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GoldenBraid-compatible infectious clone of apple latent spherical virus (ALSV) and its use for virus-induced gene silencing

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Abstract

Virus-induced gene silencing (VIGS) using self-replicating viral vectors is a powerful technique for analysing plant genes and proteins. Conversely, analysing gene silencing provides insights into the infectivity, replication, movement and stability of viral vectors. This study investigates the VIGS of phytoene desaturase gene (*PDS*) in different *Nicotiana* species using a vector based on apple latent spherical virus (ALSV, species *Cheravirus mali*, ICTV 2023). The ALSV genome consists of RNA1 and RNA2, which were cloned separately into plasmids using the GoldenBraid 3.0 system and transformed into two *Agrobacterium* strains. A third plasmid containing p19, a known gene silencing suppressor, was also introduced. These three *Agrobacterium* cultures are traditionally grown separately and then inoculated together (as a mixture) in plants. Here, we describe a novel All-in-One system in which RNA1, RNA2 and p19 are co-expressed in plants after inoculation of a single *Agrobacterium* strain. A RT-qPCR analysis comparing both methods revealed that *PDS* expression is significantly different in the early phase of inoculation (6 dpi), but becomes statistically similar at later time points. The All-in-One approach therefore enables efficient co-expression of multiple targets, minimises variations in gene expression and reduces handling complexity, space requirements and costs compared to conventional co-inoculation methods.

Keywords: agroinfiltration, apple latent spherical virus, GoldenBraid system, phytoene desaturase gene, RT-qPCR, virus-induced gene silencing.

Introduction

Virus-induced gene silencing (VIGS) is a technological process in which the expression of a plant target gene is down-regulated by inoculating a plant with a recombinant virus-based vector carrying part of the coding sequence of the target gene (Baulcombe, 1999a; Burch-Smith et al., 2004). VIGS uses an RNA silencing-based defence mechanism in which double-stranded RNAs (dsRNAs) of viral origin, as templates, are processed into small interfering RNAs by Dicer-like enzymes. The resulting siRNA is incorporated into an RNA-induced silencing complex, which leads to the degradation of the RNA (viral RNA, mRNA) with sequences complementary to the siRNA. Thus, VIGS utilises foreign plant genes/targets harboured by a viral vector to produce dsRNA,

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Abbreviations: ALSV - apple latent spherical virus; dpi - day post inoculation; MMA - infiltration medium (4-morpholineethanesulfonic acid, $MgCl_2$, acetosyringone); Nb*PDS* - *PDS* gene/its part from *Nicotiana benthamiana*; *PDS* - phytoene desaturase gene; VIGS - virus-induced gene silencing.
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a source of siRNAs that triggers RNA-mediated silencing of the corresponding target gene. VIGS has proven to be a powerful and cost-effective method for functional genomics studies in plants (Rössner et al., 2022).

A large number of VIGS vectors based on plant RNA and DNA viruses for dicotyledons or monocotyledons or both have been used extensively for gene function studies in plants (Zhou et al., 2022; Zulfiqar et al., 2023). The single-stranded RNA viruses are frequently used for the development of VIGS systems due to their low molecular weight and high infection penetrance. VIGS vectors such as tobacco rattle virus (Dinesh-Kumar et al., 2003), potato virus X (Ruiz et al., 1998), cucumber mosaic virus (Inaba et al., 2011) and apple latent spherical virus (ALSV) (Igarashi et al., 2009) have proven to be efficient. In VIGS, the cDNA copy of the viral genome is introduced into the plant cell through transient *Agrobacterium tumefaciens*-mediated transformation. The strong constitutive plant promoter, frequently derived from 35S promoter of cauliflower mosaic virus, then drives host cell-dependent transcription to produce genomic viral RNA or RNAs. This viral RNA is transported to the cytoplasm where it initiates viral protein synthesis and replication. Presence of dsRNA in cytoplasm of infected cells is essential for the replication of viral genomes. These dsRNA intermediates are potent elicitors of sequence specific RNA silencing. Genes responsible for viral transmission and the elicitation of hypersensitive response are often deleted from the VIGS vectors. Otherwise, the hypersensitive reaction would mask the resulting phenotype and the vector could be transmitted from sap-sucking pests to other individuals (for a review, *see* Rössner et al., 2022).

An ideal vector for VIGS would elicit no or minimal symptoms of virus infection, have the largest possible host range, can be transmitted mechanically, be genetically stable with large coding capacity for inserts and have simple, standardised way of cloning of various inserts. In this respect, the ALSV-based vectors have many advantages over other VIGS platforms, as the virus causes latent, asymptomatic infection in most host species (Sasaki et al., 2011), has a very large host range, most inserts tend to be stable in the viral genome, induces a uniform knockout phenotype in infected plants and the VIGS persists throughout plant growth in infected plants. The ALSV-based vectors have been constructed for the expression of foreign target genes in more than 20 plant species (Igarashi et al., 2009) for various studies such as the induction of transcriptional gene silencing of a transgene and an endogenous gene by DNA methylation (Kon and Yoshikawa, 2014), the development of viral vaccines to plant viruses (Taki et al., 2013; Satoh et al., 2014) and the promotion of flowering in rosaceous fruit or legume species (Yamagishi et al., 2011; Yamagishi and Yoshikawa, 2011).

