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Selection of suitable lily cultivars by using needle agroinfiltration for blue flower production

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ABSTRACT

Lily is one of the most important flower crops that it lacks blue-purple flowers due to the absence of the flavonoid 3′,5′-hydroxylase gene and deficiency of delphinidin accumulation. In this study, the possibility of transient expression of F3′5′H gene and delphinidin accumulation were examined by agroinfiltration method in petals of six commercial lily cultivars. Three sizes of the flower buds and new blossomed flowers were harvested and measured for anthocyanidin content by the high-performance liquid chromatography to determine the appropriate stage for agroinfiltration. Then, the petals were infiltrated at the best stage with two vectors namely pBI121-35S carrying only F3′5′H and pBIH-35S-Del2 harbouring F3′5′H, DFR and ANS genes. The results indicated that the largest buds and new blossomed flowers showed the highest contents of anthocyanins in all subjected cultivars. Therefore, new blossomed flowers were injected with two different expression vectors. The HPLC analyses showed the highest delphinidin contents were detected in the two cultivars 'Brindisi' and 'Gaucho' by 77.63 and 43.60 $\mu g\ g^{-1}$ fresh weights, respectively, when they were infiltrated with the pBIH-35S-Del2 construct. Real-Time PCR confirmed the results of anthocyanin measurements properly. Consequently, these two cultivars were selected as the appropriate hosts for lily stable transformation programmes.

Abbreviations: ANS: Anthocyanidin synthase; CaMV: Cauliflower mosaic virus; CHS: Chalcone synthase; CTAB: Cetyl trimethyl ammonium bromide; DFR: Dihydroflavonol 4-reductase; DHK: Dihydrokaempferol; DHM: Dihydromyricetin; F3′H: Flavonoid 3′-hydroxylase; F3′5′H: Flavonoid 3′, 5′-hydroxylase; FW: Fresh weight; GUS: β-glucuronidase; HPLC: High performance liquid chromatography; OD: Optical density; T-DNA: Transfer deoxyribonucleic acid.

Introduction

Lilies (species and cultivars of the genus *Lilium*) are one of the most important floral crops worldwide used as cut flowers, pot plants and garden plants because of their large and attractive flowers (De Hertogh et al., 2013). They consist of diverse hybrids that have been produced through interspecific hybridisation among various species of the genus *Lilium* and also crosses among these hybrids (Karlov et al., 1999). Nowadays, they are categorised into several groups based on the name or origin of the mainly used species for the breeding or flower shape as Longiflorum (L), Oriental (O), Trumpet (T), and Asiatic (A; Lim et al., 2008; Yamagishi & Akagi, 2013). The three major anthocyanins are delphinidin, cyanidin, and pelargonidin that influence colour of flowers (Figure 1). The delphinidin confers magenta, violet and blue colours in flowers like *Delphinium grandiflorum* (Ishii et al., 2017). In *Lilium* spp., cyanidin derivatives give petals pink to red colours (Suzuki et al., 2016). The pelargonidin derivatives are responsible for orange-red colour in flowers of orange *Euphorbia pulcherrima* (Nitarska et al., 2018).

Generally, blue flowers tend to have 3′5′ hydroxylated anthocyanins (delphinidin derivatives) that are produced in the presence of the flavonoid 3′5′ hydroxylases (F3′5′H) enzyme (Forkmann & Martens, 2001; To & Wang, 2006). Over-expression of F3′5′H gene stimulated delphinidin accumulation and lead florets to produce blue flowers (Holton & Lester, 1996; Holton & Tanaka, 1994; Katsumoto et al., 2007). Moreover, another important enzyme in the anthocyanin biosynthetic pathway is anthocyanidin synthase (ANS), which is involved in the conversion of colourless leucoanthocyanidin to anthocyanin and proanthocyanidins. Colourless or white plants are produced if ANS enzyme is deficient or absent (Forkmann & Martens, 2001). Chen et al. (2018) reported that according to the
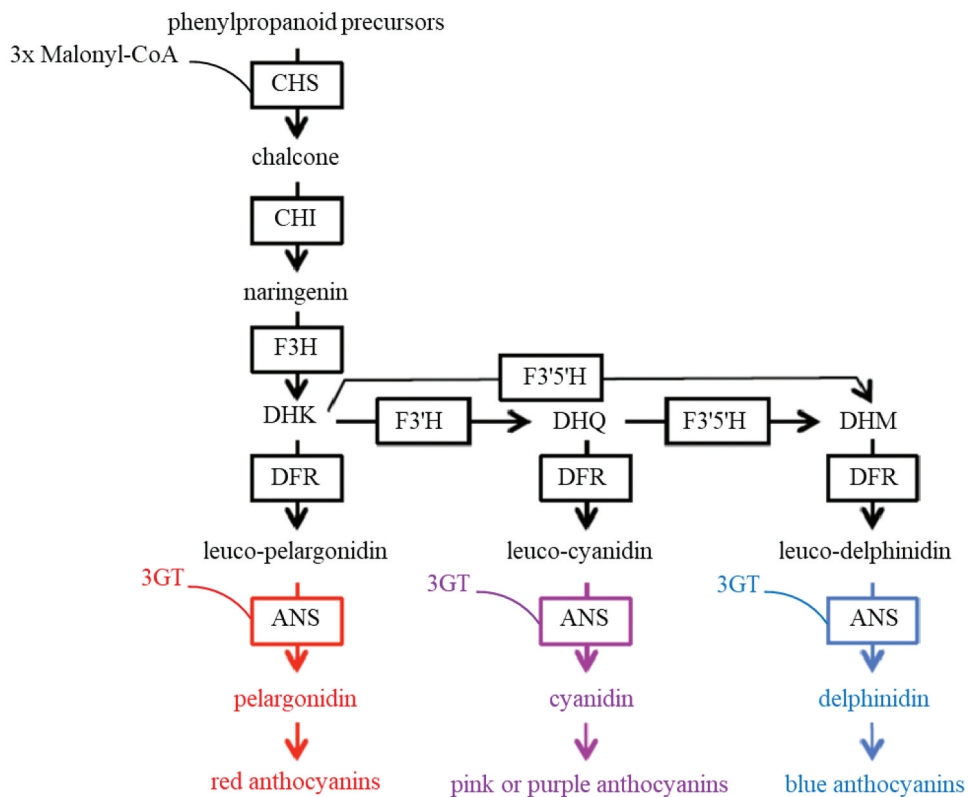
Figure 1. The biosynthesis pathway of anthocyanidin. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′- hydroxylase; F3′5′H, flavonoid 3′5′hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, 3-glucosyl transferase. Cited from Wessinger and Rausher (2014).

