., 2016). In addition, clinical trials suggested that capsaicinoids show anti-cancer, anti-inflammatory and antioxidant activities (Yang et al., 2010; Choi et al., 2011; Henning et al., 2011).

The putative aminotransferase (*pAMT*) gene is involved in the synthesis of capsaicin in chili pepper (Curry et al., 1999; Blum et al., 2003; Sutoh et al., 2006; del Rosario Abraham-Juárez et al., 2008; Lang et al., 2009; Aza-González et al., 2011; Tanaka et al., 2017). The fruit of the non-pungent chili pepper cultivar 'CH-19 Sweet', a spontaneous mutant of *C. annuum* 'CH-19' (Yazawa et al., 1989), contain higher levels of capsinoids (such as capsiate) than those of 'CH-19', concomitant with a substantial decrease in capsaicinoid content (Kobata et al., 1998, 1999). The *pAMT* gene is mutated and has lost its function in 'CH-19 Sweet'. This mutation leads to the synthesis of capsinoids, instead of capsaicinoids (Lang et al., 2009). Independent mutations in the *pAMT* gene were also identified in other 'sweet' cultivars, further suggesting the involvement of *pAMT* in capsaicinoid synthesis (Tanaka et al., 2018). Two metabolic pathways are involved in capsaicinoid biosynthesis, namely the phenylpropanoid pathway and the branched-chain fatty acid pathway. The *pAMT* enzyme is considered to catalyze the formation of vanillylamine from vanillin in the final step of the phenylpropanoid pathway. Vanillylamine is then combined with 8-methyl-6-nonenoyl-CoA, synthesized in the branched-chain fatty acid pathway, to form capsaicin (Curry et al., 1999; Stewart et al., 2005; Sutoh et al., 2006; del Rosario Abraham-Juárez et al., 2008; Gururaj et al., 2012; Weber et al., 2014).

VIGS is a useful tool for studying gene functions. For example, the tobacco rattle virus (TRV) and cucumber mosaic virus (CMV) vectors were used to silence the putative acyltransferase gene *Pun1* in chili pepper and provided direct evidence that *Pun1* is involved in capsaicin accumulation (Stewart et al., 2005; Ogawa et al., 2015). In addition, VIGS of the *pAMT* gene was achieved using the pepper huasteco yellow vein virus (PHYVV) as a vector. This virus vector contained a 148 base pairs (bp) fragment of the mRNA sequence of *pAMT* and caused a reduction in *pAMT* expression and capsaicinoid contents in the fruit of the

mildly pungent 'serrano chili pepper' (*C. annuum* 'Tampiqueño 74') upon infection, providing direct proof for the function of *pAMT* in capsaicinoids synthesis (del Rosario Abraham-Juárez et al., 2008).

In the present study, ALSV infectivity of the highly pungent chili pepper cultivar 'Habanero' was tested. This trial revealed that ALSV infects chili pepper at a high frequency when the viral RNA is inoculated to cotyledons by gold particle bombardment. In addition, two ALSV vectors were successfully used for VIGS of *pAMT*.

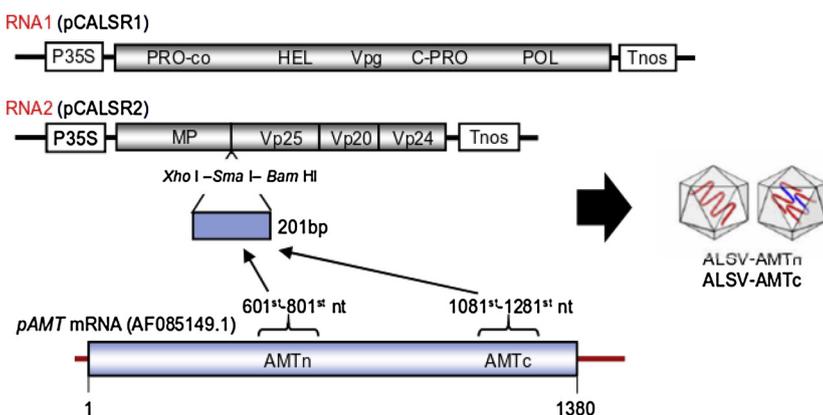
## 2. Materials and methods

### 2.1. Plant materials and virus(es)

*Capsicum chinense* 'Habanero' was used in the present study. Seeds were germinated in a petri dish on moist filter paper at 25 °C under illumination with a white fluorescent lamp. After germination, seedlings were transferred to soil in a small pot (5 cm diameter), then transferred to a larger pot (12 cm diameter) when the plants were larger. Plants were grown in a sunlit greenhouse after transfer to the small pot. ALSV (Li et al., 2000, 2004; GenBank ID ViralMultiSegProj15367) was prepared from pCALSR1 and pCLASR2 plasmids (Kon and Yoshikawa, 2014), encoding RNA1 and RNA2 genome of ALSV respectively, as described below.

