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Original article

Investigation of *Agrobacterium*-mediated Transient dsRNA Expression in Tobacco

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Abstract The *Agrobacterium tumefaciens* mediated gene transfer is widely used to generate genetic transformation of plants and transient assay of temporal exogenous gene expression. Syringe infiltration system into tobacco (*Nicotiana benthamiana*) leaves is a powerful tool for transient expression of target protein to study protein localization, protein-protein binding and protein production. However, the protocol and technical information of transient gene expression, especially double strand RNA (dsRNA), in tobacco using *Agrobacterium* is not well known. Recently, dsRNA is crucial for insecticidal effect on destructive agronomic pest such as Corn rootworm. In this study, we investigated the factor influencing the dsRNA expression efficiency of syringe agro-infiltration in tobacco. To search the best combination for dsRNA transient expression in tobacco, applied two *Agrobacterium* cell lines and three plant vector systems. The efficiency of dsRNA expression has estimated by real-time PCR and digital PCR. As a result, pHellsgate12 vector constructs showed the most effective accumulation of dsRNA in the cell. These results indicated that the efficiency of dsRNA expression was depending on the kind of vector rather than *Agrobacterium* cells. In summary, the optimized combination of transient dsRNA expression system in tobacco might be useful to *in vivo* dsRNA expression for functional study and risk assessment of dsRNA.

Key words: dsRNA, transient expression, tobacco, real-time PCR, digital PCR

INTRODUCTION

To express heterologous gene and protein in plant, genetic transformation is a powerful tool used in a variety of plant molecular biology researches, such as gene function study and *in vivo* protein production. In modern biotechnology, stable genetic transformation is required to develop genetically modified organisms (GMO) for expression stability and heritability of target gene. Although these advantages and needs are well known, stable transformation in plant is time and cost consuming and labor intensive process. By contrast, transient transformation is an easy and effective system for heterologous expression to study gene and protein molecular functions within short periods (Wroblewski *et al*., 2005). Among the transient expression system in tobacco, syringe infiltration which mediated by *Agrobacterium* is a simple, rapid, and most commonly used technique (Santi *et al*., 2008). This method lead to wide range of research such as protein localization and protein-protein interaction, functional study of gene silencing and overexpressing, and

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promoter assay (Yang *et al*., 2000; Johansen *et al*., 2001; Dugdale *et al*., 2014). Many studies of syringe agro-infiltration had focused on improving the transformation efficiency and production yield (Bakhsh *et al*., 2017), however little is known about double strand RNA expression in tobacco using transient expression system.

RNA interference (RNAi) pathways are present in the most organisms and first studied in *Caenorhabditis elegans* (Fire *et al*., 1998). Recently, a new agro-biotechnology using RNAi technique has developed to protect from pests without the expression of specific insecticidal proteins (Baum *et al*., 2007; Worrall *et al*., 2019). The pest Snf7 dsRNA in plant cell is absorbed in the insect intestine and inhibits the expression of *Snf7* mRNA, which encodes endosomal sorting complexes, required for transport (ESCORT) (Cazzonelli *et al*., 2006). MON87411 which developed by Monsanto can effectively inhibit the growth of western corn rootworm (*Diabrotica virgifera*) (Bachman *et al*., 2013) and approved in South Korea in 2016 for food, feed and processing (FFP). Many ecological risk assessments (ERA) of RNAi GMO were implemented during R&D and most experiments of dsRNA in Lab scale was performed using *in vitro* RNA transcripts or massive dsRNA product in *E. coli* system (Baronti *et al*., 2018; Choi *et al*., 2018). To conduct the risk assessment of dsRNA to target organisms, it was necessary to establish the optimal conditions of dsRNA expression in plant cells (Niehl *et al*., 2018).

