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Development of a *Broad bean wilt virus 2*-based expression vector for gentian☆

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ABSTRACT

Japanese gentian is one of the most important ornamental flowers in Japan. Its most attractive trait is the vivid blue flower, whose color results from polyacylated anthocyanins. Other important traits in gentian breeding include flower shape, flowering time, and overwintering characteristics. To show the molecular mechanism by which these traits are regulated, transgenic gentian plants are often generated by *Agrobacterium*-mediated transformation. However, this method is not suitable for all gentian cultivars because of the difficulties in regeneration and/or transformation. Also, it takes a long time to obtain transgenic plants and analyze their phenotypes. Therefore, it is important to develop a more efficient method to analyze gene function in gentian. In the present study, we developed a novel viral expression vector for efficient gene functional analysis in gentian using *Broad bean wilt virus 2* (BBWV-2) infectious cDNA. First, we expressed two reporter genes, *GFP* and *GUS*, in gentian using the BBWV-2 vector. Fluorescence from GFP was observed in various organs including the leaf, flower, root, and overwintering bud. β-Glucuronidase (GUS) activity was detected in the inoculated leaf, but not in upper uninoculated leaves because the *GUS* insert was unstable in the BBWV-2 vector. Next, we inoculated the BBWV-2 vector carrying *GtMYB3*, a transcription factor regulating anthocyanin biosynthesis, fused with *SRDX*, which encodes the EAR transcriptional repression domain. Expression of *GtMYB3-SRDX via* the BBWV-2 vector partly changed the gentian petal color from blue to white. The expression levels of genes encoding anthocyanin biosynthetic enzymes (flavanone 3-hydroxylase, dihydroflavonol-4-reductase, anthocyanidin synthase, flavonoid 3′,5′-hydroxylase, and anthocyanin 5,3′-aromatic acyltransferase) were markedly lower in the white parts of petals than in the blue parts. These results clearly indicated that *GtMYB3-SRDX* expressed *via* the BBWV-2 vector was functional in gentian flowers. This BBWV-2-based gene expression system enabled us to analyze a phenotypic change in gentian flower color within a few months.

1. Introduction

Gentians belong to the genus *Gentiana* in the family Gentianaceae. There are more than 300 gentian species, which are distributed worldwide (Struwe, 2014). Gentians have been widely used for ornamental and medicinal purposes, and as an ingredient in some beverages (Köhlein, 1991). Gentian is one of the most important ornamental flowers in Japan. Japanese gentian is a perennial plant that produces vivid blue flowers from July to October in Japan. Most gentian cultivars have been bred from *Gentiana triflora* and *Gentiana scabra* over the last 30 years. The blue color of gentian flowers results from the accumulation of polyacylated anthocyanins such as gentiodelphin (Goto et al., 1982; Hosokawa et al., 1997). The biosynthetic pathway of flavonoids, including anthocyanins, in gentian flowers has been extensively studied at

Abbreviations: BBWV-2, *Broad bean wilt virus 2*; GFP, green fluorescent protein; GUS, β-glucuronidase; CRES-T, Chimeric REpressor gene Silencing Technology; CHS, chalcone synthase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthase; F3′5′H, flavonoid 3′,5′-hydroxylase; 5/3′AT, anthocyanin 5,3′-aromatic acyltransferase; MES, 2-(*N*-morpholino)ethanesulfonic acid.