### 2.2. Plasmid construction

To construct ALSV vectors for VIGS, a pair of 201-bp fragments of *pAMT* (GenBank accession AF085149.1) of chili pepper were prepared. The first fragment extended from nucleotide (nt) 601 to 801, and the second fragment extended from nt 1081 to 1281, of the coding region of *pAMT*. These DNA fragments were chemically synthesized and introduced into plasmids (Fasmac, Atsugi, Japan). The DNA fragments were amplified by Ex Taq DNA polymerase (Takara, Kusatsu, Japan) with primer pairs attached to the *Xho*I or *Bam*HI recognition sites. The primers Xho-hab601 (5' – CCA CTC GAG CTC AAA GAG GGG CCT GAA ACA-3') and BamH-hab801 (5' – CGA GGA TCC AAT GTT GTA TTT ATC ACT GCC AAA C-3') were used for amplification of the first fragment, and the primers Xho-Hab1081 (5' – CCA CTC GAG GGG GAG ATA AGG GGA ACT GGT TT-3') and BamH-Hab1281 (5' – CGA GGA TCC CTC ATC AAG TTC TTC AAG ACT CAA-3') were used for amplification of the second fragment. After simultaneous digestion with *Xho*I and *Bam*HI, the PCR products were purified using the MonoFas DNA purification kit (GL Sciences, Tokyo, Japan). These fragments were then inserted into the *Xho*I-*Sma*I-*Bam*HI (XSB) cloning site of the pCALSR2-XSB binary plasmid encoding the RNA2 genome of ALSV (Kon and Yoshikawa, 2014), which had been digested with *Xho*I and *Bam*HI (Fig. 1). The resulting plasmids were designated pCALSR2-XSB(AMTn) and pCALSR2-XSB(AMTc), respectively.



**Fig. 1.** Schematic representation of the ALSV vectors. The structure of the expression vectors of RNA1 and RNA2 of ALSV are shown. The 201-nucleotide (nt) fragmental sequences of the coding sequence of *pAMT* were prepared at the indicated positions and introduced into the XSB (*Xho*I-*Sma*I-*Bam*HI) cloning site in the ALSV-AMTn and ALSV-AMTc vectors, whereas there is no insertion in wtALSV (wild-type ALSV). P35S, cauliflower mosaic virus 35S RNA promoter; Tnos, *A. tumefaciens* Nopaline Synthase terminator; PRO-co, protease cofactor; HEL, NTP-binding helicase; Vpg, viral protein genome-linked; C-PRO, cysteine protease; POL, RNA polymerase; MP, movement protein; Vp25, Vp20, and Vp24, three capsid proteins.

### 2.3. Preparation of ALSV vectors

For agro-inoculation, the binary plasmid pCALS1 containing the RNA1 genome of ALSV (Kon and Yoshikawa, 2014), and the pCALS2-based plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101. Agro-infiltration of *Nicotiana benthamiana* leaves was carried out at the 6–8 true leaves stage by the mixture of three *A. tumefaciens* clones transformed with pCALS1, pCALS2, or pBE2113–HCPro for transient expression of the silencing suppressor HCPro (Li et al., 2014; Yaegashi et al., 2007). ALSV infection of *N. benthamiana* was tested by reverse transcription-polymerase chain reaction (RT-PCR) analysis as described in Section 2.5. The ALSV vectors derived from pCALS2-XSB (AMTn) and pCALS2-XSB(AMTc) were designated ALSV-AMTn and ALSV-AMTc, respectively.

Crude sap of the infected leaves of *C. quinoa*, partially purified ALSV, or RNA extract from partially purified ALSV was used for inoculation of chili pepper. Partial purification of ALSV and extraction of its RNA were performed as described previously (Kasajima et al., 2017b). Briefly, the upper ALSV-infected leaves of *N. benthamiana* (10 g fresh weight) were ground in ‘ALSV buffer’ (0.1 M Tris–HCl; 0.1 M NaCl; 0.005 M MgCl<sub>2</sub>, pH 7.8) and rub-inoculated to *Chenopodium quinoa*. Infected *C. quinoa* leaves were homogenized and treated with bentonite. Virus particles were precipitated in 8% polyethylene glycol (PEG) solution. The precipitate was suspended in 2 mL ALSV buffer to produce the partially purified virus preparation. Total RNA was extracted from the virus preparation by treatment with phenol-chloroform (1:1), precipitated with ethanol, and dissolved in RNase-free water. The final RNA concentration was 2–3 µg µL<sup>-1</sup>.

### 2.4. Inoculation of ALSV to chili pepper seedlings

The three ALSV preparations were used for inoculation (Table 1). First, chili pepper seedlings at the 3–4 true leaves stage were rub-inoculated with ‘crude sap’. Crude sap of *C. quinoa* leaves, ground in three times the volume of ALSV buffer, was rub-inoculated onto the upper surface of the true leaves together with carborundum, then immediately rinsed by spraying with tap water. Second, ‘partially purified virus’ was inoculated in the same manner as ‘crude sap’. Third, the expanded cotyledons of chili pepper seedlings were inoculated by particle bombardment of ‘RNA from partially purified virus’ using a PDS-1000/He Particle Delivery System gene gun (Bio-Rad Laboratories, Hercules, CA, USA) as described previously (Yamagishi et al., 2014). The inoculated seedlings were incubated at 4 °C in the dark for 2 days, then transferred to a small pot.

### 2.5. RT-PCR

RNA was extracted from leaves (approximately 0.1 g fresh weight) of ALSV-inoculated *N. benthamiana*, *C. quinoa*, or chili pepper plants using the cetyltrimethylammonium bromide (CTAB) method as described previously (Chang et al., 1993) with slight modification. In summary, leaves were crushed with two metal beads in a Micro Smash 100R cell disrupter (Tomy, Tokyo, Japan) in a 2-mL specialized tube in 500 µL ‘CTAB buffer’ (2% CTAB; 100 mM Tris–HCl, pH 8.0; 25 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0; 2% polyvinylpyrrolidone K-30; 2 M NaCl; 2% 2-mercaptoethanol) and incubated for 15 min at 60 °C. This solution was treated with an equal

volume of chloroform. After centrifugation, one-third volume of 7.5 M LiCl was added to the recovered water phase and incubated at –20 °C for at least 1 h. RNA was precipitated by centrifugation and rinsed in 70% ethanol, then dissolved in RNase-free water at the concentration of 1 µg µL<sup>-1</sup>.