Digital PCR have been widely applied for various molecular assay and biochemical study. It has developed to overcome limitations of relative quantification of real-time PCR techniques and increased the sensitivity to detect the small amounts of mutation and the copy number of target gene (Jones *et al*., 2016; Pavšič *et al*., 2016). Droplet digital PCR (ddPCR) is a new method of counting the number of copies according to amplification of the DNA by dividing a sample into thousands to tens of thousands of compartments (Collier *et al*., 2017; Ricchi *et al*., 2017). Recent advances of ddPCR for biochemistry and molecular biology led to application for Genomics, Proteomics, Cellomics, Metabolomics, and biomedical diagnosis (Barea *et al*., 2019).

In this study, first assessed three dsRNA generation plasmids and two *Agrobacterium* cell lines to investigate the efficiency of GFP and Snf7 dsRNA expression in *N. benthamiana*. In addition, the expression level of dsRNA were measured by real-time PCR and droplet digital PCR. Thus, this novel transient expression system of dsRNA in tobacco leaves might be useful for risk assessment of dsRNA to target or non-target organisms and dsRNA function in plant and pest science.

MATERIALS AND METHODS

1. Plant material and syringe agro-infiltration

Wild-type *Nicotiana benthamiana* were grown under standard green house conditions in a 16 h light/8 h dark cycle at 24°C. For all assays, 5-week old *N. benthamiana* plants were used for Syringe agro-infiltration. *Agrobacterium* cell line, GV3101 and EHA101 (EHA105) have selected for syringe infiltration. The transformed *Agrobacterium* was cultured in YEP medium containing antibiotics and incubated until absorbance of 0.4-0.8 at OD_{600} at 30°C. *Agrobacterium* cell were harvested at 4,000 rpm for 10 min and re-suspended with infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 100 μM Acetosyringone). The optical density adjusted to 0.2 at OD_{600} and co-infiltrate with p19 cell using 1 mL syringe into the leaf surface of the *N. benthamiana*. After agro-infiltration, the plants placed onto plant growth chamber (16 h light/8 h dark cycle at 24°C) from 48 h to 120 h and harvested the infected leaves each indicated time. Three independent experiments have perform and each experiment contains at least three leaves.

2. Construction of dsRNA expression

For transient expression of dsRNA in tobacco leaves, *GFP* gene and *Snf7* gene have cloned into the plant vector. Three different vectors have selected for experiment: pCAMBIA1300-multi, pK7GWIW (2), and pHellsgate12. Partial fragments of *Snf7* and *GFP* genes have inserted to face each other in the opposite direction and a spacer (intron) was located in the middle to construct pCAMBIA1300-multi GFP and pCAMBIA1300-multi Snf7. For the pK7GWIW (2) vector and pHellsgate12 vector cloning, *GFP* and *Snf7* gene cloned into the pENTR-TOPO vector and performed LR reaction using the Gateway system.

3. RNA extraction and cDNA synthesis

Two or three tobacco leaves be collected after infiltration at 48, 72, 96, and 120 h and frozen at −70°C until RNA extracted. Total RNA was extraction from finely grinded the tobacco leaves using RNeasy mini kit (Qiagen, Germany) following manufacturer's manual. Total RNA amount was measured using NanoDrop ND-2000 (ThermoScientific,

USA) and electrophoresed on 2% agarose gel to confirm that RNA quality (data now shown). One microgram of total RNA were synthesized cDNA using ReverTra Ace® (TOYOBO, Japan) in a 5 μL total volume. The cDNA synthesis have carried out following protocol by the manufacturer.

4. Quantitative real time PCR

The synthesized cDNA (1 μg) was diluted five times with distilled water. The EF-1α primer used as a reference gene for GFP and Snf7 dsRNA amplification and primers used in this study are listed in Table 1. The PCR mixture contained 1 μL cDNA, 12.5 μL of 2×SYBR Green PCR Master Mix (Applied Biosystems, USA) in a final volume of 25 μL. Real-time PCR was performed in 96-well plate with StepOne Plus Real-time PCR system (Applied Biosystems, USA) and PCR condition was 1 cycle of 50°C 120 sec, 95°C 600 sec, and 40 cycles of 15 sec at 95°C and 60 sec at 60°C.

