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Short communication

Phenotypic effects and the quantification of transcript abundance in *Petunia hybrida* 'Fantasy Blue' with virus-induced *GA$_2$ox* gene silencing

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ABSTRACT

The aim of this study was to establish an efficient virus-induced gene silencing (VIGS) protocol for the gibberellin 2-oxidase gene (*GA$_2$ox*) in *Petunia hybrida* 'Fantasy Blue' to measure the reduction in GA$_2$ox transcript levels by quantitative RT-PCR (qRT-PCR) and to investigate the phenotypes of the infected plants. *In vitro* multiplied *Petunia* plants were subjected to VIGS using tobacco rattle virus vectors. An 838-bp fragment from the *N. tabacum* gene (*NtGA$_2$ox*) was cloned in the TRV2 vector. Control plants were also infected with a TRV2 vector containing a fragment of the *E. coli β-glucuronidase* (*GUS*) gene as a nonsense sequence. The abundance of the *GA$_2$ox* and *cyclophylin* (for normalization) transcripts was determined by qRT-PCR four weeks after inoculation. Shoot tips with the five youngest leaves were used. Stem lengths were measured weekly from the day of inoculation. After four weeks, significant increases in stem elongation were observed in *GA$_2$ox*-TRV2-infected plants compared with *GUS*-TRV2-infected plants. In accordance with this observation, the normalized abundance of the *GA$_2$ox* transcript in *GA$_2$ox*-TRV2-infected plants was significantly reduced compared with *GUS*-TRV2-infected plants.

1. Introduction

Virus-induced gene silencing (VIGS) is a method used for functional gene analysis (Lu et al., 2003; Chen et al., 2004; Purkayastha and Dasgupta, 2009). Several possible virus-derived vectors may be used for VIGS, but *Tobacco Rattle Virus*-derived vectors (TRVs) are generally useful in diverse plant species given the wide host range of TRV and minimal side effects in the treated plants (Ratcliff et al., 2001). TRV vectors silence gene expression in vegetative and floral meristems. Compared with vectors derived from other viruses, TRV vectors only induce mild disease symptoms. In addition, TRV vectors infect large areas of adjacent cells. TRV is two-partite virus with two separately encapsidated RNA genomes, RNA1 and RNA2. RNA1 encodes a movement protein, replicase proteins, and a cysteine-rich protein; RNA2 encodes two non-structural proteins and the coat protein. TRV RNA1 can replicate and move systemically in the plant in the absence of RNA2; thus, it is possible to substitute a portion of RNA2 with a sequence corresponding to genes targeted for silencing (Liu et al., 2002a; Senthil-Kumar and Mysore, 2014). The TRV-based VIGS method uses binary *Agrobacterium tumefaciens* transformation vectors with T-DNA encoding TRV-RNA1 and TRV-RNA2 as well as 35S promoters for the transcription of viral sequences after the transfer of T-DNA to the plant cells. In the vector used, the sequence for the non-structural proteins in RNA2 is replaced with a multiple cloning site into which fragments of target genes can be inserted (Ratcliff et al., 2001,b; Liu et al., 2002a,b).

Petunia is one of the top-selling outdoor bedding plants worldwide with approximately 12 million plants sold in 2013 (Facts and figures 2013, Flora Holland, 2013). For comparison, sales of all other ornamental bedding plants consisted of 30 million pots in 2013 (Facts and figures 2013, Flora Holland, 2013). Various bedding plant species, including *Petunia* 'Fantasy Blue' or 'Picobella Blue', exhibit a compact growth habit; stem elongation may be beneficial for other commercial uses, including use in baskets or as ornamental ground cover plants. VIGS is a convenient method for rapid analysis of gene function through silencing and has been used to silence the *Petunia MKS1* gene (Gargul et al., 2015). This work describes the effect of *GA$_2$ox* VIGS on stem-length and other aspects of the phenotype of *Petunia hybrida* 'Fantasy Blue' as well as the associated reduction in *GA$_2$ox* transcript abundance as determined by qRT-PCR.