RNA was reverse transcribed to complementary DNA (cDNA) using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) with oligo-(dT)<sub>20</sub>. cDNA was PCR amplified by using Ex Taq DNA polymerase (Takara). Here, a portion of the RNA2 genome of ALSV, including the insertion of the partial *pAMT* coding sequences, was amplified using the primers ALSR2-1363(+) (5′-GCG AGG CAC TCC TTA TCC TAT CAA-3′) and ALSR2-1511(–) (5′-ATA ACT CTT GCA AGG TGG TCG TGA -3′).

### 2.6. Western blot

Infected leaves of chili pepper plants were homogenized in ALSV buffer (0.1 M Tris–HCl; 0.1 M NaCl; 0.005 M MgCl<sub>2</sub>, pH 7.8), separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% gel, and transferred to a Hybond-P nitrocellulose membrane (GE Healthcare, Chicago, IL, USA). The anti-ALSV serum was used for western blotting as described previously (Li et al., 2014).

### 2.7. Northern blot

For RNA extraction, placenta and septa tissues of immature chili pepper fruit (4 weeks after anthesis) or mature chili pepper fruit (8 weeks after anthesis) were collected and rapidly frozen in liquid nitrogen. Total RNA was extracted from about 0.1 g of frozen tissue using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) in accordance with the manufacturer’s instructions. The total RNA samples (about 10 µg) were separated in 1% agarose gel containing 6% formaldehyde, then transferred to a Hybond-N membrane (GE Healthcare). The mRNA of *pAMT* was detected as described previously (Yaegashi et al., 2007). Here, a digoxigenin (DIG)-labeled antisense RNA probe complementary to nt 1 to 600 of the coding region of *pAMT* was used for hybridization.

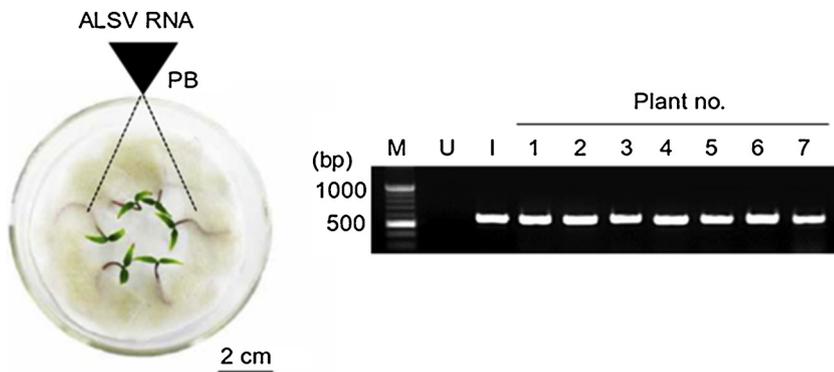
### 2.8. Quantitative RT-PCR

The harvested immature fruit (4 weeks after anthesis) were freeze-dried for 2 days using a FDU-2100 lyophilizer (Eyela, Tokyo, Japan). Dried fruit were ground in a blender at room temperature. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands) following the manufacturer’s instructions. The RNA was treated with Recombinant DNase I (Takara) for DNA digestion and dissolved in RNase-free water. An aliquot of 500 ng RNA was converted to cDNA using ReverTra Ace reverse transcriptase (Toyobo) with a random hexamer. Quantitative PCR was performed with a Thermal Cycler Dice Real Time System (Takara) or Eco Real-Time PCR System (Illumina, San Diego, CA, USA) using SYBR Premix Ex Taq DNA polymerase (Takara) as described previously (Lang et al., 2009). The 18S *rRNA* gene was used for normalization, which was amplified using the primers ‘18S rRNA F’ (5′-CTG CCA GTA GTC ATA TGC TTG TC-3′) and ‘18S rRNA R’ (5′-GTG TAG CGC GCG TGC GGC CC-3′). The *pAMT* gene was amplified using the primers ‘pAMT F’ (5′-TAC TGA GGT GGT GAA CAA AAT ATC A-3′) and ‘pAMT R’ (5′-CAT TCA TAT GGA AAG GGA TCA TTA G-3′).

**Table 1**

Infection efficiency of ALSV vectors on chili pepper ‘Habanero’ seedlings with different inocula, inoculation methods, and at different growth stages.

| Preparation of inocula            | Growth stage of seedlings | Inoculation methods       | ALSV vector                | Infected / inoculated seedlings (%) |
|-----------------------------------|---------------------------|---------------------------|----------------------------|-------------------------------------|
| Crude sap                         | Three to four true leaves | Rub-inoculation           | wtALSV ALSV-AMTc           | 0/10 (0) 0/10 (0)                   |
| Partially purified virus          | Three to four true leaves | Rub-inoculation           | wtALSV ALSV-AMTc           | 8/8 (100) 2/7 (29)                  |
| RNA from partially purified virus | Expanded cotyledons       | PDS-1000/He <sup>TM</sup> | wtALSV ALSV-AMTc ALSV-AMTn | 5/5 (100) 9/10 (90) 8/10 (80)       |



**Fig. 2.** Inoculation of chili pepper by particle bombardment. Viral RNA (ALS<sub>V</sub>-AMTc) was inoculated by gold particle bombardment (PB) to chili pepper seedlings at the cotyledon stage (left). ALS<sub>V</sub> infection of the cotyledon was tested in independent inoculated plants (No. 1 through 7) by RT-PCR at 7 days post inoculation (dpi) in this experiment (right). M, DNA ladder marker BRG-100-02. U, uninoculated chili pepper (negative control). I, infected *C. quinoa* (positive control). Figures on the left of the gel image indicate DNA size in base pairs (bp).