5. Droplet digital PCR

Digital PCR mixture containing 10 μL Eva Green® Supermix (Bio-Rad, USA), each primer set (final concentration of 250 nM), DNase/RNase-free water, and 2 μL diluted cDNA was performed in a 20 μL final volume. Each ddPCR mixture was loaded into each sample well of the DG8 droplet generator cartridge (Bio-Rad, USA) and 70 μL of droplet generation oil for EvaGreen (Bio-Rad, USA) was loaded into each oil well of the DG8 cartridge. The cartridge was placed inside a QX200 droplet generator (Bio-Rad, USA) and approximately 20 thousand of droplets were generated in each droplets well. The PCR plates were sealed and placed in a C1000 Touch thermal cycler with deep-well (Bio-Rad, USA). Thermal cycling conditions were 5 min at 95°C, 40 cycles of denaturation for 30 sec at 95°C and annealing/extension for 1 min at 58°C, final three step at 4°C for 5 min, 90°C for 5 min, and 4°C infinite hold. The no template control (NTC) and five negative control (distilled water) were included in each assay. After thermal cycling, the PCR plate included droplets was loaded onto the QX200 Droplet Reader (Bio-Rad, USA), identifying the fluorescent intensity of each droplet for EvaGreen fluorophore using multi-pixel photon counter. This study used a QuantaSoft software version 1.7.4 (Bio-Rad, USA) to determine the concentration of target gene in copies/μL.

RESULTS AND DISCUSSION

GFP and Snf7 dsRNA construct carrying *Agrobacterium* was infiltrated into the intercellular space of the tobacco tissue, allowing the delivery of genes into plant genomes (Grimsley *et al*., 1986). The most popular method of

Table 1. List of primers used for PCR.

Primer name		Primer sequence (5′-3′)	GC (%)	Size (bp)
Plasmid cloning	GFP Kpn1 5′	ggggtaccATGGTAGATCTGACTAGTAAAG	48.4	31
	GFP BamH1 3′	gggggatccATCTGGGTATCTTGAAAAGC	51.7	29
	GFP Sph1 5′	ggggcatgcATGGTAGATCTGACTAGTAAAG	48.4	31
	GFP Xba1 3′	gggtctagaATCTGGGTATCTTGAAAAGC	44.8	29
	GFP GW 5′	CACCATGGTAGATCTGACTAGTAAAG	42.3	26
	GFP GW 3′	ATCTGGGTATCTTGAAAAGC	40	20
	Snf7 Kpn1 5′	ggggtaccATCCATGATATCGTGAACATC	50	30
	Snf7 BamH1 3′	gggggatccGCAAAGAAAAATGCGTCGAA	51.7	29
	Intron BamH1 5′	gggggatccAAGTACTGCGATCGCGTTAAC	56.7	30
	Intron Xba1 3′	gggtctagaGCTCCTGCAGGCGGCCGCGTG	73.3	30
	Snf7 Sph1 5′	ggggcatgcATCCATGATATCGTGAACATC	50	30
	Snf7 Xba1 3′	gggtctagaGCAAAGAAAAATGCGTCGAA	44.8	29
	Snf7 GW 5′	CACCATCCATGATATCGTGAACATC	44	25
	Snf7 GW 3′	GCAAAGAAAAATGCGTCGAA	40	20
Quantitative PCR	EF1α 164F	CTCTCAGGCTCCCACTTCAG	60	20
	EF1α 164F	AAGAGCTTCGTGGTGCATCT	50	20
	GFP 76F	GATGGTGATGTTAATGGGCAC	47.6	21
	GFP 76R	GGGTAAGTTTTCCGTATGTTGC	45.5	22
	Snf7 86F	GCGTCGAAAAATAAAAGAGTTGC	39.1	23
	Snf7 86R	GTTGTAAGGGTTCCATCTATTTGTAG	38.5	26

agro-infiltration was syringe infiltration and applied to introduce the gene of interest for biochemical and molecular research. In here, investigate the efficiency of transient dsRNA expression using three different plant vectors and two *Agrobacterium* lines to establish the best system for dsRNA production in plant cell (Fig. 1).

