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Original Research

RNAi-induced silencing in floral tissues of *Petunia hybrida* by agroinfiltration: a rapid assay for chalcone isomerase gene function analysis

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Abstract: Variegation in flower color is commonly observed in many plant species and also occurs on Petunia (*Petunia hybrida*) as an ornamental plant. Variegated plants are of highly valuable in the floricultural market. Agroinfiltration is an Agrobacterium-mediated transient assay for the analysis of gene function and genetic modification in leaves, flowers and fruit tissues of various plants. Transient RNAi-induced silencing by agroinfiltration has been developed in leaves and fruits of several plant species. Here we report the establishment of a transient hairpin RNAi-induced silencing system for color modification assay in floral tissues of Petunia with different colors. *chi*RNAi construct was cloned into the pBI121 vector under the control of 35S promoter. Transient RNA silencing of *chi* in the floral tissues of Petunia was induced by delivering 530 bp *chi* hairpin RNAs (hpRNAs) into the petals of flowers using agroinfiltration. Impaired anthocyanin accumulation and reduction of endogenous mRNAs of the corresponding targets were observed in the infiltrated areas of the petals of four colors of Petunia. Silencing of the endogenous *chi* mRNAs was highly effective in reduction of *chi* gene and anthocyanin accumulation. This transient silencing system is a prototype for modification of the anthocyanin biosynthetic pathway in Petunia through *chi* gene suppression.

Key words: Transient Transformation, Transient RNAi, Gene silencing, Anthocyanin degradation, Flower color, Petunia.

Introduction

Biotechnology provides plant breeders an additional tool to improve various traits desired by growers and consumers of horticultural crops. It also offers genetic solutions to major problems affecting horticultural crops and can be a means for rapid cultivar improvement (1). Studies carried out in model systems such as Arabidopsis (2, 3), Petunia (4, 5) and Snapdragon (6, 7) have provided ample molecular information on the regulation and biosynthesis of flower pigments in the past decades. A great number of non-classical plants are offering distinctive insights into molecular mechanisms involved in flower pigment formation; thereby leading to further understanding of the extent flower color varies among plants (8). Flower color is one of the most significant characteristics in ornamental plant breeding. New varieties of various plants in relation to their flower color have been obtained by monitoring the expression levels of genes involved or regulating the flavonoid and anthocyanin biosynthesis pathway (9, 10).

Flavonoids possess significant and diverse biological functions. They are the major pigments for flowers, fruits, seeds, and leaves (11, 12). They are natural products that contain a C6-C3-C6 carbon framework (13) and are synthesized by a branched pathway that yields both colored and colorless compounds. Some of the colorless compounds, however, enhance visible light absorption and therefore act as co-pigments (14, 15). Flavonoids comprise several different classes of compounds such as chalcones, flavones, flavonols and anthocyanins (16, 17). Anthocyanins are a major group of pigments involved in a large range of plant functions. They are largely responsible for diverse pigmentation from shiny orange to pink, red, violet and blue colors in flowers and fruits (18, 19). Their biosynthesis, as part of the larger phenylpropanoids pathway, is well characterized at both the biochemical and molecular level (2). The gene encoding chalcone isomerase (CHI) is among the genes and enzymes identified in the flavonoid pathway. This enzyme catalyzes the isomerization of naringenin chalcone into the corresponding flavanone (20). CHI enzyme belongs to the family of isomerases, specifically the class of intramolecular lyases. Chalcone isomerase has a core 2-layer alpha/beta structure and has attracted much attention recently due to its role in stress response and pigment production (21, 22).

RNA interference (RNAi) technology has been used to silence anthocyanin related genes in many plant species in order to produce new color flowers. Silencing of any gene in the anthocyanin biosynthetic pathway can result in reduced or inhibited anthocyanin production (1). RNAi technology is an effective gene silencing method and a powerful tool for studying gene function and development of new traits by transformation of viral RNA or hairpin RNA (hpRNA) constructs into

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plants. The processing of dsRNA into 21-23-nt small interfering RNAs (siRNAs), and the mediators of RNAi, triggers cognate mRNA degradation (23). The hpRNAi methodology simply requires a transgene construct containing an inversely-repeated sequence of the target gene flanked with a promoter and terminator which effectively function in plants. The comparative simplicity of hpRNAi has made it to be more widely used for transient and stable gene silencing (3).