### 2.9. HPLC analysis of capsaicin and capsate

The same freeze-dried and ground fruit as used for quantitative RT-PCR were used for high-performance liquid chromatography (HPLC). Capsaicin and capsate were extracted and quantified as described previously (Lang et al., 2009).

### 2.10. Bisulfite sequencing

Genomic DNA was prepared from the fruit or leaves of chili pepper using the DNeasy Plant Mini Kit (Qiagen). An aliquot of 500 ng genomic DNA was treated with bisulfite using the EpiTect Fast Bisulfite Kit (Qiagen), then purified using the MonoFas DNA Purification Kit (GL Sciences). The DNA extract was used for amplification of a portion of the *pAMT* gene using KOD Multi & Epi DNA polymerase (Toyobo). The first-round PCR primer pair was pAMTcintF1(+) (5'-GTT ATT AAG GAA ATT TGG AAT GTG AAG AAA-3') and pAMTcintR1(-) (5'-CCT AAT TTA TCT ACC TCC ACA ACT TC-3'). The second-round PCR (nested PCR) primer pair was pAMTcF2(+) (5'-GTT TAT AAG TAA AAG GAT AGT TAT GAA TTG-3') and pAMTcintR2(-) (5'-CAT ACT AAA AAA CAT CTT AAA ATA TTC TAA CAA AT-3'). Thus, a 477-nt region of the plus strand of genomic sequence of *pAMT* (nt 8929 to 9405 of LC032106.1: 5'-TCA TGA ATT ... TTA CTT TAC-3'), which includes 15th and 16th exons, was amplified. The 3' end of the PCR products was attached with dATP (deoxyadenosine triphosphate) using the 10 × A-attachment Mix (Toyobo) and then cloned into the T-vector pMD20 (Takara). For each PCR product, ten plasmid clones were sequenced for determination of the ratio of DNA methylation.

## 3. Results

### 3.1. Preparation of ALS<sub>V</sub> vectors

For VIGS of the *pAMT* gene in chili pepper, 201-nt fragments were prepared that corresponded to nt 601–801 or 1081–1281 of the coding region of *pAMT*. These nucleotide sequences were designated 'AMTn' and 'AMTc' in the present study. The AMTn and AMTc sequences were independently introduced into ALS<sub>V</sub>. Wild-type ALS<sub>V</sub> (wtALS<sub>V</sub>) was without insertion. Given that AMTn and AMTc sequences were introduced into the XSB site in-frame with the polyprotein of RNA2, all proteins encoded by RNA2 (MP, Vp25, Vp20, and Vp24) were expected to be properly expressed in plant cells. The viruses wtALS<sub>V</sub>, ALS<sub>V</sub>-AMTn, and ALS<sub>V</sub>-AMTc were successfully propagated in *N. benthamiana*, and further amplified in *C. quinoa*.

Viral inocula for inoculation to chili pepper were prepared in three different forms as described in 'Materials and methods'. This is because infectivity of ALS<sub>V</sub> to host plants is relatively low compared with the other virus vectors. The first preparation was 'crude sap', which was a simple homogenate of *C. quinoa* leaves. The second preparation was 'partially purified virus', which was the homogenate of *C. quinoa* treated with bentonite for purification. The third preparation was 'RNA

from partially purified virus', which was viral RNA extracted from 'partially purified virus' (Table 1).

### 3.2. Inoculation of ALS<sub>V</sub> to chili pepper seedlings

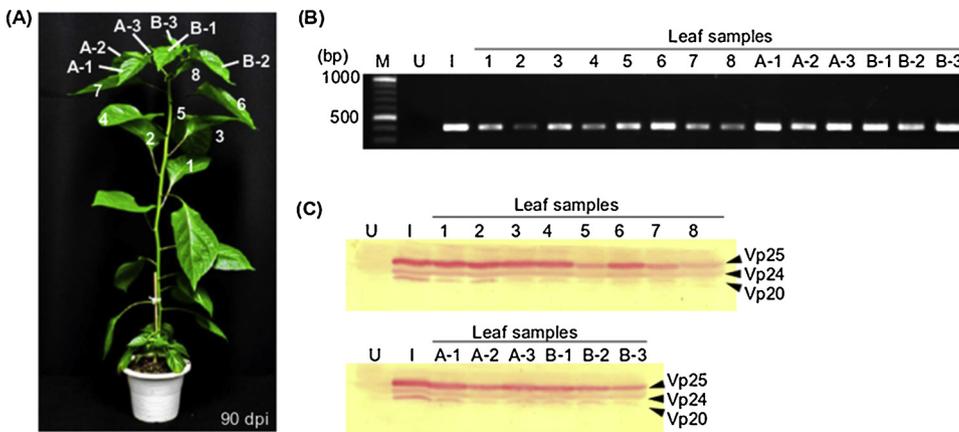
Chili pepper 'Habanero' was used throughout the present study. To examine whether ALS<sub>V</sub> is capable of infecting chili pepper, 'crude sap' was rub-inoculated onto the true leaves of young seedlings. Upper leaves were analyzed by RT-PCR to detect ALS<sub>V</sub> infection at 1 month post inoculation (mpi). Both wtALS<sub>V</sub> and ALS<sub>V</sub>-AMTc were tested in this trial, but none of the inoculated plants were infected with ALS<sub>V</sub> (Table 1). In contrast, all chili pepper plants were infected with wtALS<sub>V</sub> when 'partially purified virus' was rub-inoculated onto true leaves, but the infection frequency was only 29% when ALS<sub>V</sub>-AMTc was inoculated with 'partially purified virus' (Table 1). To efficiently infect ALS<sub>V</sub> with insertions at the XSB site, particle bombardment of 'RNA from partially purified virus' to cotyledons was also tested (Fig. 2). This protocol was suitable to infect wtALS<sub>V</sub>, ALS<sub>V</sub>-AMTc, and ALS<sub>V</sub>-AMTn for 100%, 90%, and 80% of inoculated chili pepper plants, respectively, as examined in upper leaves at 1 mpi (Table 1). Fig. 2 shows an example of the inoculation of 'RNA from partially purified virus' to juvenile chili pepper seedlings by particle bombardment.