1. Agro-infiltration into *N. benthamiana*

In plant, RNAs and Proteins can be produced by two general approaches; stable transgenic plants or transient expression. Despite of the transgene becomes inheritable and stable expression of gene, transient expression without integrating into the plant genome applied for transient production of the desired protein. This strategy had intensively studied to improve protein expression; however, the method of dsRNA production in tobacco using agro-infiltration was not well known. To investigate the efficiency of dsRNA production in tobacco cells, three different vectors were selected (pCAMBIA1300-multi, pK7GWIW (2), pHellsgate12) (Fig. 2) and infiltration was performed on using two *Agrobacterium* cell lines (GV3101, EHA101 (EHA105)).

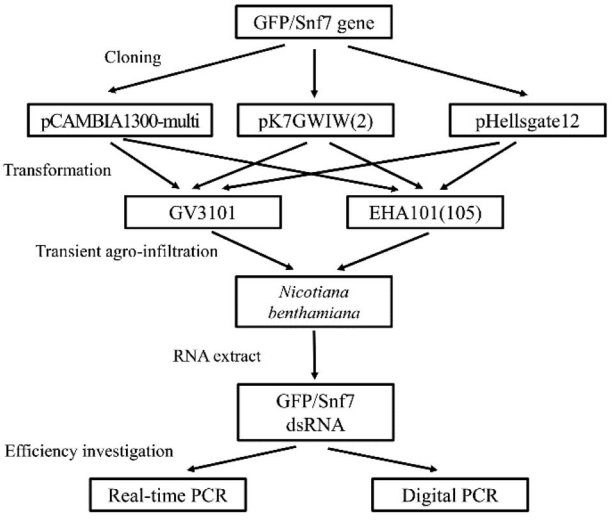

Fig. 1. Strategy of transient dsRNA expression using syringe agro-infiltration in *Nicotiana benthamiana*.

2. Real-time PCR analysis

To investigate the relative expression level of GFP and Snf7 dsRNA compare to reference gene, we tested six combination of vectors and *Agrobacterium* cell. The expression pattern of GFP and Snf7 dsRNA in three plant transformation plasmid and two *Agrobacterium* cell line was investigate using real-time PCR. The expression level of dsRNA increased with time course after agro-infiltration and the highest level was observer at 72 to 96 h and decreased after 96 to 120 h. The relative expression of each dsRNA target gene was similar in each *Agrobacterium* cells, however pHellsgate 12 GFP and Snf7 construct showed the highest expression pattern compare to pCAMBIA1300-multi and pK7GWIW (2) (Fig. 3). These results indicated that the expression level of GFP and Snf7 dsRNA did not differ from gene type and *Agrobacterium* cell line, but the plant expression vector affected the dsRNA expression in tobacco (Fig. 3). Spacer (or intron) is essential for generation of dsRNA in plant cell. The difference of each plant vector was the length of intron between target gene, pCAMBIA1300-multi (110 bp), pK7GWIW (2) (677 bp), and pHellsgate12 (957 bp). Moreover, overexpression of unnecessary dsRNA in the plant tissue is recognized as energy consuming in the cells, therefore the expression level is decreased or decomposed by various types of RNase present in plant cells (Okano *et al*., 2014). Therefore, co-infiltrated *Agrobacterium* cell which carrying the p19 to enhance the accumulation of dsRNA in infected tobacco cells (Zhao *et al*., 2017).