bioinformatic study and gene expression analysis, a mutation in *ANS* gene is responsible for white flowers production. Hydroxylation of the B-ring of anthocyanin needs a presence of flavonoid 3'-hydroxylase (F3′H) and F3′5′H enzymes that requires naringenin and dihydrokaempferol (DHK) as the main substrate, respectively (Forkmann & Martens, 2001). The *F3′H* gene encodes the enzyme that converts DHK to dihydroquercetin. Also, the enzyme produced by F3′5′H changes DHK to dihydromyricetin. Then, the intermediates produced by these two enzymes were reduced by dihydroflavonol–4 reductase (DFR) to cyanidin and delphinidin, respectively (Nishihara & Nakatsuka, 2010; Tanaka, 2006). Usually, in each plant species, a limited amount of these kind of anthocyanin accumulates, resulting in a limited number of colours based on the expression of a specific set of genes. Many ornamental plants such as rose, carnation, chrysanthemum and lily lack purple-to-blue cultivars due to the lack delphinidin that has been attributed to the absence of *F3′5′H* gene (Hu et al., 2013; Noda, 2018; Qi et al., 2013). Although, there are some modifications such as glycosylation and acylation to produce different anthocyanidin derivatives that are effective in flower colour variations in different species and environmental conditions (Tanaka et al., 2010).

In ornamental plants, one of the most important aims of breeding is to produce novel flower colours. In lilies, numerous cultivars with various flower colours have already been produced, but no blue-purple cultivars have so far been produced despite the extensive efforts of breeders. Like many other important ornamental plants such as carnation, rose and chrysanthemum, lilies lack the ability to produce delphinidin, which is an anthocyanidin responsible for blue colour production in flower cells (Noda, 2018). The common reason for the difficulty to produce blue-purple cultivars in these crops is the lack of available germplasm which could be hybridised by conventional cross breeding technologies. To overcome the difficulties, transgenic technologies have successfully been employed and the resulted cultivars with blue-purple flowers have already been available in the market with carnation, rose and chrysanthemum (Qi et al., 2013; Tanaka & Brugliera, 2013). However, there has been no report on the successful production of blue-purple lily cultivars so far. Two reports on the production of transgenic bluish lily have been published (Nakano et al., 2016; Tanaka et al., 2013).

Selection of appropriate lily cultivars as targets for ensuring the expression of the objective genes, is one of the important points for the successful production of blue-purple flower colouration by transgenic technology. For this purpose, *Agrobacterium*-mediated transient expression of the objective gene, namely agroinfiltration method, might provide useful information on the suitable plant materials for the appropriate expression of the target gene. As syringe

agroinfiltration is a simple and speedy transient transformation method that does not need expensive equipment, it has been widely used in many molecular studies in plants (Santi et al., 2008; Wroblewski et al., 2005; Zeinipour et al., 2018). In this study, therefore, we tried to find suitable lily cultivars for the expression of *F3′5′H* gene, which is a key gene for delphinidin production, through agroinfiltration method.

Materials and methods

Plant materials

Six commercial lily cultivars (OT hybrids; 'Paraguay', 'Arvandrud', 'Gaucho', LA hybrids; 'Brindisi', 'Amiga', and Asiatic hybrid; 'Royal trinity') with different flower colours were used (Figure 2). The entire lily bulbs of these cultivars were cultivated in March 2019 in pots with 20 cm diameter in the greenhouse under 16-hour light and 8-hour dark photoperiod with day and night temperatures of 25°C and 18°C, respectively. The relative humidity was set at 70%. The soil used in this study was a mixture of peat, perlite and garden soil (2:1:1). Forty to 45 days after planting bulbs, flower buds appeared and the pots of plants were transferred to the growth chamber just before anthesis.

Developmental stages of flower

Flower buds were harvested in three developmental stages before bloom: Stage 1, the young bud of 4–6 cm long with almost colourless petals; Stage 2, the medium size bud of 6–8 cm long whose
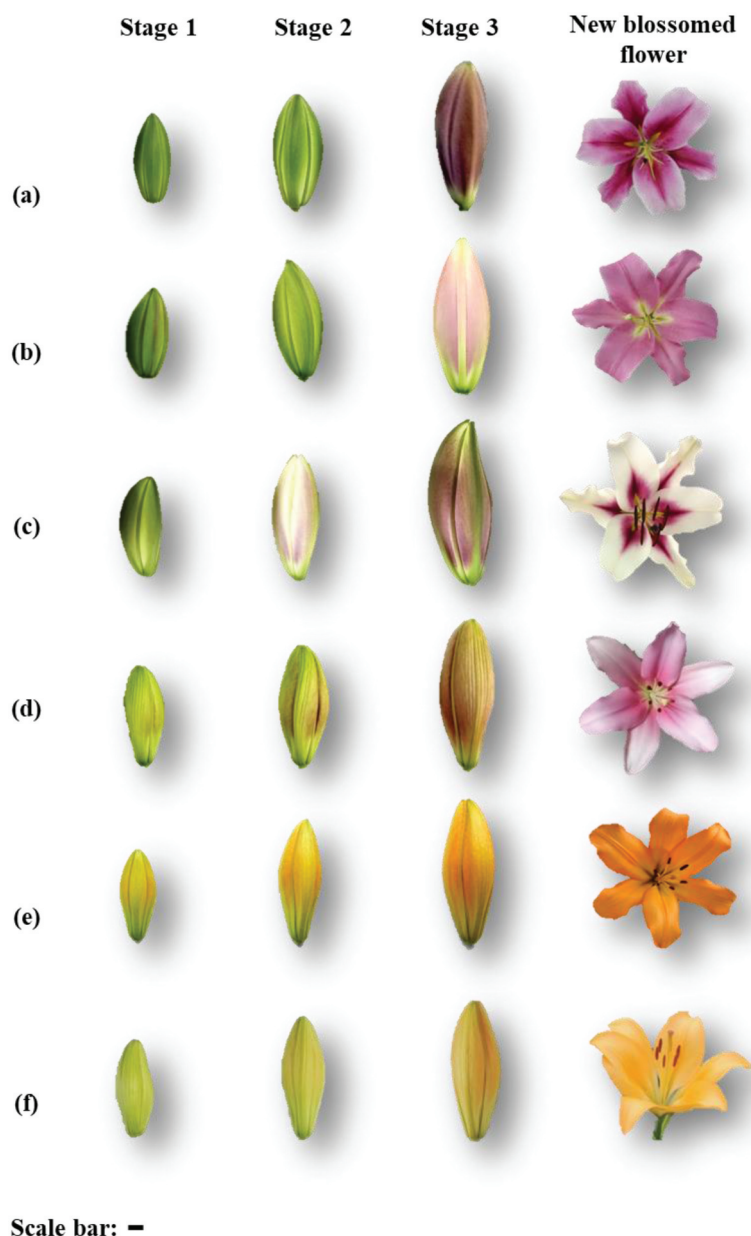