To verify systemic infection of ALS<sub>V</sub> in chili pepper, ALS<sub>V</sub> infection was tested in 14 leaves of a plant inoculated with wtALS<sub>V</sub> (Fig. 3A). This included eight leaves formed on the stem, and three leaves each on two branches, respectively. RT-PCR analysis of these leaves revealed that ALS<sub>V</sub> had spread to all of the tested leaves (Fig. 3B). This result was reconfirmed by detection of three capsid proteins in the same leaves by western blot analysis (Fig. 3C).

### 3.3. Expression analysis of *pAMT*

In preparation for VIGS of *pAMT* in chili pepper, natural *pAMT* expression in 'Habanero' fruit was first analyzed. In previous reports, *pAMT* is expressed in placental tissue of unripe fruit of chili pepper except for the cultivar 'Trinidad Moruga Scorpion Yellow' (Lang et al., 2009; Tanaka et al., 2017). The placenta and septa from immature and mature fruit of 'Habanero' were tested for expression of *pAMT*-mRNA by northern blot analysis (Fig. 4A). The hybridization signal of *pAMT*-mRNA, approximately 1.6 kilo-bases (kb), was detected in immature fruit but not in mature fruit of 'Habanero' (Fig. 4B), consistent with the previous report (Arce-Rodríguez and Ochoa-Alejo, 2017). The same result was obtained by RT-PCR analysis (Fig. 4C).

The expression of *pAMT*-mRNA in ALS<sub>V</sub>-infected plants was tested by northern blot analysis (Fig. 5). The hybridization signal of *pAMT*-mRNA in the placenta/septa of immature fruits was decreased in the six plants infected with ALS<sub>V</sub>-AMTc compared with that of healthy (uninoculated) plants. Chili pepper plants were also analyzed that were infected by ALS<sub>V</sub>-AMTc, but the insertion sequence (AMTc) was deleted in the infection process (indicated as 'ALS<sub>V</sub>-AMTc/del' in Fig. 5). The expression of *pAMT*-mRNA was detected, at a similar level to that of



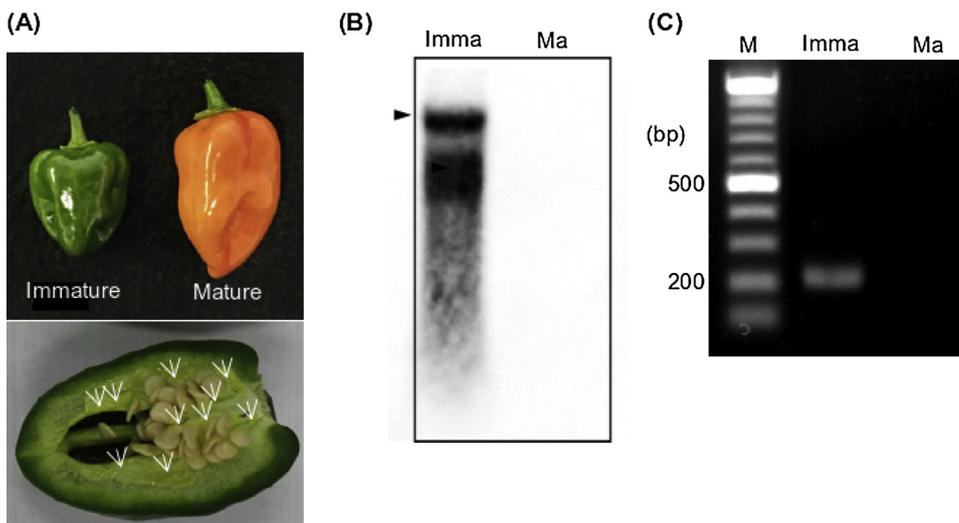
**Fig. 3.** Systemic infection of wtALSIV in chili pepper. (A) A chili pepper plant photographed at 90 days post inoculation (dpi). The numbers 1–8 indicate independent true leaves on the stem, whereas ‘A-1’, ‘A-2’, ‘A-3’, ‘B-1’, ‘B-2’, and ‘B-3’ indicate true leaves on two different branches. (B) RT-PCR analysis for detection of ALSIV infection/penetration of the indicated leaves in (A). M, DNA ladder marker BRG-100-02. U, uninoculated chili pepper (negative control). I, infected chili pepper (positive control). Figures on the left of the gel image indicate DNA size in base pairs (bp). (C) Western blot analysis for detection of ALSIV infection/penetration of the indicated leaves in (A). U, uninoculated chili pepper (negative control). I, infected chili pepper (positive control). Capsid proteins (Vp25, Vp24, and Vp20) are indicated by arrowheads.

healthy plants, in these infected plants with deletion of the AMTC sequence, indicating that ALSIV itself does not influence *pAMT* expression.