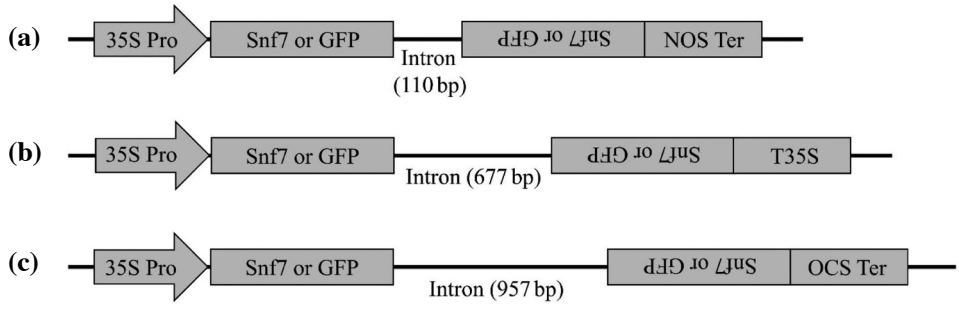

Fig. 2. Schematic diagrams of the dsRNA expression vectors. (a) pCAMBIA1300-multi GFP or Snf7, (b) pK7GWIW (2) GFP or Snf7, (c) pHellsgate12 GFP or Snf7. Pro and Ter represents promoter and terminator.