In this study, we aimed to address some of the remaining obstacles hindering the use of ALSV as a universal VIGS vector, namely cloning and agroinfiltration, to improve the efficiency of *Agrobacterium*-based delivery of the target gene. We developed a system with four different recombinant sequences in one *Agrobacterium* strain and tested it in plants. In this case, the *Agrobacterium* was first transformed with a plasmid encoding the RNA1 of ALSV. These *Agrobacterium* cells were then transformed with a plasmid encoding three targets – RNA2 of ALSV together with part of the phytoene desaturase gene (*PDS*, nt 856-1059, *GenBank*: LC543532.1) from *N. benthamiana* (Nb*PDS*) as a marker for gene silencing (Ratcliff et al., 1997; Baulcombe, 1999b; Lu et al., 2003) and a protein p19 as a suppressor of gene silencing from tomato bushy stunt virus (TBSV) to increase the virus content (Park et al., 2004; Canto et al., 2006; Mohammadzadeh et al., 2016; Jay et al., 2023). We expected that the presence of Nb*PDS* would cause virus-induced gene silencing leading to a *PDS*-deficient/bleached phenotype of the experimental plants (Gedling et al., 2018; Liu et al., 2020; Zaulda et al., 2022). We tested this expression system in comparison to standard expression by infiltration with a mixture of three different *Agrobacterium* clones carrying RNA1 of ALSV, RNA2 including Nb*PDS*, and p19, respectively. We monitored the development of viral infection using one-step RT-PCR and specific ALSV primers. In addition, we monitored the development and progression of the *PDS*-deficient phenotype both subjectively, by observing the appearance of bleached leaves, and objectively by quantitative analysis of the expression of the *PDS* gene by qPCR.

Materials and methods

Plant material: *Nicotiana benthamiana*, *N. occidentalis*, *N. glutinosa*, *N. rustica* and *N. tabacum*, cv. SR1 plants were grown in a growth chamber under controlled conditions (20°C, 16 h day/8 h night photoperiod) and used for agroinfiltration at their four-leaf stage.

Modification of ALSV vector system for GoldenBraid compatibility and generation of VIGS constructs: The original vectors containing the infectious clone of ALSV were constructed by Prof. N. Yoshikawa, Iwata University, Japan, and were designed for biolistic infection. Further rationalisation was achieved by the group of Prof. Qu and Prof. Dorrance (Department of Plant Pathology, The Ohio State University, Ohio, USA), who transformed the infectious clones into the binary plasmid pBinPlusARS resulting in the constructs pYL-AR1 (ALSV RNA1), pYL-ALSV-RNA2-Nb*PDS* (ALSV RNA2 with Nb*PDS*), allowing the initiation of infection by direct infiltration of *N. bethamiana* leaves with a mixture of three *Agrobacterium* strains carrying ALSV RNA1, ALSV RNA2 and tomato bushy stunt virus p19 suppressor of gene silencing, respectively (Gedling et al., 2018).

All used vectors were domesticated to be compatible with the GoldenBraid 3.0 system (Dusek et al., 2020). The creation of a new delta plasmid allows the simple insertion of a part (in our case the Nb*PDS* sequence) into the remaining part (in our case the ALSV vector) in a single restriction/ligation step. They were subsequently transformed into *E. coli* Top10 (*Thermo Fisher Scientific*,

Table 1. Used plasmids and strains. Kan - kanamycin, Spec - spectinomycin.

Original plasmid	Simplified term of plasmid	Concentration of antibiotics (mg/L)	*A. tumefaciens* strain	Designation of the final *A. tumefaciens*
pYL-AR1	AR1	Kan (100)	EHA105	AR1
pYL-ALSV-RNA2-Nb*PDS*	AR2-Nb*PDS*	Kan (100)	EHA105	AR2-Nb*PDS*
p19	p19	Spec (100)	EHA105	p19
pLXΔ-ALSV-RNA2-p19	pLXΔ-AR2-p19	Kan+Spec (80+100)	EHA105-AR1	AR1:AR2-p19
pLXΔ-ALSV-RNA2-Nb*PDS*-p19	pLXΔ-AR2-Nb*PDS*-p19	Kan+Spec (80+100)	EHA105-AR1	AR1:AR2-Nb*PDS*-p19

Waltham, USA) and selected by plating on Petri dishes with the appropriate antibiotics (Table 1). The next day, two colonies per plate were grown in liquid LB medium and DNA was isolated. The sequences of the plasmids were confirmed by sequencing *via* a commercial service (*Eurofin Genomics*, Ebersberg, Germany).

All used plasmids are summarized in Table 1 and schematically shown in Fig. 1*A*, the scheme of restriction/ligation step is shown in Fig. 1*B*.

***Agrobacterium* transformation:** Two batches of *Agrobacterium tumefaciens*, strain EHA105, were prepared for agroinfiltration: the first for classical agroinfiltration using a multiple mixtures of different *Agrobacterium* clones, the second for a variant in which all required plasmids/targets are contained in one *Agrobacterium* clone (All-in-One system; *see* Table 1, Fig. 1*C1*,1*C2*). For both approaches, *A. tumefaciens*-competent cells were transformed with the appropriate plasmid using the freeze-thaw method (Wise et al., 2006) and then plated on LB agar with appropriate antibiotics (Table 1) and incubated at 28°C for 2 days. Two colonies per plate were prepared for sequencing: in brief, the cells were transferred from the colony by pipetting into 100 µl PBS containing 5% *Tween20*. After incubation at 80°C for 20 min followed by centrifugation (10 000 *g* for 10 min at room temperature), the supernatant was used as template for PCR using the *Phusion*® high-fidelity DNA polymerase kit (*Thermo Fisher Scientific*) and the target DNA was amplified with the appropriate primers (Table 1). The amplified PCR products were then purified using the *QIAquick® PCR Purification* Kit (*Qiagen*, Germantown, MD, USA) and sequenced *via* a commercial service (*Eurofin Genomics*).