☆ The BBWV-2 vectors are available from Masahiro Nishihara (mnishiha@ibrc.or.jp)

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the molecular level (Nakatsuka et al., 2010, 2014, 2005a, 2005b). In addition to flower color, other traits such as flower shape, flowering time, and overwintering characteristics are important for gentian breeding, and their molecular mechanisms have also been investigated (Hikage et al., 2010; Imamura et al., 2011, 2014; Nakatsuka et al., 2015; Takahashi et al., 2014). The production of stable transgenic plants with over-expression or knockdown of a target gene is frequently used to evaluate gene function (Nishihara et al., 2014). Two transformation systems, *Agrobacterium* (Mishiba et al., 2005) and particle bombardment (Hosokawa et al., 2000), have been established to produce stable transgenic gentians. However, there are several problems when these methods are applied to gentians. First, *Agrobacterium*-mediated transformation is unsuitable for many gentian cultivars because they are difficult to regenerate and/or show low transformation efficiency. Second, it takes a long time, usually more than one year, to obtain transgenic plants. It takes even longer to observe the floral phenotypes in stable transgenic gentian because gentian is a perennial plant with a long juvenile growth phase. Finally, strong transgene silencing is frequently observed in transgenic gentians (Mishiba et al., 2005). Therefore, it is important to develop a more efficient method to analyze gene function in gentian.

Viral vectors have been used for studies on gene functions (Purkayastha and Dasgupta, 2009; Robertson, 2004) and have been used to produce foreign proteins including vaccines (Canizares et al., 2005) in plants. This system does not require regeneration of the plant, and the target gene can be knocked down or over-expressed simply by inoculating the virus into the plant. The virus-induced gene silencing (VIGS) method has been used to knock-down mRNA expression in several families of ornamental plants. For example, *Tobacco rattle virus* was used for VIGS in California poppy (*Eschscholzia californica*) in the family *Papaveraceae* (Wege et al., 2007), and *Thalictrum* (Di Stilio et al., 2010) and *Aquilegia coerulea* (Gould and Kramer, 2007) in the family *Ranunculaceae*. Similarly, *Cucumber mosaic virus* (CMV) was used for VIGS in petunia in the family *Solanaceae* (Koseki et al., 2005). Recently, a VIGS system using an *Apple latent spherical virus* (ALSV) vector was developed for gentian. The suppression of the class C *MADS-box* gene *GsAG1* resulted in a morphological change from a single to a double flower (Nakatsuka et al., 2015). Several viral vectors have been developed to over-express a target gene. For example, CMV for *Nicotiana benthamiana*, *Arabidopsis thaliana,* and soybean (Fujiki et al., 2008; Matsuo et al., 2007; Zhao et al., 2000), *Tobacco mosaic virus* for *N. benthamiana* and *Nicotiana tabacum* (Culver et al., 1993; Dawson et al., 1989; Rabindran and Dawson, 2001), *Potato virus X* for *N. benthamiana* and *Nicotiana clevelandii* (Baulcombe et al., 1995; Chapman et al., 1992), *Clover yellow vein virus* for *N. benthamiana* and broad bean (Masuta et al., 2000), and *Tobacco etch virus* for tobacco (Dolja et al., 1992).

Previously, we isolated and analyzed the *Broad bean wilt virus 2* (BBWV-2) Ty isolate from gentian plants cultivated in Iwate Prefecture, Japan (Atsumi et al., 2013). The BBWV-2 Ty isolate does not induce symptoms in gentian under our experimental conditions. We constructed an infectious cDNA from the BBWV-2 Ty isolate and used it to develop an easy and efficient system to inoculate *N. benthamiana* using *Agrobacterium* (Atsumi et al., 2013). In this study, we developed a viral expression vector from an infectious cDNA of BBWV-2 for gentian. The BBWV-2 vector was assessed by expressing reporter genes encoding green fluorescent protein (GFP) and β-glucuronidase (GUS). We successfully changed gentian flower color from blue to white within a few months by expressing the artificial chimeric transcriptional repressor, GtMYB3-SRDX, which is known to suppress the transcription of anthocyanin biosynthetic genes. These results demonstrate that the BBWV-2 vector is an effective tool for gene functional analysis in gentian.