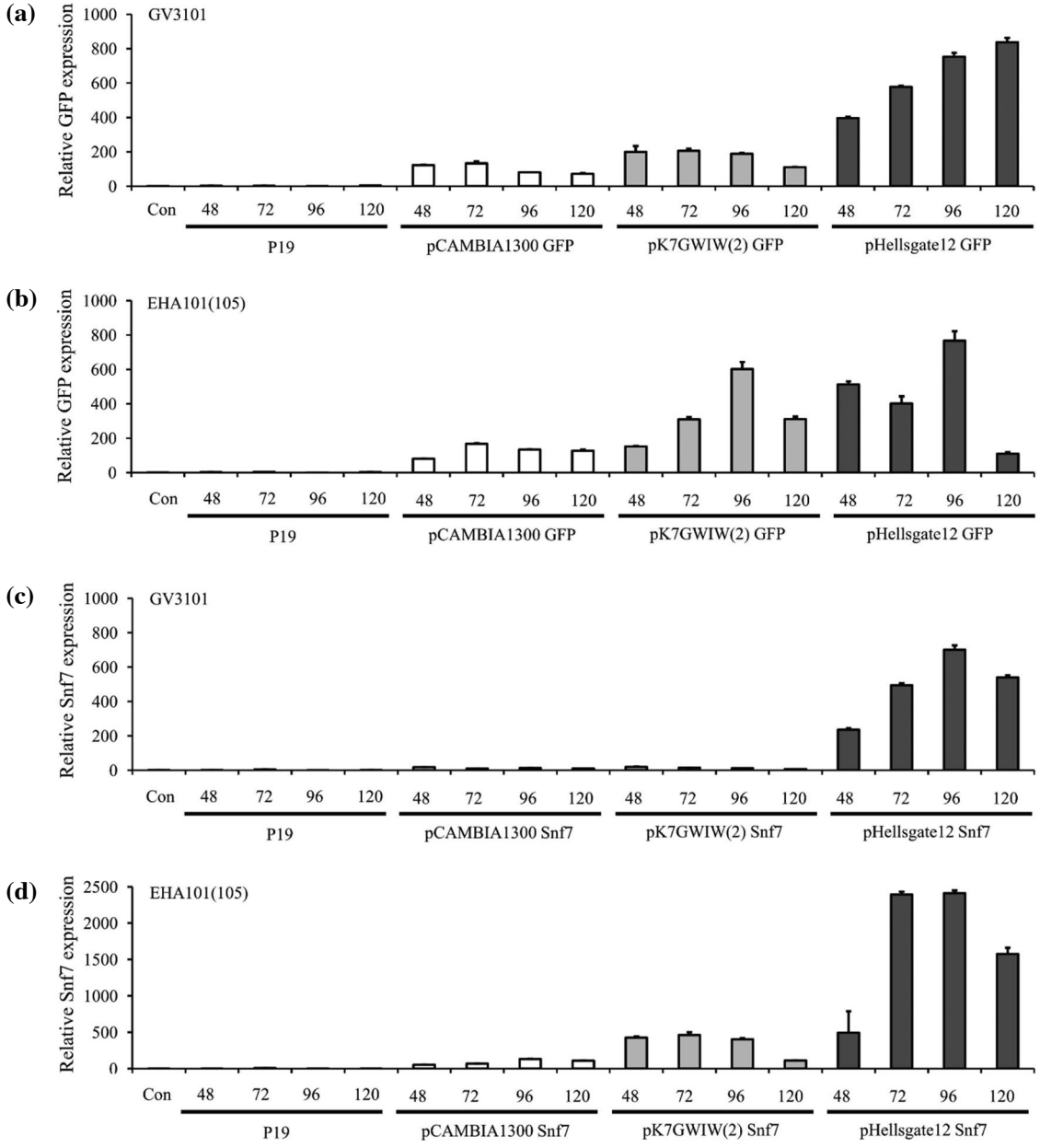

Fig. 3. Relative expression of GFP or Snf7 dsRNA in tobacco by Real-time PCR. Relative GFP or Snf7 dsRNA expression using each plant expression vector in GV3101 (a, c) or EHA101 (105) (b, d). P19 was used for negative control. Error bar represent standard error.

3. Estimation of transgene copy number by ddPCR

Supposing that the real-time PCR is a test for all genes in a single tube, the ddPCR has divided into many small individual PCRs to estimate real copy number in the samples. Digital PCR has an advantage of detecting a trace amount of variation due to segmentation in a sample (Zmienko *et al*., 2015; Taylor *et al*., 2017). To verify the real-time PCR results, we employed the ddPCR, the most precise, sensitive and accurate PCR systems. Each infiltrated samples analyzed, which were used for real-time PCR, with same primer sets. The data from duplicate experiments had separately processed in Quanta Soft version 1.7.4 software. To determine the *GFP* and *Snf7* gene copies in reaction volume, the expression value of each gene was calculated as the ratio of droplet numbers for the *GFP* or *Snf7* gene vs. the reference gene (EF-1α). The results of the statistical analysis, performed in R Bioconductor, have presented (Fig.