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2. Materials and methods

2.1. Preparation of VIGS-vector constructs for plant infection.

A TRV-based vector system (Liu et al., 2002a; Ratcliff et al., 2001) was used to investigate the effect of *GA$_2$ox* gene silencing on the phenotype of *Petunia hybrida* 'Fantasy Blue'. A 1268-bp fragment of the *Nicotiana tabacum GA$_2$ox1* gene (acc. no.: AB125232.1) was amplified by PCR (forward primer, 5′CGGCAAATACTTGTCACTGAT3′; reverse primer, 5′TTTGCCTATGAACTTGTTTCATTAAC3′) and cloned into the T-easy vector (Promega Co., Madison, WI, USA) according to the manufacturer's protocol. The plasmid containing the *GA$_2$ox* fragment was digested with *Eco*RI (Thermo Scientific/Fermentas, Vilnius, Lithuania). The 838-bp fragment was purified by agarose gel electrophoresis and inserted into *Eco*RI-digested TRV2 vector. The ends of the *Eco*RI-digested TRV2 vector were dephosphorylated with FastAP^{TM} Thermosensitive Alkaline Phosphatase (Thermo Scientific/Fermentas) according to the manufacturer's protocol. Another TRV2 vector was prepared with a 798-bp fragment of the *β-glucuronidase* gene that was obtained from the pBI121 binary vector (acc. no. AF485783) by PCR amplification (forward primer, 5′TTTTTGTCACGCGCTATCAG3′; reverse primer, 5′CAACGAACTGAACTGGCAGA3′) to serve as a nonsense sequence (NS). As a control for the infection procedure, the *PDS* (*phytoene desaturase*) gene from *N. tabacum* was used as a reporter that causes leaf photo-bleaching (data not shown). All of the TRV2 constructs were assembled as described above and used to transform *Agrobacterium tumefaciens* strain GV3101. The bacterial cultures with TRV2 constructs and helper TRV1 constructs were prepared as described in Gargul et al. (2015). Plant infection was performed with harvested *Agrobacterium* cultures re-suspended in 10 mM $MgCl_2$ with 150 μM acetosyringone. Equal volumes of TRV1- and TRV2-becterial cultures were mixed and used for *Agro*-infiltration at the abaxial side of the leaf. All of the fully developed leaves on each plant were infiltrated. The experiments were performed twice with 20 plants per treatment.

2.2. Evaluation of phenotypes and the degree of gene silencing

Plants were maintained at 20 °C day/18 °C night temperatures and an average relative humidity of 70% under approximately 150 μmol m^{-2} s^{-1} of white lite with 16 h light/8 h dark cycles. Stem lengths were measured weekly after infection with *Agrobacterium*. After four weeks, infected tips, including the five latest leaves, from selected *Petunia* plants were picked for RNA isolation. RNA extraction and first strand cDNA synthesis was performed as described in Gargul et al. (2013). Quantitative RT-PCR mixtures were prepared at a final volume of 20 μL containing 0.5 ng of cDNA template, 0.25 μM forward primer and reverse primer, 2 U DCSHot DNA Polymerase (DNA Cloning Service, Hamburg), 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl, 0.15 mM each dNTP (Jena Bioscience, Jena, Germany), and SYBR Green diluted 1:20,000 from the originally supplied stock solution SYBR Green I (Roche Applied Science Co. Mannheim, Germany). The following primers were used: 5′GAAGCCATCAAATTCTTCTCCTC3′ (forward) and 5′TTCGACCAAACCAACATCG3′ (reverse) for *GA$_2$ox* transcript; 5′AGGCTCATCATTCCACCGTGT3′ (forward) and 5′TCATCTGCGAACTTAGCACCG3′ (reverse) for the *CYP* (*cyclophylin*, Mallona et al., 2010) housekeeping reference gene transcript. The amplicon sizes were 117 bp for the *GA$_2$ox* primers and 111 bp for the *CYP* primers. To normalize the transcript levels, *CYP* and *GA$_2$ox* expression levels were detected concomitantly in the *GA$_2$ox*-TRV2- or NS-TRV2-treated samples using appropriate primer combinations. The DSCHot polymerase was thermally activated at 95 °C for 10 min to prevent nonspecific amplification, the extension of non-