Stable plant transformation either by *Agrobacterium*-mediated transformation or by biolistics is required tissue culture to regenerate transgenic plants. Transient transformation offers a fast and simple method in many plant species and is used in testing gene function and the impacts of genetic modification on phenotypes as compared to the generation of stable transformed plants (24). Transient transformation which is an alternative to the generation of stably transformed plants provides rapid and facile functional analysis of transgenes. This method involves the transferring of T-DNA into plant cells by *Agrobacterium tumefaciens.* Transient expression of a transgene can be analyzed directly in infiltrated tissues within a few days (25). Agroinfiltration has been proven very useful for a real success story in the induction of local and eventually systemic silencing. Agroinfiltration is an Agrobacterium-mediated transient assay used for the analysis of gene function and genetic modification in leaves, flowers and fruit tissues of various plants. However, transient RNAi-induced silencing by agroinfiltration has been developed in leaves and fruits of several plant species (26).

In this study, we report the establishment of a transient RNAi-induced silencing system by agroinfiltration with hpRNA construct in floral tissues of Petunia with blue, red, pink and white flowers. The *chi* in petals was targets of gene silencing induced by T-DNA construct producing *chi* hpRNAs. Coloration and reduction of the target RNA were determined in infiltrated flowers.

Materials and Methods

Plant material

Potted plants of *P. hybrida* were grown under standard greenhouse conditions (16-17°C night temperature and 21-24°C day temperature and photoperiod 16/8 (light/dark)). Expanded white, pink, red and blue petals of Petunia were separately used in this experiment.

Construction of hpRNAi binary vectors

The RNAi construct including the 530 bp (corresponding to nucleotides 1430–1960 of X14589) cds of the chalcone isomerase (*chi*) gene and 741 bp of *pdk* gene as intron between *chi* sense and antisense were used for transient RNAi-induced silencing (Figure 1). Designed RNAi construct was cloned into pBI121vector (Bioneer, South Korea).

Agrobacterium transformation

The pBI121-*chi*530 plasmids were introduced into *A. tumefaciens* strain LBA4404 by electroporation method (27). Colonies of *A. tumefaciens* carrying the desired plasmid were screened by PCR with specific primers for *chi* gene. The sequences of forward and reverse primers were 5' CTATCCTTCGCAAGACCC 3' and 5'CAG-

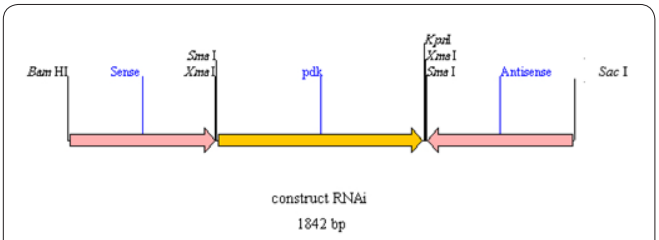

Figure 1. Schematic map of hpRNA expression construct for transient RNAi-induced silencing in *Petunia hybrida* flowers.

CATTCGCAGTGCCAGTT 3', respectively. Primers contain section of 35S promoter and sense sequence of the *chi* gene. PCR was performed as follow: based on the following temperature profile: 94ºC 1 min, 58ºC 1 min, 72ºC 1 min for 34 cycles.

Agroinfiltration

A single colony of *A. tumefaciens* containing *chi*RNAi construct was cultured in LB medium supplemented with 50 mg/l kanamycin and 100 mg/l rifampicin at 28 °C for 48 h and agitated at the speed of 250 rpm. After attaining a cell density of OD_{600}=1.5, the cultured cells were collected by centrifugation at 4500 rpm for 10 min. The supernatant was discarded and the pellet was re-suspended in infiltration medium (2 mM NaH_2PO_4, 0.5% glucose, 50 mM MES, 100 µM acetosyringone and pH= 5.6) adjusted to OD_{600}=0.5. The suspension was then incubated at room temperature for 2 h. In order to increase the transformation efficiency, 0.05% Silwet L-77 was added as surfactant to the agroinfiltration medium. Petals were treated with cotton soaked in agrobacterium cell suspension. In order to assess if the observed phenotypic changes resulted from the recombinant agrobacterium containing *chi*RNAi or were created by the scratches with needles on the petals, a number of samples in four colors of Petunia flowers were treated with infiltration free bacteria suspension. The infiltrated flower plants were placed in a growth chamber for a day at 25ºC before being analyzed. Each agroinfiltration experiment was repeated three times.