Agroinfiltration: Two different basic principles were used for agroinfiltration. Firstly, a classical one: *A. tumefaciens* EHA105 transformed with the plasmid AR1, AR2-Nb*PDS* and p19, respectively, were cultured separately in LB media supplemented with appropriate antibiotics (Table 1). After centrifugation (3 700 *g* for 30 min at 20°C), the pellets were resuspended in 20 mL of infiltration medium (King et al., 2015) and diluted to the required absorbance at 565 nm. The absorbance at 565 nm of the infectious particles for agroinfiltration of *N. benthamiana* by syringe is suggested to be 0.2 (Moran-Bertot et al., 2021). Due to the task of the inoculum to obtain all three components (pYL-AR1, pYL-ALSV2-Nb*PDS*, and p19), the subcultures were diluted to an absorbance of 0.6 at 565 nm and then mixed in a 1:1:1 ratio (v:v:v) so that the final effective absorbance of the infectious particles is theoretically 0.2 (Lombardi et al., 2010). Secondly, the newly developed All-in-One approach, in which *A. tumefaciens* was subsequently transformed with two different plasmids (one of which contains three target parts) was tested and compared with the classical approach above. In this approach, the *A. tumefaciens* already originally including the AR1 plasmid were transformed with pLXΔ-AR2-p19 or pLXΔ-AR2-Nb*PDS*-p19 plasmid, respectively, and subsequently the cultures were grown in LB media containing appropriate antibiotics (*see* Table 1). After confirming the respective sequence in each transformed *Agrobacterium* batch as described above, the selected clones were grown in LB media at 28°C for 2 d. After centrifugation (3 700 *g* for 30 min at 20°C), the pellets were resuspended in 20 mL of infiltration medium (King et al., 2015) and diluted to an absorbance of 0.2 at 565 nm. Groups of 8 plants of *N. benthamiana*, *N. occidentalis*, *N. rustica*, *N. glutinosa*, and *N. tabaccum* cv. SR1 were each agroinfiltrated using a 1-mL syringe without a needle, with the suspensions of *Agrobacterium* cultures pressed onto the abaxial surface of the leaves (Sparkes et al., 2006). After infiltration, the plants were stored in the dark for 24 h and then returned to the growth chamber under the conditions described above. Plants used as a negative control were infiltrated with the pure infiltration medium (MMA).

The monitoring of the *PDS*-silencing phenotype: After agroinfiltration, all plants were visually checked three times per week up to four weeks and the phenotype of the plants associated with *PDS* silencing was assessed and recorded (Table 2).

Sampling for virus detection and quantification of *PDS*: Samples were collected from the upper leaves of the plants, both for monitoring the progress of virus infection by One-enzyme RTX-PCR (Hoffmeisterová et al., 2022) and for subsequent virus titer analysis by RT-qPCR (Dráb et al., 2014). One upper leaf disc (using *Uni-core*™, 6 mm; Harris, USA) per plant, *i.e.*, 8 discs per group per pooled sample corresponding to approximately 45 - 50 mg of plant tissue were placed directly into the 2 mL microtubes (*Sarstedt*, Nümbrecht, Germany) containing approximately 0.4 g of pre-weighted *SiLibeads*® (type ZS, 1.2 - 1.4 mm, *Sigmund Lindner GmbH*, Warmensteinach, Germany). The samples were immediately placed in liquid