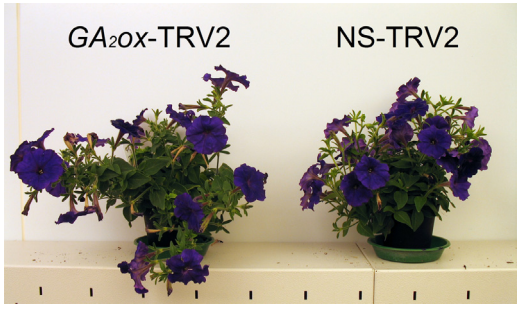

Fig. 1. Phenotypic comparison of *Petunia* plants with *GA$_2$ox*-TRV2 and NS-TRV2 (nonsense sequence) vectors at day 32 after infection. The stem length of *GA$_2$ox*-TRV2 treated plants was 13 cm and 22 cm in NS-TRV2 treated plants.

specifically annealed primers, and the formation of primer-dimers at low temperatures during PCR setup. Thermal activation was followed by 45 cycles of denaturation for 10 s at 94 °C, annealing for 30 s at 60–70 °C, and elongation for 30 s at 72 °C. Following the final PCR cycle, the specificity of PCR amplification was assessed by performing a melting curve analysis (from 68 to 95 °C). The PCR conditions were optimized for high amplification efficiency. Reactions were performed with the use of a Rotor Gene 3000 real-time thermal cycler (Corbett Life Science Co./Qiagen, Sydney, Australia), and data analysis was performed using the Rotor Gene software (6.1.81). The fold change in the *GA$_2$ox* transcript normalized to the *CYP* transcript between samples from *GA$_2$ox*-TRV2- and NS-TRV2-infected plants was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). A total of three independent repetitions of the qRT-PCR reactions were performed for three independent, randomly chosen plant samples. Each sample was represented by three technical replications (three reaction tubes) during the qRT-PCR procedure.

3. Results and discussion

The main stem lengths of the *Petunia* plants infected with *GA$_2$ox*-TRV2 and NS-TRV2 constructs clearly differed (Figs. 1 and 2). A statistically significant ($P < 0.05$) difference regarding main stem length, which averaged 1.6 cm, was observed on day 28 after infection. On day 42, the average difference had increased to 3.44 cm

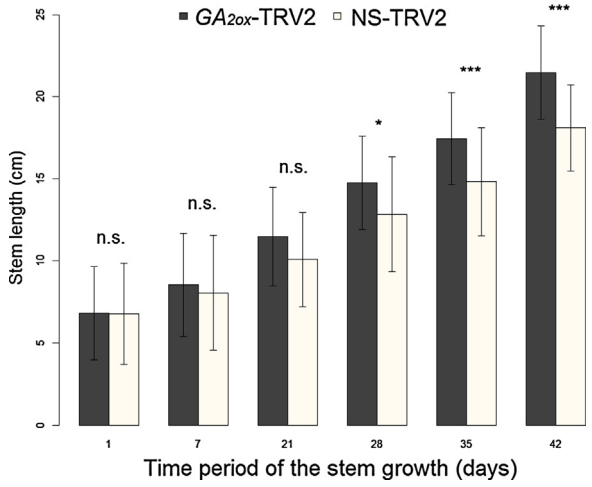

Fig. 2. Stem length comparison (cm) of *Petunia* plants infected with *GA$_2$ox*-TRV2 and NS-TRV2 vectors. Statistical analysis of stem length measurements in VIGS-treated plants was performed as previously described by Gargul et al. (2013); significance codes: '***', $P < 0.001$; '**', $P < 0.01$; '*', $P < 0.05$; '.', $P < 0.1$ and ' ' $P < 1$ based on log-transformation and a two-factorial analysis of variance. The length of the stems was measured weekly. Mean ± SD ($n = 20$) are shown.