Figure 2. Lily cultivars with different flower colours and their developmental stages. (a) Paraguay. (b) Arvandrud. (c) Gaucho. (d) Brindisi. (e) Amiga. (f) Royal trinity. Bud length in Stage 1, 4–6 cm; Stage 2, 6–8 cm; and Stage 3, 8–10 cm. New blossomed flower, a flower bud just after anthesis. (scale bar: 1 cm).

coloured part was less than 50% of the whole surface of the bud; Stage 3, the pre-bloom bud of 8–10 cm long whose petals were colorated up to 90% of the surface. Also, in each cultivar, a new blossomed flower with full colour was harvested to measure anthocyanin content (Figure 2). The collected buds and new blossomed flowers in six cultivars were immersed in liquid nitrogen and stored at −80°C for clarifying anthocyanin compositions during floral development according to Deng et al. (2019).

Agrobacterium strain and the genetic constructs

The *Agrobacterium* strain LBA4404 harbouring three different expression vectors was used in this study, including (A) pCAMBIA 2301–35S-GUS-intron, carrying *npt*II gene conferring kanamycin resistance and GUS reporter gene, (B) pBI121-35S-Viola F3′5′H, containing *Viola* x *wittrockiana* flavonoid 3',5' hydroxylase (*F3′5'H*) gene, and (C) pBIH-35S-Del2 containing *Commelina communis F3′5'H* gene, *Torenia fournieri DFR* and *ANS* genes (Yuki et al., 2013), which was obtained from Professor Mii. (A) and (B) vectors carried kanamycin resistance gene (*NPT*), whereas pBIH-35S-Del2 contained hygromycin-resistance gene (*HPT*), all under the control of CaMV35S promoter and *NOS* terminator as a selectable marker (Figure 3). *Agrobacterium* strain was grown overnight in liquid LB medium containing antibiotics kanamycin (50 mg L^{-1}) and rifampicin (70 mg L^{-1}) at 28°C. Chloramphenicol (25 mg L^{-1}) was used to select the bacterial strain with vector pBIH-35S-Del2. The vector pBI121-35S-Viola F3′5'H was constructed in this study as described below.
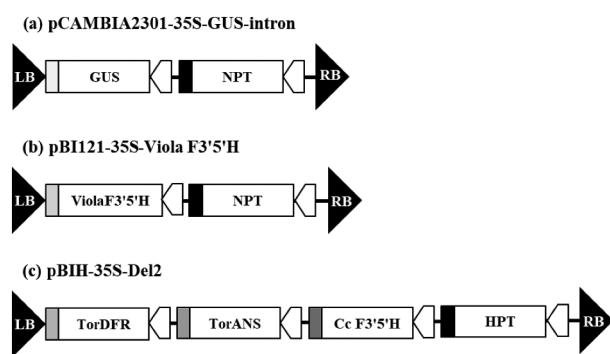

Figure 3. Schematic representation of T-DNA structure of the expression vectors. (a) and (b) vectors contain the *neomycin phosphotransferase II* (*nptII*) as the selection marker that confers kanamycin resistance and (c) vector contain the *hygromycin phosphotransferase* (*hpt*) that confers hygromycin resistance as the selection marker. (a) pCAMBIA 2301–35S-GUS-intron binary vectors carrying *GUS* reporter gene. (b) pBI121-35S-Viola F3′5'H binary vectors harbouring Pansy F3′5'H, and (c) pBIH-35S-Del2 binary vectors harbouring *Commelina communis F3′5'H*, *Torenia fournieri DFR* and *ANS*. All genes are under the control of CaMV35S promoter of the cauliflower mosaic virus and *NOS* terminator.

Cloning of F3′5'H Viola x wittrockiana and construction of pBI121-35S-Viola F3′5'H plasmid

Viola x *wittrockiana*, which is a garden pansy derived from the hybridisation among several species of the genus *Viola*, was used a source of *F3′5'H* gene (Noda, 2018). The violet pansy flowers were collected and the genomic DNA of them was isolated from fresh petals using CTAB protocol (Doyle & Doyle, 1990). Primers were designed based on conserve regions of *F3′5'H Viola* x *wittrockiana* (*GeneBank accession Number*: AB332097.1). Restriction site of *Bam*HI and *Sac*I were added to end of 3' and 5' site, respectively. The gene was isolated by mentioned enzymes and incorporated into pTZ57R/T vector, followed by checking the size and gene orientation. Primers used for PCR amplification are given in Table 1. Amplification cycle was as follows; 94°C for 5 minutes of initial denaturation, 40 cycles of 94°C for 1 minute of denaturation, 55°C for 1 minute of annealing, and 72°C for 5 minutes of extension. The PCR products were subjected to 1% agarose gel electrophoresis (Figure 4). Sequencing of the PCR products was done by Bioneer Corporation and the data was converted into FASTA file using BIOEDIT software, and MEGALIGN software was used to align amplifies fragments sequences. BLAST program was used to align the sequence with reference genes sequences from gene bank (NCBI) and ensure the query product (99% similarity). Finally, the confirmed sequence was cut by the enzymes, inserted to pBI121 vector and introduced into *A. tumefaciens* strain LBA4404.