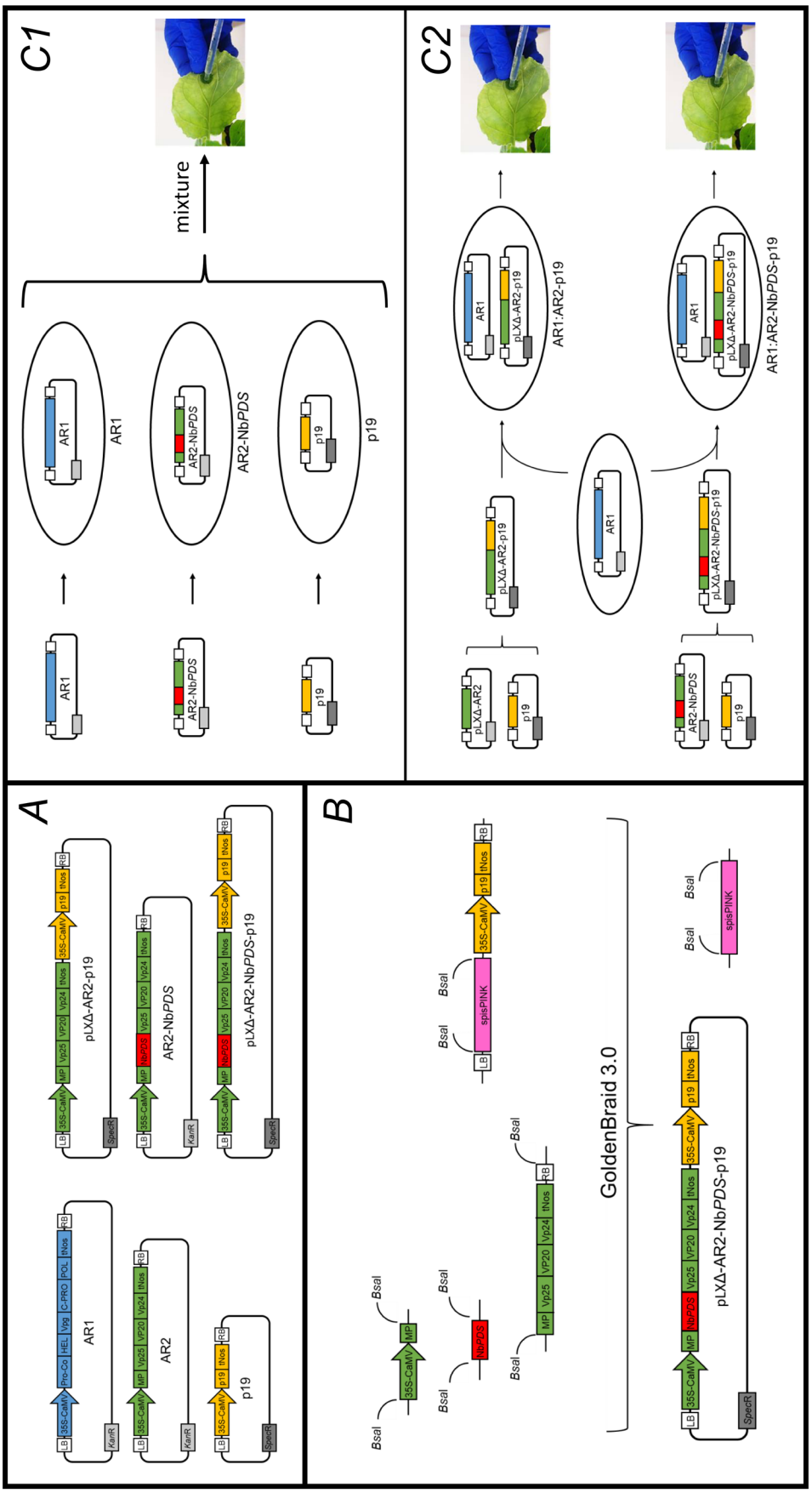

Fig. 1. Schematic illustration of used constructs, cloning using GoldenBraid (GB) 3.0 method and the processing of constructs before *Nicotiana* inoculation. *A*: All original constructs are schematically illustrated on the left, all newly cloned constructs are on the right. *B*: The principle of GoldenBraid reaction using *Bsa*I restriction enzyme. *C*: Schematic illustration of classical co-infection using mixture of three different *Agrobacterium* clones (*C1*) and the subsequent transformation of *A. tumefaciens* using different constructs and/or their combinations in the relevant order (*C2*). In blue – parts of the original pYL-AR1 (AR1) plasmid, in green – parts of the original pYL-ALSV-RNA2 (AR2) plasmid, in yellow – parts of the original p19 plasmid, in red – a 208 nt long sequence from *N. benthamiana-PDS*, in light grey – kanamycin gene of resistance (*Kan*R); in dark grey – spectinomycin gene of resistance (*Spec*R), in white – left (LB) and right (RB) borders in used plasmid, in pink – cassette including the gene for pink chromoprotein from *Stylophora pistillata* for the pink/white selection of *E. coli* colonies. 35S-CaMV - cauliflower mosaic virus 35S promoter; Pro-Co - protease cofactor; HEL - NTP-binding helicase; Vpg - virus-genome-linked protein; C-PRO - cysteine protease; POL - RNA polymerase; tNos - nopaline synthase terminator; MP - movement protein; Vp25, Vp20, and Vp24 - capsid proteins; p19 - suppressor of gene silencing from tomato bushy stunt virus.

nitrogen and stored at -80°C until processed. Samples were collected in parallel in duplicates.

The general screening of virus infection: A few days after the observation of the *PDS* deficiency phenotype on several plants (*N. benthamiana* and *N. occidentalis* at 14 dpi, *N. rustica* and *N. glutinosa* at 24 dpi) a general screening of virus infection was performed by One-enzyme RTX-PCR (Hoffmeisterová et al., 2022) using appropriate primers (Table 3) to detect the modified virus. In brief, plant samples in 2-mL microtubes containing *SiLibeads*® as described above were placed in liquid nitrogen. The plant tissue was grounded using *FastPrep*®*-24* (*MP Biomedicals*, Solon, OH, USA) – first directly after removal from the liquid nitrogen and a second time after addition of the extraction buffer (1:40, m:v). After centrifugation (10 000 *g*, 10 min, room temperature), the supernatant was used directly as a template for one-step RT-PCR using RTX as enzyme. For rapid virus detection, the primers framing the insert in the ALSV-based vector were used (RNA-2-1364_F and RNA-2-1523_R, *see* Table 3).The 25 µL reaction mixture contains 5× RTX buffer, 400 µM dNTPs, 4 µM $(NH_4)_2SO_4$, 0.4 µM 5'-primer, 0.4 µM 3'-primer and 1.2 U RTX polymerase. The reactions were carried out under the following conditions: 10 min 68°C (an equivalent to RT) directly followed by 33 cycles of PCR (98°C 10 s, 58°C 20 s, 72°C 20 s) and terminal elongation (72°C 5 min). The products were separated on a 2% agarose-TBE gel with the addition of *MidoriGreen* stain (*NIPPON Genetics EUROPE GmbH*, Düren, Germany) and transilluminated using the *ChemiDocTM MP* Imaging System (*BioRad*, Hercules, CA, USA).

RNA isolation: RNA was isolated from 45-50 µg of plant material using *RNAzol*® (*Sigma-Aldrich*, MI, USA) according to the manufacturer's instructions. In brief, plant tissue stored at -80°C was crushed in 2-mL microtubes containing *SiLibeads*® using *FastPrep*®*-24* as described above. After addition of *RNAzol*®, homogenization was performed using *FastPrep*®*-24*. After addition of H_2O and centrifugation (12 000 *g* for 15 min at 4°C), the supernatant was removed and the total RNA was precipitated with isopropanol. After centrifugation (8 000 *g* for 3 min at room temperature), the RNA/pellet was washed twice with 75% EtOH and finally resuspended in 40 µL of DEPC-treated H_2O. The concentration, spectrum and A_{260}/A_{280} ratio were measured using *NanoDrop* (*Thermo Scientific*, Wilmington, DE, USA).

***DNase*I-treatment and cDNA synthesis:** Prior to reverse transcription (RT), *DNase*I treatment was performed using the *DNase*I, *RNase*-free kit (*Thermo Scientific*) according to the manufacturer's instructions. RT was performed using the *RevertAid* RT Kit (*Thermo Scientific*) including Random Hexamer Primers according to the manufacturer's instructions. The amount of RNA was adjusted to 1 000 ng/50 µL of the RT reaction mixture and the run conditions were 42°C for 60 min, followed by 70°C for 5 min.