Detection of *chi*RNAi vector in transformed petals

PCR analysis was performed to evaluate the presence of foreign gene in petal tissues of transformed Petunia plants. Genomic DNA was extracted from the petal tissues of transgenic plants and used as template for PCR analysis, using specific primers (mentioned above). In this experiment, the DNA of the treated plants, bacterial plasmid (positive control) and the DNA of wild type and treated plants with free agrobacterium infiltration medium (negative control) were used. PCR was performed based on the following temperature profile: 94ºC 1 min, 58ºC 1 min, 72ºC 1 min for 34 cycles.

RNA Extraction and cDNA synthesis

Total RNA was extracted separately from control and infiltrated Petunia petal tissue using Denazist Column RNA Isolation Kit (#S-1020, Iran). Extracted RNA was treated with DNase I (Thermo Scientific DNase I #EN0525, USA) at 37°C for 30 min to remove probable DNA residues. The synthesis of first strand cDNAs was carried out using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (#K1622) based on the ma-

Table 1. Used primers for the amplification of *chi* and *ef1A* genes.

Primers/Genes	chalcone isomerase (*chi*)	elongation factor (*ef1A*)
Forward primer (5'→3')	TCTCCTCCAGTGTCCGTTAC	TAAGTCTGTTGAGATGCACC
Reverse primer (5'→3')	ACAAACTTCCCTTCTATCTCCAG	CTGGCCAGGGTGGTTCATGAT

nufacturer's protocol.

Quantitative real-time PCR

To perform the real-time quantitative PCR (qrtPCR), primers for the amplification of *chi* gene were designed to amplify a 137 bp fragment (Table1). Expression of the *chi* gene was quantitatively analyzed using a Real-Time PCR system (BioRad). The qrtPCR was carried out with the SYBR® Premix Ex Taq TM II (Perfect Real Time) kit (TaKaRa #RR820L). Each reaction contained 2 µL of the first-strand cDNA as template, in a total volume of 20 µL reaction mixture. The amplification program was carried out at 95°C/10 min followed by 95°C/15sec, 60°C/15sec and 72ºC/30sec (40 cycles).

Elongation factor (*ef1A*) was selected as housekeeping gene in order to normalize the qPCR data and the following specific primers with product size of 180 bp were designed and used (Table1).

The experiments were repeated three times on independently isolated mRNA preparation as biological repeats. Real time PCR experiments were done with two identical technical replications in order to increase the reliability of gene expression analysis. The calculation of relative gene expression was done based on methods that explain expression ratio equal to $2^{-\Delta\Delta Ct}$ (28).

Results

In this study, agroinfiltration was used to assess the transient expression of gene silencing with the *chi*RNAi construct in *P. hybrida*. Due to the delicacy and thin tissue of petals, treatment of samples using syringe without needle for transferring the bacterial suspension was not possible. Instead, some crashes were created on the petals using a needle, thereafter the petals were treated with cotton wool soaked with bacterial suspension.

Morphological characteristics of four colors of *P. hybrida* after agroinfiltration

Each petal of Petunias with various colors (white, pink, red, and blue) was considered for two sections. Half of the petals were proposed as control and the other half were treated with a solution infiltration. After a day of post infiltration, the infiltrated petals with *A. tumefaciens* strain LBA4404 containing *chi*RNAi started to develop impaired anthocyanin accumulation. Areas with markedly reduced pigmentation were clearly observed in both the upper and lower surfaces of the petals. The silencing phenotype was evenly observed in all replications. After infiltration solution inoculation, no change was observed in petals of white flowers. Conversely, color change was observed in other treated petals with different colors. The most color change was observed in the pink flower. So that the most sections of treated petals displayed a white color (Figure 2).

Also, in the red and blue flowers, phenotypic changes were observed though with less severity than those in pink flowers. Additionally, the treated parts of the petals were brighter than the control sections (Figure 3). This can be attributed to the pigment intensity created in the two types of flowers which made the observation of phenotypic change difficult.

Furthermore, no phenotypic changes were observed in petals of treated plants with free agrobacterium infiltration medium (Figure 4).