Preparation of Agrobacterium

All of *Agrobacterium* strains were grown overnight in YEP liquid medium (10 g L^{-1} bacto tryptone, 10 g L^{-1} yeast extract and 5 g L^{-1} NaCl, pH 7.2) supplemented with specific antibiotics (25 mg L^{-1} chloramphenicol and 50 mg L^{-1} kanamycin) at 28°C and 180 rpm according to Wroblewski et al. (2005). When OD$_{600}$ of the overnight cultured bacteria reached to 0.5–0.6, bacteria were collected by centrifugation at 3000 rpm at 22°C for 10 min. The pellet was washed once with the infiltration buffer (5 g L^{-1} D-glucose, 500 mM 2-(N-morpholino) ethane sulphonic acid (MES) and 50 mM Na$_2$HPO$_4$, pH 5) according to the modified method of Sparkes et al. (2006). Subsequently, the washed pellet was resuspended in sterile infiltration buffer at final OD$_{600}$ = 0.5–0.6. The bacterial suspension was used for injection into the petal surface.

Table 1. List of primers used for amplifying *Viola F3′5'H* gene.

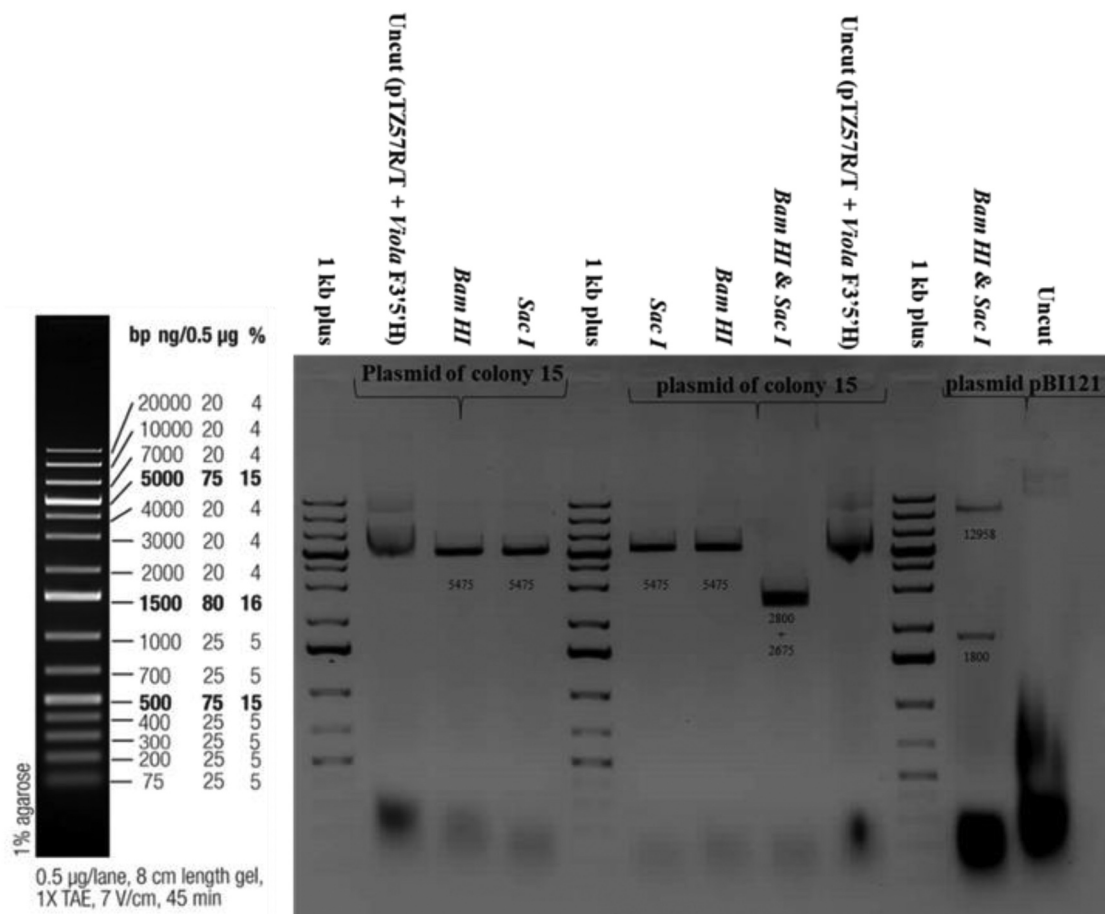| Primer pairs	Sequence (5'-3')	Amplicon size
Forward primer	ATGGCAATTCTAGTCACC	1800
Reverse primer	CCATTAGCTCAGGTTGCG	

Figure 4. Electrophoresis visualisation of amplified fragments in construction of construct B in order to confirm the size and orientation of the ligated gene. The constructed plasmid carrying the *Viola F3′5′H* gene was single digested with *Bam*HI and *Sac*I to see the desirable bands (5475 bp). Then, double digestion was done with *Bam*HI and *Sac*I to see the expected bands (2800 bp + 2675 bp). After sequence confirmation, *F3′5′H* gene was inserted into the *Bam*HI and *Sac*I sites of the binary vector pBI121 and checked to see the desirable bands (1800 bp + 12,958 bp).

Agroinfiltration and incubation conditions

Before blooming, the pots of lilies with flower buds were transferred from the greenhouse to the growth chamber under the conditions of 80% relative humidity, 16 h light/8 h darkness photoperiod at 21 ± 1°C air temperature. The flowers on stem just after opening were chosen for agroinfiltration. The 1 mL sterile syringe with hypodermic needle was used to infiltrate bacterial suspension (0.5 mL in per petal) from a hole punctured at the base of the adaxial surface of the petals, thereby dispersing the suspension onto the petal surface, according to the protocol of Tripathi et al. (2009). The pots of all injected flowers were kept in the growth chamber (21 ± 1°C) with a 16-h light photocycle for 6 days according to the method of Fatihah et al. (2019). In addition, a pot of tobacco (*Nicotiana benthamiana*) was placed in the growth chamber under similar conditions to inject the leaves with vector A as a positive control for agroinfiltration. The infiltrated tobacco leaves and lily petals were harvested after 6 days of infiltration for GUS histochemical assay or were frozen in liquid nitrogen and stored at −80°C until anthocyanin extraction. For each lily cultivar, a flower infiltrated by buffer solution was used as a control. A flow diagram from lily petal agroinfiltration to HPLC analyses is presented in Figure 5.