Quantitative PCR (qPCR): Primers detecting *PDS* were used to monitor changes in *PDS* expression while protein phosphatase 2A (*PP2A*) and the corresponding primers were used as the reference gene and for its detection (Table 3). PCR efficiency values (E) were calculated for each gene from the given slope after generating standard curves (10-fold dilutions of pooled cDNA samples) using the formula E (%) = [–1/(10slope – 1)] × 100 and considering 100% = 2 (Table 3) (Radonić et al., 2004). Amplifications were performed with a *LightCycler 480 Instrument II* (*Roche*, Basel, Switzerland) in 96-well plates (*96 Well PCR Microplate*, white, with *Sealing Film*, both *Axygen*, Reynosa, Mexico) with 10 µL reaction solutions per well using 5 µL *LightCycler*® *480 SYBR Green I Master* 2× concentrated mixture, appropriate primers (Table 3; 0.42 µM) and 1 µL cDNA template (diluted 20-fold). The cycling conditions were: 95°C for 10 min, followed by 45 cycles of 95°C for 5 s, 56°C for 30 s and finally 72°C for 10 s followed by a classical melting curve analysis. To check reproducibility, each assay was performed with two technical replicates for each of the two biological samples. The resulting cycle threshold (Ct) values were normalised to the expression of the reference gene to calculate the double delta Ct value ($2^{-\Delta\Delta Ct}$) and obtain the relative gene expression values (Livak and Schmittgen, 2001). The mean values of the expressions of *PDS* from two independent biological experiments were statistically evaluated by two-way Analysis of Variance (*ANOVA*). Significant differences are at the $P<0.05$ level.

Results

Evaluation of *PDS* silencing based on symptoms and One-enzyme RTX-PCR: Individual plants were monitored by visual observation of the appearance of the *PDS* silencing phenotype in two biological experiments in all groups three times per week (8 plants per group, Table 2). In the agroinfiltrated areas of *N. tabacum* cv. SR1 leaves necrotic lesions were observed at 4 dpi and during the time these areas become completely damaged (Fig. 2*E*). After repeating the experiment with *N. tabacum* cv. SR1, the samples from upper leaves were collected at 14 dpi from both experiments and tested by One-enzyme RTX-PCR for the presence of appropriate constructs. All *N. tabacum* cv. SR1 were PCR-negative, and therefore *N. tabacum* cv. SR1 plants were finally excluded from subsequent experiments. All mock-inoculated (MMA) plants and plants inoculated with the viral vector without the Nb*PDS* sequence (AR1:AR2-p19) showed no phenotype associated with *PDS* silencing till 29 dpi (the end of the experiment). Both tested viral vectors containing the Nb*PDS* fragment (*i.e.*, groups inoculated with the mixture of the AR1 + AR2-Nb*PDS* + p19 culture and with the modified GB All-in-One vector AR1:AR2-Nb*PDS*-p19) were able to infect the remaining *Nicotiana* plants and displayed the *PDS* silencing phenotype (bleached upper leaves, Fig. 2*A-D*). In *N. benthamiana* the symptoms appeared at 11 dpi on all plants and remained

Table 2. Monitoring of *PDS*-deficient (bleached) phenotypes of the tested *Nicotiana* plants after inoculation with the infiltration medium (MMA) or culture of *A. tumefaciens* containing AR1:AR2-p19, AR1 + AR2-Nb*PDS* + p19 or AR1:AR2-Nb*PDS*-p19 during the given time frame. dpi - day post inoculation.

Plant species	*Agrobacterium* strain	Number of *PDS*-deficient (bleached) phenotype-plants/number of plants in group in total										
		6 dpi	8 dpi	11 dpi	13 dpi	15 dpi	17 dpi	20 dpi	22 dpi	25 dpi	27 dpi	29 dpi
N. benthamiana	MMA	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
	AR1:AR2-p19	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
	AR1 + AR2-Nb*PDS* + p19	0/8	0/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8
	AR1:AR2-Nb*PDS*-p19	0/8	0/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8
N. occidentalis	MMA	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
	AR1:AR2-p19	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
	AR1 + AR2-Nb*PDS* + p19	0/8	0/8	1/8	4/8	7/8	8/8	8/8	8/8	8/8	8/8	8/8
	AR1:AR2-Nb*PDS*-p19	0/8	0/8	1/8	4/8	6/8	8/8	8/8	8/8	8/8	8/8	8/8
N. rustica	MMA	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
	AR1:AR2-p19	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
	AR1 + AR2-Nb*PDS* + p19	0/8	0/8	2/8	4/8	7/8	8/8	8/8	8/8	8/8	8/8	8/8
	AR1:AR2-Nb*PDS*-p19	0/8	0/8	0/8	0/8	0/8	1/8	3/8	3/8	3/8	3/8	3/8
N. glutinosa	MMA	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
	AR1:AR2-p19	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
	AR1 + AR2-Nb*PDS* + p19	0/8	0/8	0/8	0/8	0/8	3/8	7/8	8/8	8/8	8/8	8/8
	AR1:AR2-Nb*PDS*-p19	0/8	0/8	0/8	0/8	0/8	0/8	1/8	1/8	4/8	4/8	4/8