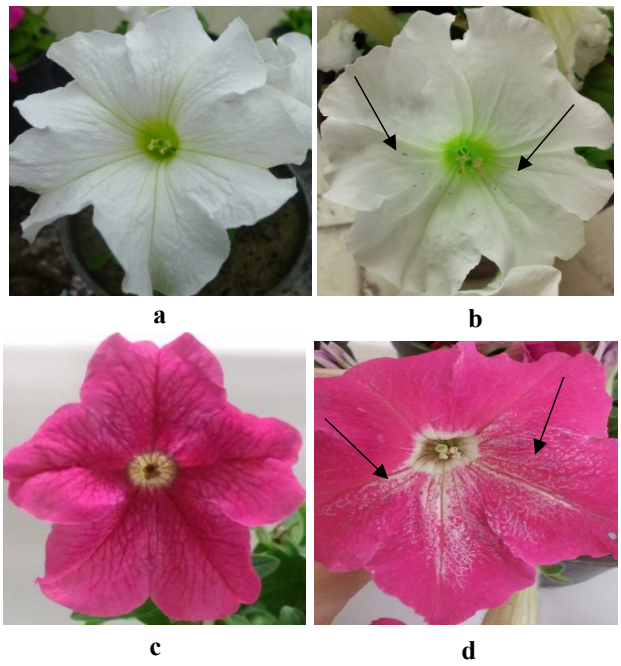

Figure 2. Observed phenotypic changes in white and pink Petunia flowers, 1 day after agroinfiltration. a) Control of white flowers; b) treated white flowers with agroinfiltration medium; c) Control of pink flowers d) treated pink flowers with agroinfiltration medium.

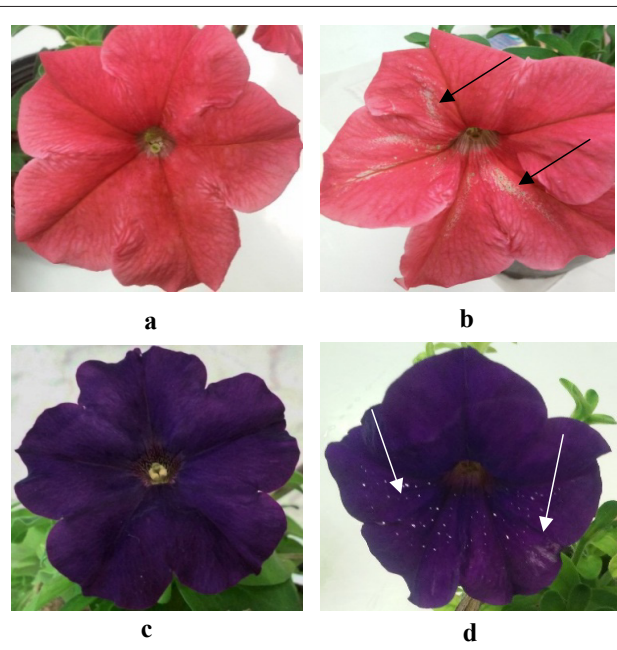

Figure 3. Observed phenotypic changes in white and pink Petunia flowers, 1 day after agroinfiltration. a) Control of red flowers; b) treated red flowers with agroinfiltration medium; c) Control of blue flowers d) treated blue flowers with agroinfiltration medium.

Figure 4. Treated flowers with free agrobacterium infiltration medium in four colors of Petunia. No phenotypic changes were observed after treatment with infiltration medium free agrobacterium.

DNA extraction and PCR tests

In the treated petals, amplified fragment was observed which is similar to bacterial plasmid, whereas no bands were observed in the wild type (Figure 5). In transient transformation, foreign DNA is not inserted in the genome of the host; therefore, the observed band in PCR was due to the bacterial plasmids. In this method, the PCR test was conducted to ensure that the bacterial suspension was carried into the plant tissue but this does not confirm the insertion of the transgene in the genome of the host plants.

Quantitative real-time PCR analysis

To examine the suppression of the *chi* gene at the transcriptional level, quantitative RT-PCR was carried out to analyze the level of the endogenous *chi* transcripts in the petals agroinfiltrated with *chi*RNAi. The expression levels of the endogenous *chi* at the reduced pigmentation areas were markedly lower than the normal pigmentation areas of the same infiltrated petals. So that, in the treated petals of pink, red and blue colors of Petunia, 3.4, 3.9, and 4.9 reduction in gene expression respectively, was observed compared to their controls (Figure 6).