GUS histochemical staining

The histochemical assay was performed according to Jefferson et al. (1987). The *Agrobacterium* infiltrated petals were collected 6 days after *Agrobacterium* infiltration and incubated in staining solution overnight at 37°C in darkness situation, followed by submerging in 70% (v/v) ethanol. Leaves of *Nicotiana benthamiana* were used as a positive control for agroinfiltration and blue spots were visualised under a light microscope (Zeiss, Axiostar Plus, Germany).

Anthocyanin extraction

Frozen petals were ground into a fine powder in liquid nitrogen by a pestle and mortar. Anthocyanins of 1 g of petal powder were extracted by 1 mL of methanol
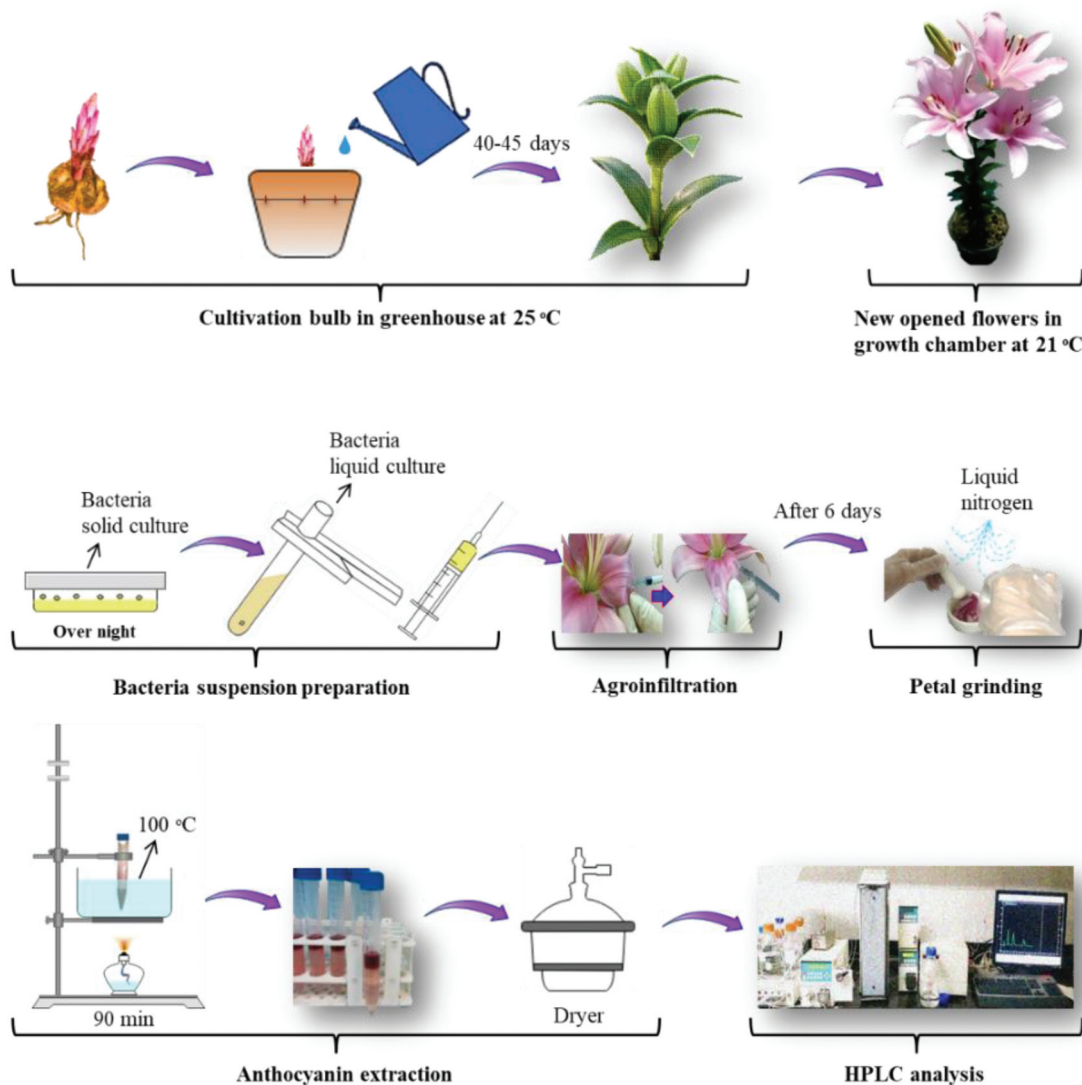
Figure 5. Flow diagram of lily petal agroinfiltration and anthocyanin analysis. Cultivation of bulb in green house: The bulbs were cultivated in the greenhouse under semi-controlled conditions at 25°C. Transfer to growth chamber: 40–45 days after bulbs planting, buds appeared and plants with flower buds were transferred to the growth chamber under the conditions of 80% relative humidity, at 21°C before opening. Bacteria suspension preparation: A colony of agrobacteria freshly grown on agar plates were grown overnight in YEP liquid medium at 28°C and 180 rpm. The bacterial suspension was prepared for injection into the petals. Agroinfiltration: The bacterial suspension (0.5 mL in per petal) was slowly infiltrated into the petals via needle syringe injection. Petal grinding: The infiltrated petals were ground to a fine powder using liquid nitrogen 6 days after injection. Anthocyanin extraction: Anthocyanin was extracted from petal powders by using methanol containing 1% HCl (v/v) and 3 M HCl, the extracts were hydrolysed by boiling for 90 min in a water bath, which were added with 1 mL pentanol and vortexed. This step was repeated twice. Then, the upper phase was transported to a new tube and the pentanol fraction was evaporated in the freeze dryer: The analysis of anthocyanins was performed using the HPLC.

containing 1% HCl (v/v) according to the method described by Nakamura et al. (2010). After that, the samples were centrifuged at 12,000 rpm for 15 min. Then, 4.5 mL of 3 M HCl was added to each supernatant and the extracts were hydrolysed by boiling for 90 min in a water bath, followed by adding 1 mL pentanol and vortexing. This step was repeated twice. Then, the upper phase was transported to a new tube and the pentanol fraction was evaporated in the freeze dryer. Hydrolysed powder was re-dissolved in 500 μL methanol containing 0.1% HCl (v/v) and filtered through a 0.45 μm polytetrafluoroethylene filter to prepare for injection into the HPLC.

