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Research article

RNAi-mediated suppression of *dihydroflavonol 4-reductase* in tobacco allows fine-tuning of flower color and flux through the flavonoid biosynthetic pathway

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ABSTRACT

To examine flux regulation in the flavonoid pathway of tobacco flowers, we suppressed two genes for dihydroflavonol 4-reductase (*NtDFR 1* and *2*) by RNA interference (Ri)-mediated post transcriptional gene silencing in pink-flowered tobacco. Two phenotypes were observed, pale pink (DFR-Ri_PP)- and white (DFR-Ri_W)-flowered lines. The relative mRNA levels of *NtDFR* genes in DFR-Ri_PP and DFR-Ri_W lines were reduced by 79%–95% relative to non-transformed (NT) plants. DFR-Ri_W lines had five-fold higher levels of small interference RNAs compared to DFR-Ri_PP lines. Expression of eight structural genes in the flavonoid pathway was significantly increased in DFR-Ri_W lines but not in DFR-Ri_PP lines based on quantitative RT-PCR. Anthocyanin contents correlated with flower color, with a reduction of 72%–97% in DFR-Ri_PP and DFR-Ri_W lines. Decreases in anthocyanin in flower were proportional with reductions of proanthocyanidin content in seeds. Two pale pink lines, DFR-Ri_PP 17 and 20, with anthocyanin decreases and the lowest level of *DFR* gene silencing, had higher (dihydro) flavonol production than a white flowered line, DFR-Ri_W 67. This finding suggests that suppression of *DFR* can increase the total levels of flavonoids due to (dihydro) flavonol biosynthesis. Our observations that higher suppression of *DFR* had a greater influence on the expression of flavonoid biosynthetic genes demonstrates the key role of *DFR* in the pathway and allows selection among DFR-Ri lines for plants with specific gene expression profiles to fine-tune flux through the pathway.

1. Introduction

Flower color is a main target for modification in plant breeding. Mutation breeding with ion-beam irradiation has been to obtain new flower colors in several ornamental plant species, including fragrant cyclamen (*Cyclamen hederifolium*), blue toreina (*Torenia fournieri*), and carnation (*Dianthus caryophyllus*) (Hase et al., 2012; Nakayama et al., 2012; Okamura et al., 2012). While successful, ion-beam irradiation technologies use high energy levels and can generate random mutations in addition to altering the target trait. For example, *xwf1* tobacco plants with an acyanic flower color also show growth retardation due to spontaneous mutation of dihydroflavonol 4-reductase (*NtDFR1*), deletion of the *NtDFR2* gene and semi-dwarf phenotype-related fragments (Kazama et al., 2013). Flower color changes based on genetic manipulation have been reported in a variety of plant species including rose (*Rosa hybrid*), cyclamen, and chrysanthemum (*Chrysanthemum morifolium*) (Katsumoto et al., 2007; Boase et al., 2010; Nakatsuka et al., 2010). Generally, two mechanisms are employed to modify color: alteration of the endogenous flavonoid-derived pigment profile or production of exotic pigments. Magenta flower color was achieved in gentian by down-regulating the endogenous gentian flavonoid 3′,5'-hydroxylase (F3′5′H) to decrease delphinidins and increase cyanidins (Nakatsuka et al., 2010). A blue-hued rose was produced by down-regulating the endogenous rose *DFR* while over-

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expressing an exogenous pansy *F3′5′H* and a Dutch iris *DFR* to accumulate delphinidin pigments (Katsumoto et al., 2007).

DFR is an important flavonoid biosynthetic enzyme and has long been known to contribute to the biosynthesis of the flavonoids, anthocyanins, and proanthocyanidins (Grotewold, 2006). DFR competes with flavonol synthase (FLS) for their common substrate, dihydroflavonols, which are branched metabolites of flavonols and leucoanthocyanidins. Genetic engineering to over-express or suppress expression of *DFR* genes changes the visible color pigments anthocyanins and proanthocyanidins, and can affect accumulation of colorless human health metabolites called flavonols (Luo et al., 2016; Tian et al., 2015).

In this study, we used RNAi constructs to suppress two tobacco *DFR* genes and generate transgenic tobacco plants with pale pink (PP) or white (W) flowers (as compared to wild-type pink (P) flowers). We compared the expression level of genes related to the flavonoid biosynthetic pathway and analyzed small interference RNA (siRNA) levels for endogenous *DFR* genes in these lines. Further analyses of anthocyanins, proanthcyanidins and flavonoid contents were performed to better understand the relationship between anthocyanin and flavonol accumulation under different *DFR* gene suppression levels.

2. Materials and methods

2.1. Vector construction

A RNAi-inducing vector was constructed to down-regulate *DFR* gene expression. Target sequences were identified from sequence alignment of tobacco *DFR1* (EF421429) and *2* (EF421430). *NtDFR1* and *NtDFR2* fragments were amplified using primer sets NtDFR1-P (5′-GACGCGCAGATGAATTCCC-3′/5′-GCTGGAACTGTCGGATCCAA-GAGCAC-3′), and NtDFR2-P (5′-CACCATGCCCAAGAACACCAAAAG-3′/5′-CTGGATCCCCATTGGTTGACTTTC-3′), respectively. The 719 base pair (bp) fragment from *NtDFR2* was combined in the sense orientation with the 361 bp fragment from *NtDFR1* in the anti-sense orientation by cloning into pENTR/D-TOPO (Invitrogen, Carlsbad, CA) using *Eco*RI and *Bam*HI digestion. The resulting *pENTR-DFR-Ri* construct was incorporated into the Gateway® destination vector pB7WG2D (VIB-Ghent University, Ghent, Belgium), and the final plant expression vector was designated as *pDFR-Ri* (Fig. 1).