The expression level of *pAMT* was also estimated by quantitative RT-PCR analysis using whole immature fruit crushed to homogeneity. The relative expression level of *pAMT*, which was standardized with 18S rRNA, was measured in total RNA extracts (Fig. 6). The expression level of *pAMT* in immature fruit was reduced to 12% or 20% in plants infected by ALSIV-AMTC or ALSIV-AMTN respectively, compared with that of control plants infected by wtALSIV. No obvious difference in expression levels was observed between the plants infected by ALSIV-AMTC and ALSIV-AMTN.

### 3.4. Capsaicin and capsiate accumulation in fruit

Accumulation of capsaicin and capsiate, the representative metabolites of capsaicinoids and capsinoids, were measured. These metabolites were extracted from whole-fruit homogenates and measured by HPLC. Capsaicin content in wtALSIV-infected fruit was between 16.5 mg g<sup>-1</sup> dry weight (DW). Capsaicin accumulation was dramatically reduced in fruit of plants infected by ALSIV-AMTC or ALSIV-AMTN, which was 0.6 or 1.0 mg g<sup>-1</sup> DW respectively (Fig. 7A). Capsiate content in wtALSIV-infected fruit was 0.3 mg g<sup>-1</sup> DW. The capsiate content was 1.7 mg g<sup>-1</sup> DW in ALSIV-AMTC-infected plants, and 1.3 mg g<sup>-1</sup> DW in ALSIV-AMTN-infected plants (Fig. 7B). Thus, capsiate content was increased by 6.0 or 4.6 times by infection of ALSIV vectors.



**Fig. 4.** Expression of *pAMT*-mRNA in uninoculated chili pepper. (A) Photograph of immature and mature fruits of chili pepper ‘Habanero’ (top). Cross-section of an immature fruit is also shown (bottom). Arrows indicate placenta and septa. (B) Northern blot analysis of the accumulation of *pAMT*-mRNA in placental tissues of immature fruit (Imma) and mature fruit (Ma). Arrowhead indicates approximate RNA size of 1.6 kilo-bases (kb). (C) The mRNA accumulation of *pAMT* analyzed by RT-PCR. M, DNA ladder marker BRG-100-02; Imma, immature fruit; Ma, mature fruit. DNA size is indicated on the left of the gel image.

### 3.5. Methylation state of *pAMT*

Infection of virus vectors not only causes mRNA degradation (VIGS), but also induces methylation of genomic DNA (e.g. Kanazawa et al., 2011; Kon and Yoshikawa, 2014). Then the possibility of genomic DNA methylation by the ALSIV vector in chili pepper was tested. Genomic DNA was extracted from chili pepper plants infected by wtALSIV or ALSIV-AMTC. The *pAMT* gene contains 17 exons (Tanaka et al., 2015). The 201-nt ‘AMTC’ sequence is composed of five nucleotides of the 14th exon (5’-GGG GA-3’), and the complete 15th and 16th exons of *pAMT*. The methylation state of the plus-strand DNA of the 15th and 16th exons was analyzed by bisulfite sequencing (Fig. 8). In the control wtALSIV-infected plants, the 74 cytosine residues in the PCR-amplified region were rarely methylated, except for the tail of the 16th exon and the following intron. A higher level of cytosine methylation was detected within this region in ALSIV-AMTC-infected plants. Many of the cytosine residues were highly methylated in the 15th and 16th exons, in the sequences matching ‘AMTC’, and some cytosine residues upstream of the 15th exon were methylated.

## 4. Discussion

In the present study, it was first aimed to establish a protocol for stable infection of chili pepper with ALSIV vectors. Although crude sap from leaves of ALSIV-infected *C. quinoa* did not result in infection of chili pepper, partially purified wtALSIV infected all inoculated chili pepper plants, when rub-inoculated onto true leaves (Table 1). The ALSIV-AMTC

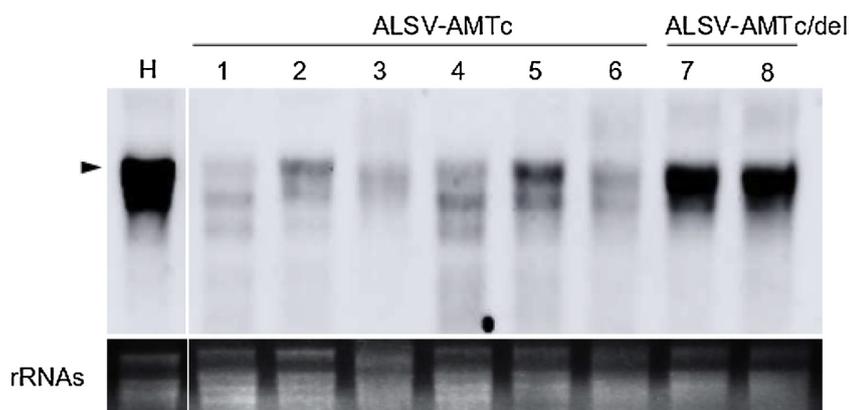


Fig. 5. Expression of *pAMT*-mRNA in ALSV-infected chili pepper. The accumulation of *pAMT*-mRNA was analyzed by northern blot analysis in placental tissues of immature fruit. H, healthy (uninoculated) plant. ALSV-AMTc, independent plants infected by ALSV-AMTc vector (1–6). ALSV-AMTc/del, independent plants infected by ALSV, although the insertion sequence of AMTc is deleted (7 and 8, as negative controls). Arrowhead indicates the approximate position of the RNA size of 1.6 kb. rRNAs, ribosomal RNA bands stained with ethidium bromide.