HPLC analysis

Anthocyanin extracts were analysed by using Knauer HPLC (Germany) with UV-detector K-2501, HPLC Knauer pump K1001 and EZchrom Elite client software (2006–2007 USA). The column used was a 250 mm × 4 mm i.d., 4 μm, P S spheribord 80–5 ODS2 (Knauer,Germany). The operating conditions were as follows: column oven at 40°C; injection volume, 20 μL; eluent flow rate, 1 mL/min. Anthocyanins were separated by solvent A (acetonitrile) and solvent B (10% acetic acid, 5% acetonitrile and 1% phosphoric

acid). A linear gradient elution program was performed as follows: 5% A, 95% B from 0 to 20 min; 20% A, 80% B from 20 to 30 min; 5% A, 95% B from 30 to 45 min. Calibration curves were constructed with delphinidin chloride, cyanidin chloride and pelargonidin chloride, which were purchased from Extrasynthese (Genay, France) as standards and anthocyanin chromatograms were monitored at 530 nm. Results of HPLC were verified by at least three independent experiments.

RNA isolation, cDNA synthesis and Real-time PCR analysis

In order to confirm anthocyanin changes of 'Brindisi' and 'Gaucho' petals infiltrated with B and C vectors and buffer solution (as control), Real-time PCR analysis was done. At first, injected petals finely powdered by grinding in liquid nitrogen and total RNA was extracted according to the method of Morante-Carriel et al. (2014). The quantity of isolated total RNA was obtained at 230, 260, and 280 nm (A260/A230 and A260/A280 ratios) with a Nano drop 1000 (Thermo Scientific). Total RNA was loaded on a 1.5% agarose gel and stained with ethidium bromide (Sambrook et al., 1968) to check the integrity of the RNA by the presence of rRNA bands. For each sample, 0.5 microgram RNA was used for synthesising cDNA after treating with RNase-free DNase I (BioLabs) using PrimerScript™ RT Reagent Kit (Takara) to synthesise single-stranded cDNA. Then, cDNAs were quantified using the Nano Drop 1000 (Thermo Scientific). The cDNAs from 'Brindisi' and 'Gaucho' were used to carry out Real-time PCR that was performed by a iCycler iQ real-time PCR (Roche, Germany) system using the iQTM Syber Green Supermix (BioRaD). Serial dilution of the cDNAs was used as relative standard. Real-time PCR reactions were performed in a final volume of 25 µL reaction mixture containing 12.5 µL of SYBR-Green, 1 µL of forward and reverse primers (10 µM), 1 µL of cDNA, and 9.5 µL of DEPC water. Real-time PCR was performed with the following cycling profile: initial denaturation at 94°C for 4 min, followed by 45 cycles at 94°C for 30s, 52°C for 30s, and 72°C for 1 min and a final extension step at 72°C for 10 min. To normalise gene expression, actin was used as the reference gene and injected petals by buffer solution were used as control. Mean values were obtained from three biological replicates. Cycle threshold values (CT) were recorded for analysis by the REST Software version 2009. The relative expression of the detected genes (targets and reference genes) were calculated according to

Table 2. List of gene-specific primer pairs used for Real-time PCR.

Gene	Primer pairs	Sequence (5'-3')	Amplicon size
Actin	Forward primer	CTTCCTTGAGCACTTTCCAAC	184 bp
	Reverse primer	CAATATCCTCAACAAGCCACC	
F3'5'H	Forward primer	ATGATCCTGAACCTGCTGACC	159 bp
	Reverse primer	GATGTCAGACTCGGAGACCAG	
F3'H	Forward primer	GTGTTAGGGTTACGGGTATTC	162 bp
	Reverse primer	ATTCTTCATCTTGCCAACCAC	

the $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak, 2008). Three pairs of primers were designed from the following three genes: *Lilium Actin* (GenBank accession number: KP861871.1); *Iris* x *hollandica F3'5'H* (GenBank accession number: KJ396194.1); *Narcissus F3'H* (GenBank accession number: JX292106.1) for Real-time PCR are listed in Table 2.

Statistical analysis

The experimental design used was a completely randomised design based on a factorial arrangement with three replications. All the data obtained were analysed using the SPSS 24.0 software. Means were compared using the Duncan's multiple range test at the 1% probability level (P-value ≤ 0.01). In Real-time PCR method, mean values were obtained from three biological replicates and outputs analysed by the REST Software version 2009.

Results

GUS expression in agroinfiltrated lily petals

In this study, the compatibility of lily petals to agroinfection was initially evaluated with *Nicotiana benthamiana* leaves and flower of *Lilium* 'Brindisi' by *GUS* expression after infiltrating construct A (Figure 6). Fatihah et al. (2019), previously revealed that higher and more intense expression has been obtained by keeping the infiltrated petals under normal light conditions and harvested flowers at day 6 after infiltration. Therefore according to the results of this study, this procedure was also applied for the agroinfection of lily petals with the vectors for blue colour expression. As in the results, petals of lilies showed *GUS* expression 6 days after agroinfection like leaves of *N. benthamiana*.

Changes in anthocyanin contents during flower bud development in different lily cultivars

The contents of anthocyanins in the buds of 6 lily cultivars at different developmental stages of flower buds were represented in Table 3. There is no
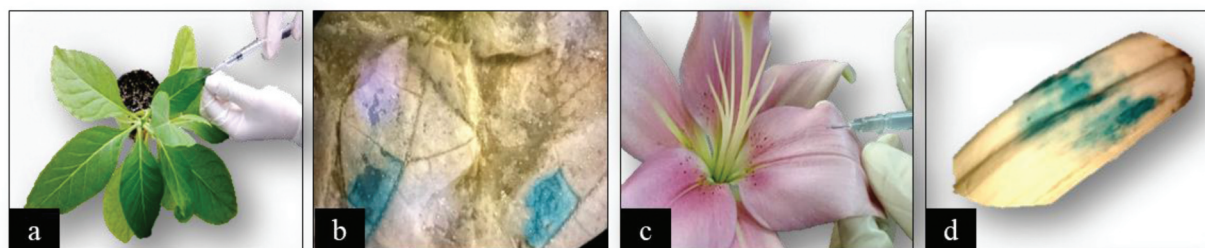
Figure 6. *Agrobacterium* infiltration with *GUS* gene by *A. tumefaciens* LBA4404 carrying pCAMBIA 2301–35S-GUS-intron: (a) *Nicotiana benthamiana* plant at infiltration treatment, (b) GUS histochemical staining on infiltrated leaf of *N. benthamiana* 6 days after agroinfection, (c) flower of *Lilium* 'Brindisi' at infiltration treatment and (d) GUS histochemical staining on petal of 'Brindisi' 6 days after agroinfection.