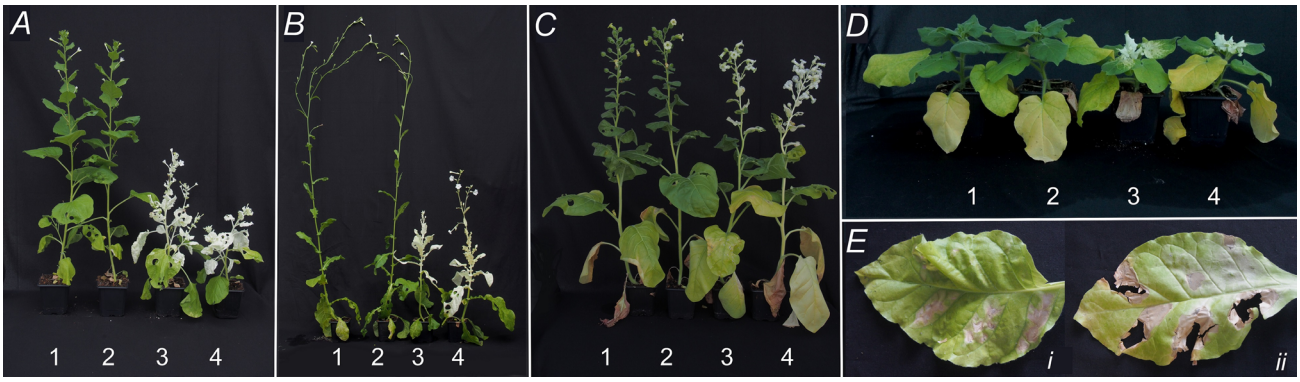

Fig. 2. Phenotype of *N. benthamiana* (*A*), *N. occidentalis* (*B*), *N. rustica* (*C*), and *N. glutinosa* (*D*) 29 dpi after inoculation with MMA (1), AR1:AR2-p19 (2), AR1 + AR2-Nb*PDS* + p19 (3) and AR1:AR2-Nb*PDS*-p19 (4), respectively. *N. tabacum* cv. SR1 leaves (*E*) after inoculation with AR1:AR2-p19 at 4 dpi (*i*) and at 7 dpi (*ii*).

unchanged until the end of the experiment (29 dpi; Table 2, Fig. 2*A*). In *N. occidentalis*, the first bleached upper leaves were detected at 11 dpi (1 plant per group inoculated with AR1 + AR2-Nb*PDS* + p19 or AR1:AR2-Nb*PDS*-p19). The amount of bleached *N. occidentalis* plants increased uniformly in both Nb*PDS*-including groups until 17 dpi, when all 8 plants of both groups were bleached and bleaching persisted until the 29 dpi (Table 2, Fig. 2*B*). In *N. rustica*, the first bleached leaves appeared at 11 dpi in 2 plants of the group inoculated with the mixture of AR1 + AR2-Nb*PDS* + p19, and the number of bleached plants in this group increased until 17 dpi, when all 8 plants were bleached. In the group of *N. rustica* inoculated with AR1:AR2-Nb*PDS*-p19, the first bleached plant appeared at 17 dpi. Thereafter, their number increased slightly to 20 dpi when 3 out of 8 plants were bleached. The remaining 5 plants stayed green until the 29 dpi (Table 2, Fig. 2*C*). In *N. glutinosa*, the first bleached leaves appeared at 17 dpi in 3 plants of the group inoculated with the mixture of AR1 + AR2-Nb*PDS* + p19, and the number of bleached plants in this group increased to 22 dpi, when all 8 plants were bleached. In the group of *N. glutinosa* inoculated with AR1:AR2-Nb*PDS*-p19, the first bleached plant appeared at 20 dpi. Thereafter, the number of bleached plants increased until 25 dpi, when 4 out of 8 plants were bleached. All 4 remaining unbleached plants remained green until 29 dpi (Table 2, Fig. 2*D*).

The end-point of presence of the virus in all plants inoculated with AR1:AR2-p19, AR1 + AR2-Nb*PDS* + p19 or AR1:AR2-Nb*PDS*-p19 and its correlation with the displaying of bleaching-symptoms was investigated by testing each plant separately by One-enzyme RTX-PCR using ALSV-2-1364_F/ALSV-2-1523_R primers (Table 3;

data not shown). The samples of *N. benthamiana* and *N. occidentalis* plants collected at 11 dpi were all tested positive, even in the case of the *N. occidentalis* plants, which were still completely green at 11 dpi. The samples of *N. rustica* and *N. glutinosa* taken at 21 dpi were positive in plants that already showed bleached leaves and also in the three *N. glutinosa* plants inoculated with AR1:AR2-Nb*PDS*-p19 that were still completely green (unbleached) at 21 dpi but showed bleached leaves after a few days (25 dpi). All AR1:AR2-p19-inoculated plants were positively tested for the presence of respectively modified virus. No positive plant was detected in the group of MMA.

The changes in *PDS* expression over time: Based on the observation of the *PDS*-deficient phenotype and analysis of the virus by the end-point One-enzyme RTX-PCR, *N. benthamiana* plants were chosen as a model because of their high susceptibility to the viral infection. Therefore, all plants per group will be most likely infected and the pooled samples in subsequent experiments will be not affected by the uninfected ones. Two further experiments were performed due to the need for qPCR analysis. Samples were collected three times per week from 6 to 20 dpi and after RNA isolation and cDNA preparation, the qPCR was performed as described above to measure the changes in *PDS* expression in plants. According to the qPCR results presented in Fig. 3, it could be concluded that the expression of Nb*PDS* was slightly reduced in plants inoculated with AR1:AR2-p19 compared to plants inoculated with MMA (not treated with any viral vector). Both groups of plants inoculated with a viral vector carrying part of the Nb*PDS* gene showed a significant decrease in the Nb*PDS* expression. In the case of plants inoculated with a mixture of the cultures AR1 + AR2-Nb*PDS* + p19, the *PDS* expression decreased more than in the plants inoculated with all in one construct AR1:AR2-Nb*PDS*-p19. Both groups of plants inoculated with AR1 + AR2-Nb*PDS* + p19 or AR1:AR2-Nb*PDS*-p19 reached the significantly similar minimum of the Nb*PDS* expression at 11 dpi and over the next few days the Nb*PDS* expression increased again, more rapidly in the plants inoculated with AR1:AR2-Nb*PDS*-p19 compared to the plants inoculated with the mixture of AR1 + AR2-Nb*PDS* + p19 cultures. Until the end of the experiment the expression of Nb*PDS* was still significantly lower in plants inoculated with AR1 + AR2-Nb*PDS* + p19 or AR1:AR2-Nb*PDS*-p19 in comparison with plants inoculated with AR1:AR2-p19 or MMA. The *PDS* expression in plants inoculated with AR1 + AR2-Nb*PDS* + p19 or AR1:AR2-Nb*PDS*-p19 was significantly different only at the beginning of the experiment (6 dpi), later the differences in *PDS* expression were similar (statistically nonsignificant at 8, 11, 15, and 20 dpi).