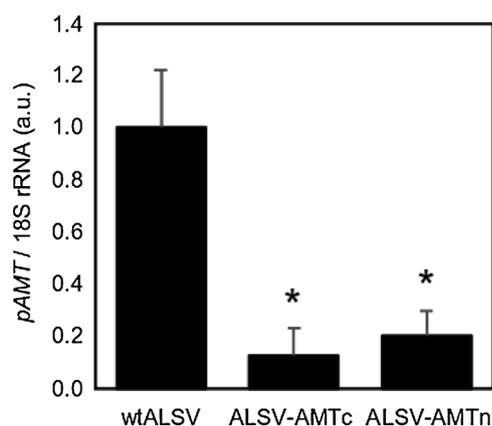


Fig. 6. Relative expression levels of *pAMT*-mRNA. The accumulation of *pAMT*-mRNA was analyzed in immature fruits by quantitative RT-PCR. Virus vectors used for inoculations are indicated on the bottom. Data represent means and standard deviations ( $n = 4$ ). Asterisks indicate statistical difference from wtALSV by Student's *t*-test ( $P < 0.05$ ).

vector, which was one of the ALSV vectors used for VIGS of the *pAMT* gene, achieved an infection frequency of 29% of inoculated chili pepper plants, when inoculated under the same conditions using the partially purified virus. A similar phenomenon has been experienced in our experiment with other plant species: the insertion sequence at the cloning sites of ALSV vectors affect infection of ALSV in some cases (Kon and Yoshikawa, 2014). Thus, a more powerful protocol for ALSV inoculation of chili pepper is required. Based on our experience, inoculation of viral RNA by gold particle bombardment was finally tested (Fig. 2). This method was quite successful and resulted in infection of 100%, 90%, or 80% of chili pepper plants respectively, with wtALSV, ALSV-AMTc, or ALSV-AMTn (Table 1). As a matter of fact, inoculation of viral RNA to cotyledons by particle bombardment tends to be more successful rather than rub-inoculation of concentrated virus preparations onto true leaves in other plant species. For example, ALSV quite rarely systemically infects apple following rub-inoculation, but the infection frequency is almost 100% when cotyledons are inoculated with highly concentrated viral RNA by particle bombardment (Yamagishi et al., 2011). An ALSV-infected mature chili pepper plant was examined by RT-PCR and western blot analyses (Fig. 3). ALSV was detected in all tested leaves of the plant, showing that ALSV systemically infected the whole shoot of the chili pepper plant. ALSV did not cause viral symptoms (i.e., disorder of plant phenotypes), so ALSV is suitable for VIGS experiments to characterize the functions of endogenous genes in chili pepper.

In northern blot and RT-PCR analyses (Fig. 4), *pAMT* was expressed in the placenta of immature 'Habanero' fruit (4 weeks after anthesis), as already reported. In addition, expression of *pAMT* was detected in

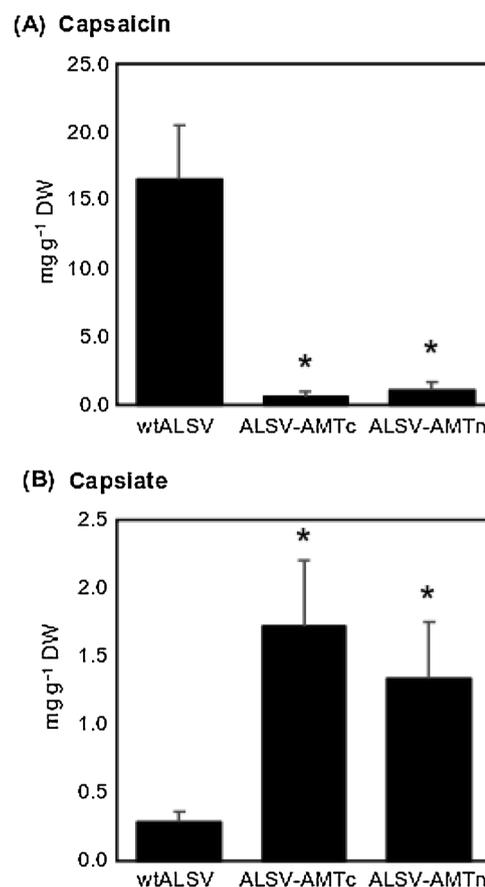
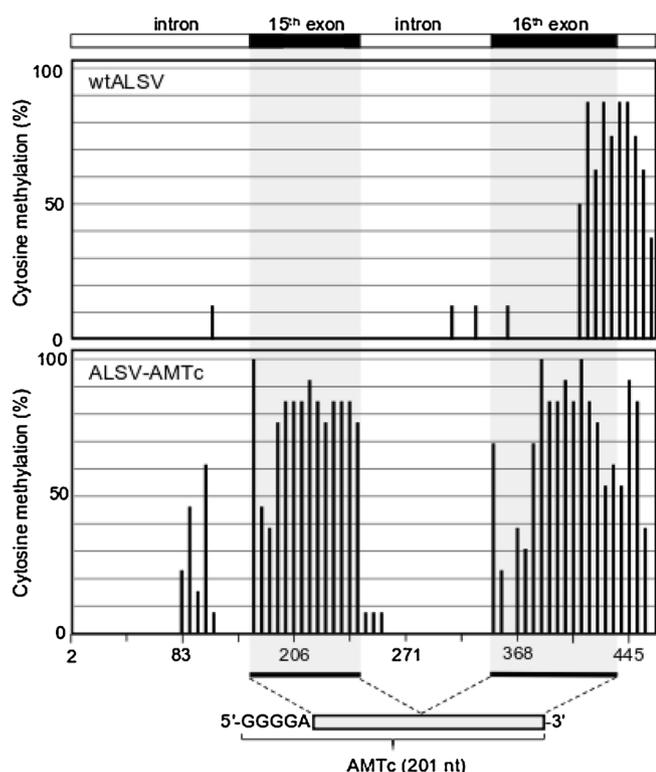