2. Materials and methods

2.1. Plant materials

The gentian cultivars ‘Giovanni’, ‘Madjiel’, ‘Maciry’ (*G. triflora*) and ‘Albireo’ (*G. triflora* × *G. scabra*), and the breeding line, Bzc-1 (the origin is unknown) were grown as pot plants in a closed greenhouse at 22 °C under a 16-h light/8-h dark photoperiod, with light supplied by sunlight and high-pressure sodium-vapor lamps (son-T Agro 400, Philips Licht GmbH, Hamburg, Germany) and/or by *in vitro* culture in an incubation room at 22 °C under a 16-h light/8-h dark photoperiod, with light supplied by cool white fluorescent lamps. Murashige and Skoog (MS) medium containing 3% (w/v) sucrose and 0.2% (w/v) gellan gum was used for *in vitro* culture. All pot plants were inoculated at the seedling stage. *In vitro* plants for inoculation were propagated by tissue culture. *N. benthamiana* plants grown in a greenhouse were used to propagate the recombinant BBWV-2 vector.

2.2. Construction of BBWV-2 vectors

To construct the BBWV-2 vector, we used two infectious cDNAs, pBBR1 and pBBR2, harboring the full-length RNA1 and RNA2 sequences of the BBWV-2 Ty isolate, respectively (Atsumi et al., 2013). We created pBBR2d, in which a cloning site was introduced between the movement protein (*MP*) and large coat protein (*LCP*) genes in pBBR2. To construct pBBR2b (the prototype vector for pBBR2d), we amplified a fragment containing an *Xba* I site, the 3′ terminal region of the *MP* gene, the first putative cleavage site, a cloning site (*Xho* I/*Sal* I), the second putative cleavage site, the 5′ terminal region of the *LCP* gene, and the *Sca* I site from pBBR2 as the template, using the primers 1475 and 1478 from a mixture of two PCR products amplified with the primer pairs 1475 & 1476 and 1477 & 1478. The amplified fragment was digested with *Xba* I and *Sca* I and introduced between the *Xba* I and *Sca* I sites of pBBR2 to generate pBBR2b. To construct pBBR2d, we amplified the *Xba* I site, the 3′ terminal region of *MP*, and the first putative cleavage site and the cloning site (*Eco* RI/*Sma* I/*Sal* I) from pBBR2b as the template using the primer pair 1475 & 1685. The amplified fragment was digested with *Xba* I and *Sal* I and introduced between the *Xba* I and *Sal* I sites of pBBR2b to generate pBBR2d. The first putative cleavage site was derived from the native viral sequence located between the *MP* and *LCP* genes. The second putative cleavage site encoded the same amino acids but most of its codons were modified to minimize the chances of homologous recombination and improve the stability of the viral RNA (Fig. 1A).

To construct pBBR2d/GFP-FLAG, we amplified *GFP* with three copies of the *FLAG* epitope tag-coding sequence (819 nucleotides) from pTA/GFP-3FLAG as the template using the primer pair 1499 & 1485. Then, pTA/GFP-3FLAG was created by introducing the *GFP* fragment into pTA/XhSp-3FLAG in which a DNA fragment harboring *Xho* I site, *Spe* I site, Gly/Ser linker, three copies of *FLAG* epitope tag coding sequence, and *Xba* I site (5′-CTCGAGatggccACTAGTggtggaagtggaggtagtggtggaagtggaggtagtATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATCATGATGGGtaaTCTAGA-3′) was introduced between *Xho* I and *Spe* I sites of pTA7001 (Aoyama and Chua, 1997). To create pBBR2d/GUS, we amplified the *GUS* (1809 nucleotides) sequence from pBI121/GUS (Jefferson et al., 1987) as the template using the primer pair 1667 & 1668. For pBBR2d/GtMYB3-SRDX, we amplified *GtMYB3-SRDX* (960 bp) from pSMABR-AtACT2pro-GtMYB3-SRDX (Nakatsuka et al., 2011) as the template using the primer pair GtMYB3-BBR2_U_EcoRI & GtMYB3 + SRDX-BBR2_L_SalI. These amplified fragments were digested with *Eco* RI and *Sal* I,