Discussion

The co-expression of recombinant genes using agroinfiltration by a mixture of *Agrobacterium* cultures

Table 3. List of primers. In the case of primers ALSV-2-1364_F and ALSV-2-1523_R the length of the PCR product varies depending on the insert (* - valid for AR1:AR2-p19; ** - valid for AR2-Nb*PDS*; *** - valid for AR1:AR2-Nb*PDS*-p19).

Gene	Primer name	Primer sequence (5'- 3')	Product length (bp)	Efficiency (%)	Amplification efficiency E	Accession number of the reference sequence	Position	Reference
PP2A	Nb-PP2A-qPCR_F	GACCCTGATGTTGATGTTCG	123	99.5	1.995	MF996339.1	1690-1812	Cook et al., 2020
	Nb-PP2A-qPCR_R	GAGGGATTTGAAGAGAGATTTC						
PDS	PDS-qPCR_F	GCCAAGTCAGACTGAACTCACG	118	99.5	1.995	LC543533.1	1019-1136	Rotenberg et al., 2006
	PDS-qPCR_R	CCACTGGAGCGGCAAACAC						
ALSV-RNA2	ALSV-2-1364_F	GAGGCACTCCTTATCCTATCAA	162*/411**/423***			NC_003788.1	1364-1523	Gedling et al., 2018
	ALSV-2-1523_R	TGCAAGGTGGTCGTGATTTCACT						

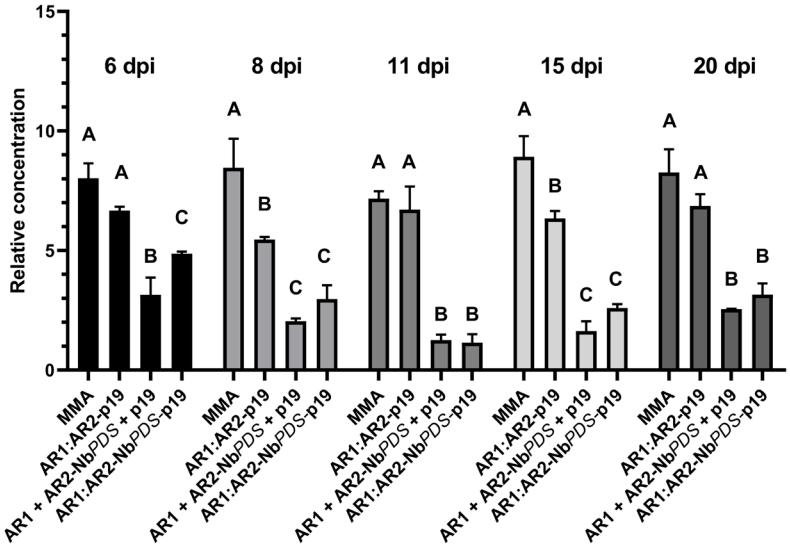

Fig. 3. Relative expression of *PDS* in *N. benthamiana* over time. Plants were inoculated with MMA, AR1:AR2-p19, AR1 + AR2-Nb*PDS* + p19 or AR1:AR2-Nb*PDS*-p19. From samples of plants collected on days 6, 8, 11, 15 and 20 were isolated RNAs, processed by reverse transcription and obtained cDNAs were analyzed by qPCR using primers to detect target Nb*PDS* and *PP2A* as a reference gene. Significant differences indicated by different letters were evaluated using two-way *ANOVA* at the $P<0.05$ level. Error bars represent the mean ± SD.

coding different vectors/targets is easily performed and widely used not only for the simple co-expression of recombinant proteins but also for studies investigating protein-protein interactions (Vézina et al., 2009; Mohammadzadeh et al., 2016; Carbonell and Daròs, 2017; Ma et al., 2021; Romsuk et al., 2022; Jay et al., 2023; Klińska-Bąchor et al., 2024). Furthermore, the co-expression of recombinant proteins is absolutely necessary in studies of some protein functions (Annamalai and Rao, 2005; Reis et al., 2018) or in the cases of expression of parts of multipartite viral genome to get the whole, functional virus as presented in this study.

In the process of necessity of co-expression *via* standard methods using the mixture of *Agrobacterium* cultures there are two basic requirements which are needed to be carried out: all used plasmids must be in the right place at the right time – they need to enter one cell all together in a short period of time to ensure the proper co-expression. In the case of two plasmids, there is a sufficient chance to target the host cell at the same time. However, if needed to use several plasmids, the probability of all of them entering the same host cell rapidly decreases.

Therefore we developed a novel method for co-expression of different recombinant proteins in *Nicotiana* plants *via* agroinfiltration using GoldenBraid-compatible parts of ALSV and compared it with the classical method of parallel expression using a mixture of *Agrobacterium* cultures described above. We chose the ALSV-based VIGS system, because the ALSV virus does not cause visually observed symptoms in plants, induces a uniform VIGS-phenotype in infected plants and the VIGS persists throughout plant growth in infected plants (Igarashi et al., 2009). ALSV has a bipartite genome containing RNA1 and RNA2, both of which are necessary for the formation, replication and systemic movement of the virus in a host plant (Li et al., 2000) and therefore for the proper folding of the infectious virus all parts of its genome must be co-expressed in the same host cell. As a marker of proper viral infection, we chose *PDS* from *N. benthamiana*, which, when knocked out, produces a distinctive bleached phenotype (Schmidt et al., 1989; Ruiz et al., 1998; Angell and Baulcombe, 1999). In addition, we also included co-expression with the protein p19, a suppressor of gene silencing widely used also as co-expressed recombinant protein (Park et al., 2004; Canto et al., 2006; Jay et al., 2023; Mohammadzadeh et al., 2023). Therefore in our newly developed system there are three components in one multifunctional plasmid (ALSV-RNA2 plasmid including Nb*PDS* and p19) transformed into *A. tumefaciens* already transformed with ALSV-RNA1 plasmid in the previous step. This robust expression system ensures the introduction of all four targets into a host cell which is crucial prerequisite for completion of the virus entry and subsequent initiation of the viral life-cycle leading to sufficient viral systemic infection and subsequent VIGS.