2.2. Plant transformation

The *pDFR-Ri* expression vector was transferred into *Agrobacterium tumefaciens* strain LBA4404. Tobacco (*Nicotiana tabacum* cv. Xanthi) transformation was performed via the leaf-disc method as described by Lim et al. (2007). Briefly, tobacco seeds were surface sterilized and grown on solidified half strength Murashige and Skoog (MS) medium. The plants were then grown in a growth room under 16 h light/8 h dark cycles at 26 ± 1 °C for two months. Tobacco leaf discs obtained from the cultured plants were submerged in the *Agrobacterium* mixture. Explants were cultured on shoot-inducing medium containing 10 mg/L phophinotricin to identify transgenic events. Regenerated shoots were subsequently transferred to root-inducing MS medium containing 10 mg/L phophinotricin prior to being transplanted in a greenhouse and cultivated to maturity. The transgenic tobacco plants were grown to maturity and seeds were obtained by self-pollination. Transgenic T_3 lines were developed by successive self-pollination of T_0 tobacco plants, which were selected based on flower color, and used for further analysis. Flower color and flower bud size of the transgenic tobacco plants was confirmed at three flower growth stages, stage 1 (S1) to stage 3 (S3) (Fig. 2).

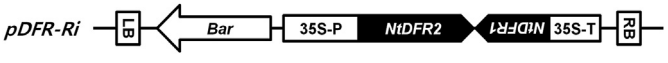

Fig. 1. Schematic representation of the binary vector used for *DFR* gene suppression with RNA interference.

2.3. Total RNA extraction and quantitative real time (qRT)-PCR analysis

Total RNA from tobacco flowers and leaves was prepared using TRIzol Reagent (Invitrogen) and first-strand cDNA was generated using the cDNA EcoDry kit (Clontech, Madison, WI). qRT-PCR conditions and gene-specific primers, with the exception of those for *flavonoid 3′-hydroxylase* (*F3′H*) and *UDP-glucose: flavonoid 3-O-glucosyltransferase* (*UFGT*) genes, were as in previous studies (Lim et al., 2013, 2016). The qRT-PCR primers for the *F3′H* and *UFGT* genes were: F3′H–P (5′-AGCCATAGTCAAGGAAACC-3′/5′-CTCA-CAACTCTCGGATGC-3′) and UFGT-P (5′-CAATGTTTGGGATGGTGTCA-3′/5′-TTCCTCCTCTGCCTCTTTCA-3′), respectively. Gene expression was normalized using the *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) gene as an internal reference. Three biological replicates were examined for each sample.

2.4. Extraction and detection of small interference RNA

The small RNAs from tobacco flowers were purified from total RNAs according to the modified method (Martin et al., 2005). Briefly, 200 mg of petal tissues from tobacco flowers was homogenized in 900 μL TRIzol Reagent (Invitrogen) with a mortar and pestle. The extract was incubated at room temperature for 10 min and then centrifuged at 14,000 g for 10 min. The supernatant was extracted with an equal volume of phenol/chloroform/isoamyl alcohol [25:24:1 v/v] solution and then re-extracted with one volume of chloroform. LiCl was added to the supernatant (2 M final concentration) and the sample was mixed and retained at −20 °C overnight. The sample was thawed, mixed, and centrifuged at 10,000 g for 5 min. One-fourth volume of isopropanol was added to the supernatant, mixed well, incubated on ice for 10 min, and centrifuged at 14,000 g for 10 min. The supernatant was transferred to a new tube, 3/5 volume of isopropanol was added, mixed, and incubated on ice for 10 min prior to centrifugation at 14,000 g for 10 min. The resulting pellet was washed with 1 mL 80% (v/v) ethanol, dried, dissolved in water, and used for siRNA expression analysis.

The small interference RNAs (siRNAs) of the tobacco *DFR* gene were detected according to the modified method of Sohn et al. (2014). The small RNA (7 μg) was fractionated by electrophoresis on a 17% (w/v) denaturing polyacrylamide gel (85 mm wide, 80 mm long, 1.5 mm thick) containing 7.5 M urea and 1 × Tris–Borate–EDTA (TBE) buffer (pH 8.0). The gel was pre-run at 60 V for 1 h. The samples were loaded and the gel was run at 150 V for 1 h and subsequently transferred onto Hybond™ N+ nylon membrane (GE Healthcare, Pittsburgh, PA) using a semi-dry transfer unit (Bio-Rad Laboratories, Hercules, CA). The transferred RNA was UV cross-linked and the membranes were dried and used for hybridization. Hybridization was conducted in Rapid-hyb™ buffer (GE Healthcare) following the manufacturer's instruction. Probes of tobacco *DFR* genes were labelled with [α-^{32}P]-dCTP (Perkin-Elmer, Waltham, MA) by the random prime labelling system (Rediprime™ II, GE Healthcare). To visualize the bands, the membrane was exposed to a Phosphor imaging screen (GE Healthcare) for 2 d and then analyzed by the Molecular Imager FX system (Bio-Rad, USA).