In other words, RNAi construct of the *chi* gene in all the examined petals acted efficiently and reduced the expression of this gene. Investigation of the *chi* gene expression of treated and untreated white flowers showed no significant differences among the samples ($p \leq 0.05$). This can be attributed to the absence of the *chi* gene activity in white color production. Alternatively, this gene is inactive in the production pathway of white pigments. The white color results from the activity of the chalcone synthase (*chs*) gene and the aggregation of chalcone in this color of Petunia flowers.

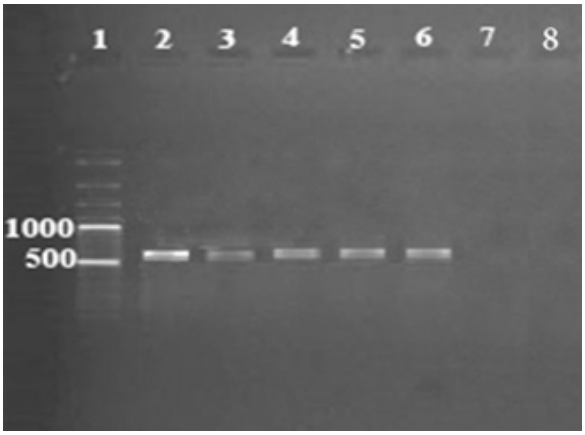

Figure 5. The 607 bp amplified fragment in treated plants of Petunia with PCR. 1) 100 bp plus DNA Ladder; 2) pBI121 plasmid containing *chi*RNAi construct; 3-6) treated plants with agroinfiltration; 7) treated plants with free agrobacterium infiltration solution; 8) wild type of Petunia.

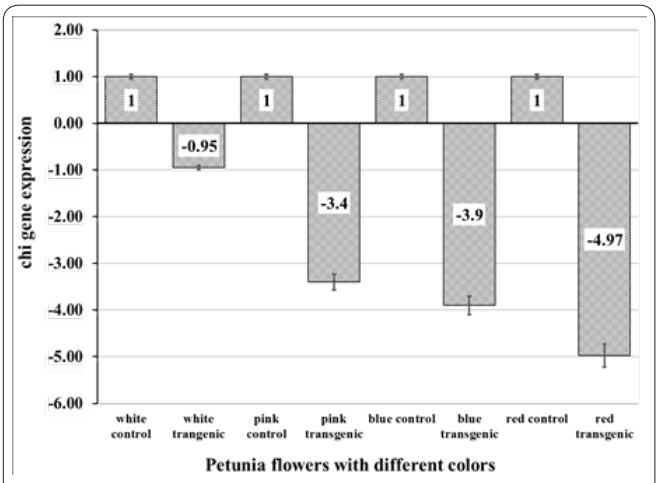

Figure 6. *chi* gene expression in treated plants with agroinfiltration containing *chi*RNAi construct in comparison with their controls.

Discussion

The aim of this study was development of transient RNAi-induced silencing in the floral tissues of Petunia by agroinfiltration with hpRNA construct for determination of flower color modification through the engineering of anthocyanin biosynthetic pathway. We have analyzed the transient hpRNAi-induced silencing of the endogenous Petunia *chi* gene. Characterization of *chi* expression profiles in four colors of Petunia petals reveals that the expression of *chi* is developmentally regulated in the flower and is associated with flower coloring. These expression patterns correspond to anthocyanin accumulation in each flower color. Anthocyanin production is stopped by the suppression of *chi* gene and anthocyanin biosynthesis related genes.

The absence of anthocyanin accumulation was also reported as a result of transient *chs*-hpRNAi-induced silencing in Antirrhinum flowers (29) and strawberry fruits (30).

In Snapdragon (*Antirrhinum majus*) two RNAi constructs for *chs* gene and RoseaI transcription factor were designed by biolistics into the petal tissue of opening flowers. Silencing led to the creation of large areas on the petal tissues that were lacking pigments (29).