We tested both expression systems, the classical one with three different plasmids in three different *Agrobacterium* clones and the All-in-One using the multifunctional plasmid in one *Agrobacterium* and compared their effectiveness both in terms of the mean levels of viral infection and in terms of the effect on *PDS* levels by VIGS. Changes in the expression of Nb*PDS* were initially monitored by simply checking the *PDS*-deficient phenotype. In *N. tabacum* cv. SR1 agroinfiltrated with all variants of *Agrobacterium*-inocula used (AR1:AR2-p19, AR1 + AR2-Nb*PDS* + p19, and AR1:AR2-Nb*PDS*-p19), necrosis occurred in the agroinfiltrated areas at 4 dpi and the affected tissue was completely necrotic at 7 dpi regardless of the inoculum. This observation agrees with

the earlier finding that the p19 of tomato bushy stunt virus in *N. tabacum* cv. SR1 leads to a hypersensitive reaction and necrosis of the affected tissue (Zhang et al., 2013). In contrast, in *N. benthamiana*, the first bleached leaves occurred 11 dpi in groups inoculated with AR1 + AR2-Nb*PDS* + p19 and AR1:AR2-Nb*PDS*-p19, and in both cases all plants per group were bleached that day. This is not surprising, as the *N. benthamiana-A. tumefaciens* tandem is known to be a highly functional system for recombinant expression, including the VIGS phenomenon (Dawson and Hilf, 1992; Rotenberg et al., 2006; Senthil-Kumar et al., 2007; Bally et al., 2018; Chuang and Franke, 2022). All three other tested *Nicotiana* species (*N. occidentalis*, *N. rustica*, and *N. glutinosa*) showed the bleached phenotype later than *N. benthamiana*. In *N. occidentalis* the first bleached leaves occurred at 11 dpi in 1 plant per group of AR1 + AR2-Nb*PDS* + p19 and AR1:AR2-Nb*PDS*-p19, and 17 dpi in all 8 plants per each group. In *N. rustica* and *N. glutinosa*, the first bleached leaves occurred at 11 dpi and 17 dpi, respectively, in the groups inoculated with the mixed infection AR1 + AR2-Nb*PDS* + p19. In both species the bleached plants appeared later in the group inoculated with AR1:AR2-Nb*PDS*-p19, namely, at 17 dpi and 20 dpi, respectively. In *N. glutinosa*, for example, the efficiency of VIGS is only about 20% and the efficiency of VIGS in *N. benthamiana* is only about 50% of the efficiency achieved using VIGS of *PDS* mediated by tobacco rattle virus (TRV) (Senthil-Kumar et al., 2007), so the lower efficiency of VIGS in *N. glutinosa* in comparison with *N. benthamiana* can fit similar. As far as we know, there are no sequencing data of *PDS* from *N. occidentalis*, *N. rustica*, and *N. glutinosa*, however, we cannot relate the differences in plant-bleaching to the lower homology of *PDS* from *N. occidentalis*, *N. rustica*, and *N. glutinosa* compared to *PDS* from *N. benthamiana*, a part of whose sequence was cloned into the viral vector AR2-Nb*PDS* and AR1:AR2-Nb*PDS*-p19, respectively. In all four used *Nicotiana* species that were inoculated with the mixture of AR1 + AR2-Nb*PDS* + p19, all eight plants per group were bleached at the end of the experiment. Therefore, we believe that the sequence of the part of Nb*PDS* undoubtedly causes VIGS. The differences between the *Nicotiana* species in the dpi where the first bleached leaves per group appeared, inoculated with AR1 + AR2-Nb*PDS* + p19, are probably due to the susceptibility of the different *Nicotiana* species to agroinfiltration and/or viral infection after the formation of the first infectious virions from the plasmids introduced by *Agrobacterium*.

From the qPCR data (Fig. 3) it could be concluded that the expression of Nb*PDS* was slightly reduced in plants inoculated with the control plasmid AR1:AR2-p19 compared to plants inoculated with MMA alone and not treated with the viral vector. This may be caused by the process of viral infection *per se* during which the expression of many plant proteins is affected including proteins involved in the photosynthesis, whereas PDS participates in the carotenoid pathway (Cunningham and Gantt, 1998; Tiedge et al., 2022).

The trend of changes in *PDS*-expression levels in both groups inoculated with AR1 + AR2-Nb*PDS* + p19 and AR1:AR2-Nb*PDS*-p19 was similar: *PDS* levels decreased until 11 dpi and then reached their minimum. In plants inoculated with AR1 + AR2-Nb*PDS* + p19, both the increase and decrease were less steep when compared to plants inoculated with AR1:AR2-Nb*PDS*-p19, nevertheless, the relative levels of *PDS* expressions were from 8 dpi not significantly different in plants inoculated with AR1 + AR2-Nb*PDS* + p19 and AR1:AR2-Nb*PDS*-p19.

Using the highly robust GoldenBraid 3.0 system, we developed a novel agroinfiltration approach that enables the co-expression of multiple targets by a single *Agrobacterium* clone. This innovative method streamlines the process by enabling the simultaneous insertion and expression of multiple genes without the delays and discrepancies in expression levels that are common with traditional multiple *Agrobacterium* co-expression methods. The All-in-One *Agrobacterium* system not only simplifies handling but also significantly reduces space requirements, making it an attractive option for laboratories with limited resources. This efficiency translates into lower operating costs.

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