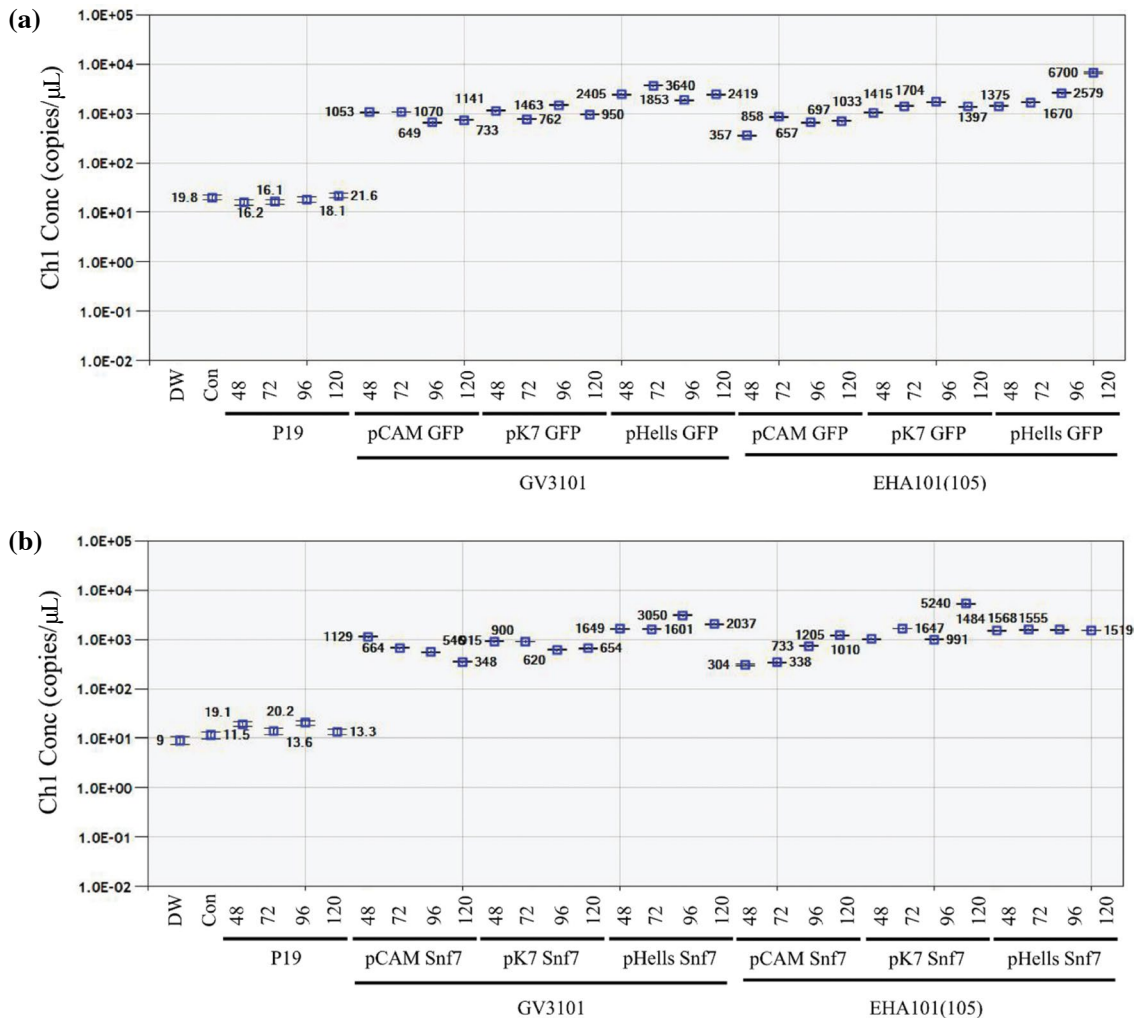

Fig. 4. Quantification of copy number by digital droplet PCR using tobacco agro-infiltration. Copy number of GFP dsRNA (a) and Snf7 dsRNA (b) using each plant expression vector and agrobacterium cell line. The concentration and poisson confidence intervals for each merged well were computed using the QuantaSoft Software 1.7.4.

4). The same as real-time PCR results, ddPCR analysis indicated that the dsRNA expression level was depend on the plant vector type, not *Agrobacterium* cell line (Fig. 5). The highest expression level of dsRNA was observed in the pHellsgate 12 *GFP* and *Snf7* construct and decreased after 96 to 120 h. The detail mechanism as to exactly how these spacer lengths regulate these difference is still required further investigation.

CONCLUSION

These novel findings showed that analysis of expression of dsRNA as a transient expression system of various combinations resulted in different expression patterns depending on the plant expression vector or *Agrobacterium*. To prove the hypothesis is that the plant vector or *Agrobacterium* cell might be affect the expression of dsRNA in tobacco. In this study, syringe infiltration were performed using three plant vector and two *Agrobacterium* cell line to investigate GFP and Snf7 dsRNA in tobacco transiently. The expression level of dsRNA was confirmed by real-time PCR and ddPRC. All PCR results indicated that pHellsgate12 vector is the most efficient system to express dsRNA in tobacco cells. The *Agrobacterium* cell type did not affect the expression pattern of dsRNA. This study suggested that the researcher or developer might be applied this *in vivo* dsRNA expression system in plant cell for ERA or biochemical study of dsRNA.