Fig. 7. Capsaicin and capsiate contents in chili pepper fruit. Capsaicin (A) and capsiate (B) contents were measured in whole fruit of plants infected by wtALSV, ALSV-AMTc, or ALSV-AMTn. Contents were calculated as milligrams capsaicin/capsiate per gram dry weight (DW). Data represent means and standard deviations ( $n = 4$ ). Asterisks indicate statistical difference from wtALSV by Student's *t*-test ( $P < 0.05$ ).

placental tissue of immature fruit of ALSV-AMTc-infected chili pepper (Fig. 5). As expected, the *pAMT* expression level was decreased in all ALSV-infected plants, indicating that *pAMT* expression was suppressed by ALSV-induced VIGS. Quantitative RT-PCR analysis showed that *pAMT* expression was reduced to 12% or 20% in ALSV-AMTc- or ALSV-AMTn-infected plants, compared with that of the control plants infected by wtALSV without any insertion (Fig. 6). Consistent with this result, the capsaicin content of the whole fruit was reduced to 3.5% or 6.5% in ALSV-AMTc- or ALSV-AMTn-infected plants, respectively. These percentages are comparable, or more severe, than the reduction in capsaicin content caused by VIGS with a PHYVV vector (7.1%) (del Rosario



**Fig. 8.** Methylation of genomic DNA in *pAMT* exon regions. DNA methylation state was analyzed by bisulfite sequencing of the plus strand sequence around the 15th and 16th exons of *pAMT* in a wtALSv-infected plant (top) and an ALSv-AMTc-infected plant (bottom). All 74 cytosine residues in the analyzed genomic region are shown in the graph. Numbers below the graphs indicate the positions of cytosine residues in the 477-nt sequenced region. Positions of the 15th and 16th exons are indicated by filled boxes up to the graphs. The genomic regions corresponding to the AMTc sequence are also indicated below graphs.

Abraham-Juárez et al., 2008). Although the host chili pepper cultivar/species differed between the studies employing ALSV and PHYVV vectors, the ALSV vector seemed to cause sufficiently strong VIGS in chili pepper, similar to that of TRV, PHYVV, and CMV vectors.

The reduction in capsaicin content observed in response to ALSV-induced VIGS provides additional direct evidence that *pAMT* is involved in capsaicin synthesis in chili pepper, similar to the response to PHYVV-induced VIGS. A moderate increase in capsate content in chili pepper plants was observed, in which *pAMT* was suppressed by VIGS. This phenomenon has been observed previously in chili pepper mutants, but the present study provides the first direct evidence for *pAMT* involvement through genetic modification of *pAMT* expression. Although capsate is synthesized in the same metabolic pathway instead of capsaicin in *pAMT*-mutated/suppressed plants, the increase in capsate content caused by *pAMT* silencing (approximately  $1 \text{ mg g}^{-1}$  DW in the present study; Fig. 7) was much lower than the concomitant decrease in capsaicin content (approximately  $10 \text{ mg g}^{-1}$  DW). Thus, *pAMT* mutation/suppression does not lead to conversion of all capsaicin to capsate, although the possibility of capsate degradation cannot be excluded (Luo et al., 2011). This observation means that mutations/suppressions of other genes should be introduced, in addition to *pAMT* mutation/suppression, to enhance the content of capsate, which is a valuable metabolite for medical purposes. As already described, vanillyl alcohol is synthesized from vanillin in the cultivar ‘CH-19 Sweet’ (which harbors a mutated *pAMT*), and is further catalyzed to capsinoids (Sutoh et al., 2006). Identification of the enzyme(s) involved in these reactions will assist in breeding high-capsate cultivars. Screening chili pepper cultivars will be also important. Suppression of *pAMT* by VIGS in the large collective genetic resource of chili pepper cultivars may

identify a genotype with a high potential capsinoid conversion rate. High capacity of capsaicin synthesis does not always indicate high capacity of capsate synthesis: strong expression of *pAMT* gene itself in placenta is one of the reasons for high capsaicin accumulation in ‘Habanero’ (Arce-Rodríguez and Ochoa-Alejo, 2017). Given that genetic transformation of chili pepper remains difficult (Chung et al., 2004), VIGS is presently a preferable choice for such experiments.

In addition to VIGS, virus vectors are known to induced gene silencing via methylation of cytosine residues in the promoter region of target genes (i.e., virus-induced transcriptional gene silencing; VITGS). VITGS is mainly reported for exogenously introduced transgenes. Although the number of reports on VITGS of endogenous genes is limited, VITGS of endogenous genes of crops has been described (Kanazawa et al., 2011; Kon and Yoshikawa et al., 2014). In preparation for future trials, the DNA methylation state of genomic DNA was analyzed in the region of the 15th and 16th exons of *pAMT* (Fig. 8.). Many portions of this genomic region were not naturally methylated, whereas infection of the ALSV-AMTc vector induced high levels of DNA methylation in these exons. Although new ALSV vectors targeting the *pAMT* promoter must be prepared and tested for the possibility of VITGS, ALSV vector is at least available for targeted methylation of genomic DNA in chili pepper.

#### Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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