significant difference between stage 3 and new blossomed flowers in anthocyanin measurements. The bud with 8–10 cm length (stage 3) and new blossomed flowers showed the highest content of both cyanidin and pelargonidin in all tested cultivars, whereas delphinidin was not detected in any cultivar as well as in any stage of buds and new blossomed flowers. The cyanidin content increased significantly along with bud growth in the four cultivars of 'Arvandrud', 'Brindisi', 'Amiga' and 'Royal trinity', and the highest content was found in 'Royal trinity' at stage 3 (96.87 µg g^{-1} FW). Pelargonidin also showed a significant increase at stage 3 and new blossomed flowers in four cultivars of 'Arvandrud', 'Gaucho', 'Amiga' and 'Royal trinity', while 'Arvandrud' gave the highest content (8.34 µg g^{-1} FW) at stage 3. Although 'Arvandrud' and 'Royal trinity' showed a drastic increase in the contents of both cyanidin and pelargonidin at stage

3, 'Royal trinity' gave the most drastic increase in the amounts, 80 folds in pelargonidin and 200 folds in cyanidin, respectively, compared to stage 1.

The effects of vector construct on anthocyanin composition in agroinfiltrated petals of 6 lily cultivars

Since the highest contents of anthocyanin were detected in the flower buds just before anthesis (stage 3) and new blossomed flowers for all the tested cultivars (Table 3), flowers just after opening were selected as the target for the agroinfiltration. Six days after agroinfiltration with two kinds of vectors B and C, delphinidin was detected besides cyanidin and pelargonidin in the petals of only two cultivars, 'Brindisi' and 'Gaucho', which originally produced light pink and white flowers pink centre, respectively (Figures 7, 8, 9). Especially, the construct C gave much higher

Table 3. Anthocyanin composition in the flower buds of 6 lily cultivars at different developmental stages (µg g^{-1} fresh weight).

Cultivar (flower colour)	Developmental stage	Delphinidin (µg g−1 FW)	Cyanidin (µg g−1 FW)	Pelargonidin (µg g−1 FW)
'Paraguay' (dark pink)	1	not detected (ND)	0.39 ± 0.007g[a]	1.61 ± 0.018e
	2	ND	0.40 ± 0.000g	1.65 ± 0.012e
	3	ND	0.41 ± 0.007g	1.21 ± 0.007f
	New blossomed flower	ND	0.44 ± 0.003fg	1.32 ± 0.007f
'Arvandrud' (pink)	1	ND	0.41 ± 0.007g	1.65 ± 0.013e
	2	ND	0.39 ± 0.007g	1.67 ± 0.013e
	3	ND	25.65 ± 0.034c	8.34 ± 0.027a
	New blossomed flower	ND	25.64 ± 0.030c	8.27 ± 0.015a
'Brindisi' (light pink)	1	ND	0.39 ± 0.007g	1.61 ± 0.007e
	2	ND	0.39 ± 0.001g	1.72 ± 0.009e
	3	ND	48.25 ± 0.067b	1.61 ± 0.004e
	New blossomed flower	ND	48.21 ± 0.107b	1.71 ± 0.143e
'Gaucho' (white with dark pink central)	1	ND	0.39 ± 0.007g	1.66 ± 0.009e
	2	ND	0.40 ± 0.003g	1.75 ± 0.008e
	3	ND	0.42 ± 0.033g	4.63 ± 0.007b
	New blossomed flower	ND	0.48 ± 0.003fg	4.61 ± 0.013b
'Amiga' (dark orange)	1	ND	0.10 ± 0.004g	0.05 ± 0.002g
	2	ND	0.55 ± 0.006f	1.19 ± 0.066f
	3	ND	1.38 ± 0.014d	2.74 ± 0.064d
	New blossomed flower	ND	1.40 ± 0.010d	2.63 ± 0.159d
'Royal trinity' (light orange)	1	ND	0.48 ± 0.014fg	0.04 ± 0.001g
	2	ND	0.95 ± 0.015e	0.19 ± 0.002g
	3	ND	96.87 ± 0.051a	3.20 ± 0.038c
	New blossomed flower	ND	96.81 ± 0.098a	3.15 ± 0.078c

[a]Different letters within columns indicate significant differences according to the Duncan's Multiple Range Test ($P \leq 0.01$). Each value represents mean ± standard error of three replicates. *ND* Not detected

content of delphinidin than the construct B in both cultivars. Delphinidin contents of 'Brindisi' and 'Gaucho' obtained by construct C were 77.63 and 43.57 µg g^{-1} FW, respectively, which were approximately 32 and 35 folds higher than the content obtained by the vector B. In these two cultivars, petal colours at the injected sites changed from pink to mauve when vector C was used as shown in Figure 7. In the remaining four cultivars of 'Arvandrud', 'Paraguay', 'Amiga', and 'Royal trinity', no delphinidin accumulation was detected in the petals treated with either B or C construct (Figure 8). However, cyanidin content increased in the 'Paraguay' and 'Arvandrud' in both constructs (B and C) compared to the contol treatment with buffer solution. In 'Brindisi', construct B increased cyanidin accumulation but construct C caused to reduce it in contrast to the accumulation of delphinidin. The amount of cyanidin in 'Gaucho' was not significantly influenced by using the two constructs of B and C. In 'Amiga' and 'Royal trinity', injection of both constructs showed a decrease in cyanidin compared to control. The pelargonidin content significantly increased in the construct C infiltrated petals of 'Paraguay', 'Arvandrud' and 'Royal trinity' but decreased in 'Brindisi', 'Gaucho' and 'Amiga'. In the use of construct B, pelargonidin content increased in 'Paraguay' whereas decreased in

other five cultivars. There was no significant difference between the anthocyanin contents in the petals treated with the construct B and those with C after 4 or 6 days (data not shown). There was a significant negative correlation between delphinidin and cyanidin content (r = −0.32, $p < 0.05$) as well as between delphinidin and pelargonidin content (r = −0.27, $p < 0.05$) in all infiltrated petals, while a significant positive correlation was observed between cyanidin and pelargonidin accumulation (r = 0.58, $p < 0.01$). Gene expression analysis of $F3'5'H$ and $F3'H$ genes was used for 'Brindisi' and 'Gaucho' after injection by B and C construct as treatments and buffer as control to evaluate the HPLC measurments of mentioned antocyanins. Results showed that relative expression level of $F3'5'H$ gene (responsible for delphinidin production) of 'Brindisi' and 'Gaucho' obtained by construct C were increased approximately 11 and 8 fold higher than the content obtained by the vector B. In 'Brindisi', construct B increased $F3'H$ gene expression (responsible for cyanidin production), but construct C showed a reduction in expression level of $F3'H$ gene. The amount of $F3'H$ gene expression in 'Gaucho' was not significantly influenced by using the two constructs of B and C (Figure 10). The results from gene expression and HPLC studies were in line and confirmed each other.