A. tumefaciens strain LBA4404 has been broadly employed for stable transformation of Petunia (31, 32), Tobacco (33), Antirrhinum (29) and Phalaenopsis (34). For RNAi-based transient gene silencing experiments, the floral tissues were infiltrated using the established agroinfiltration procedure for transient gene expression (25, 29). Transient RNAi silencing in the petals of Petunia was triggered by hpRNA derived from the hpRNA

construct driven by the 35S promoter. The CaMV35S promoter has been often employed to derive the transgene expression in many plant species (35). Agroinfiltration is a rapid and transient assessment method which employs *A. tumefaciens* for analyzing gene function and genetic modification in leaves, flowers, and fruits of various plants. Evaluation of gene function with transient RNAi-induced silencing by agroinfiltration has been developed in many plant species (36).

For example a transient expression system for Gerbera (*Gerbera jamesonii*) petals based on the Agrobacterium infiltration protocol was developed using the reporter genes β-glucuronidase (*gus*) and green florescence protein (*gfp*). Results revealed the incapability of using the *gfp* as a reporter gene for transient expression study in Gerbera flowers. However, the *gus* reporter gene was successfully utilized for optimizing and obtaining the suitable agroinfiltration system in Gerbera flowers (37).

The chalcone synthase *(chs)* gene is located upstream of the *chi* gene in the biosynthesis pathway of anthocyanin in plants. The product of the CHS enzyme activity is chalcone which leads to the production of white color in flowers. In other words, the *chs* gene is the key gene in the biosynthesis of pigments in plants (38). Therefore, it seems that the *chi*RNAi construct have been ineffective on white flowers and no changes were observed in the color of these Petunia flowers. This hypothesis is consistent with the results obtained in this study.

The CHI enzyme is required for catalyzing the conversion of chalcone into naringenin, which is essential for anthocyanidin biosynthesis. Therefore, silencing of the *chi* expression should lead to a reduction in anthocyanin production (22). *chi* gene silencing was also evident in the petals of Petunia which correspond to the phenomenon of *chi* expression in petals as mentioned above.

A transient hairpin RNAi-induced silencing system established for color modification assay in floral tissues of Dendrobium Sonia 'Earsakul' with purple and white bi-colored flowers. In this experiment, *chs* and dihydroflavonol 4-reductase (*dfr*) which are required for anthocyanin synthesis, were cloned into the hairpin-RNAi vectors. Transient RNA silencing of *chs* and *dfr* in the floral tissues of D. Sonia 'Earsakul' was induced by delivering 436 bp *chs* and 470 bp *dfr* hpRNAs into the sepals and petals of flower buds at early developmental stages using agroinfiltration. Impaired anthocyanin accumulation and reduction of endogenous mRNAs of the corresponding targets were observed in the infiltrated areas of the sepals and petals. Silencing of the endogenous *chs* and *dfr* mRNAs was highly effective at the early stages of mRNA synthesis and anthocyanin accumulation (39).

In Gerbera (Gerbera jamesonii) the vacuum agroinfiltration protocol has been applied on the cultivar 'express' for evaluating the transient expression of the two genes involved in the anthocyanin pathway (Iris-*dfr* and Petunia-*f3'5'h*), which is responsible for the color in flowers. In comparison to the control, transient expression results showed change in the anthocyanin pigment in all infiltrated flowers with color genes. Additionally, blue color was detected in the stigma and pollen grains in the infiltrated flowers (38).

In general the concentration of the target mRNA in a particular tissue could be a factor that influences silencing efficiency. At very low levels of gene expression, small amounts of the silencing target, mRNA, could be completely degraded by the RNA-induced silencing complex (RISC), whereas the presence of higher amounts of the target mRNA may result in incomplete silencing, allowing some residual functional mRNA to be translated into the corresponding protein (40).

Our results provide crucial information for the first time about RNAi silencing for the *chi* gene in *P. hybrida* with a transient expression system.

This research demonstrated that agroinfiltration for transient gene silencing using the hpRNA construct has been successfully established for floral tissues of *P. hybrida*. The hpRNA construct was developed for *chi*-RNAi silencing of one of the key genes in the anthocyanin biosynthetic pathway in Petunia flowers. The silencing effect on flower pigmentation is visible after a few days post infiltration. The silencing of the *chi* gene is a prototype for the modification of the anthocyanin biosynthetic pathway in Petunia through gene suppression. This strategy could also be useful for rapid functional analysis of other genes involved in flower development.

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