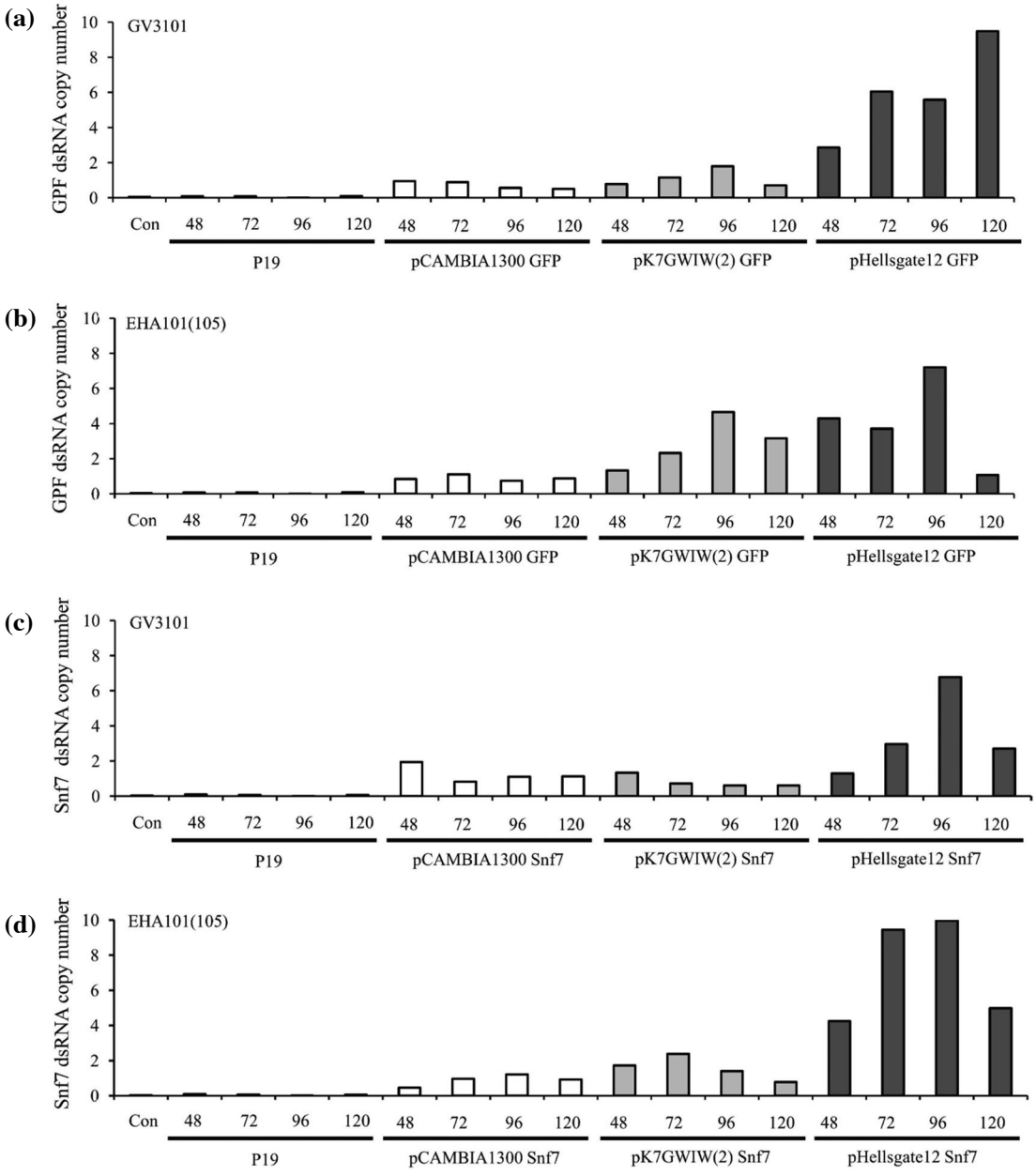

Fig. 5. Quantification of copy number by digital droplet PCR using tobacco agro-infiltration. Copy number of GFP dsRNA (a, b) and Snf7 dsRNA (c, d) using each plant expression vector and agrobacterium cell line.

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Author contribution statement DWK, WC designed the experiments. DWK, WC, HSL, HS conducted experiments. WC, DWK wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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