Figure 7. Petals of lily cultivars 6 days after agroinfiltration treatment by syringe injection: (a) and (c), 'Brindisi' and 'Gaucho' petals, respectively, 6 days after infiltrated with buffer solution (control). (b) and (d), 'Brindisi' and 'Gaucho' petals, respectively, infiltrated with *Agrobacterium* carrying pBIH-35S-Del2 construct harbouring *Commelina communis F3'5'H, Torenia fournieri DFR* and *ANS*. Areas of mauve colour pigments on adaxial side of the petals are represented by black circles.
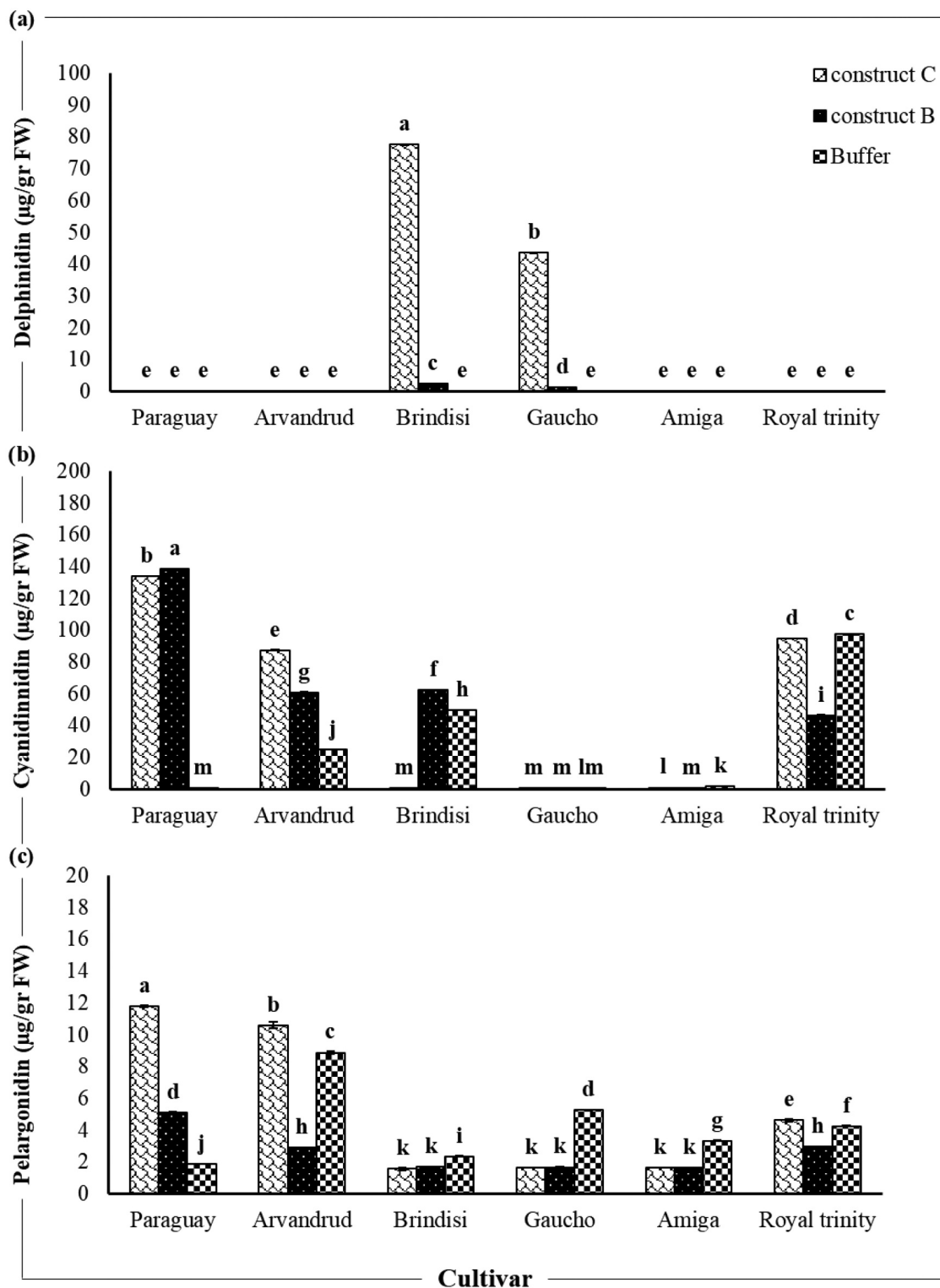
Figure 8. Mean comparison for effect of cultivar and cassette on compounds of anthocyanin in flowers of lily cultivars. (a) Delphinidin, (b) Cyanidin, and (c) Pelargonidin. In each cultivar, a flower infiltrated by buffer solution was used as a control. Different letters within columns indicate significant differences according to the Duncan's Multiple Range Test ($P \leq 0.01$)

Discussion

In genetic transformation studies, transient expression technology has been used to identify the suitable hosts for stable transformation, and agroinfiltration using the syringe injection method has been applied successfully as a rapid and reliable method in different plant species, such as grapevine (Zottini et al., 2008) and

tomato (Tai et al., 1999). In genetic transformation for changing the flower colours, selection of the suitable host is one of the most important steps (Katsumoto et al., 2007). Recently, agroinfiltration has been successfully used to select suitable rose cultivars for generation of transgenic blue rose (Zeinipour et al., 2018). In our present investigation, we presented for the first
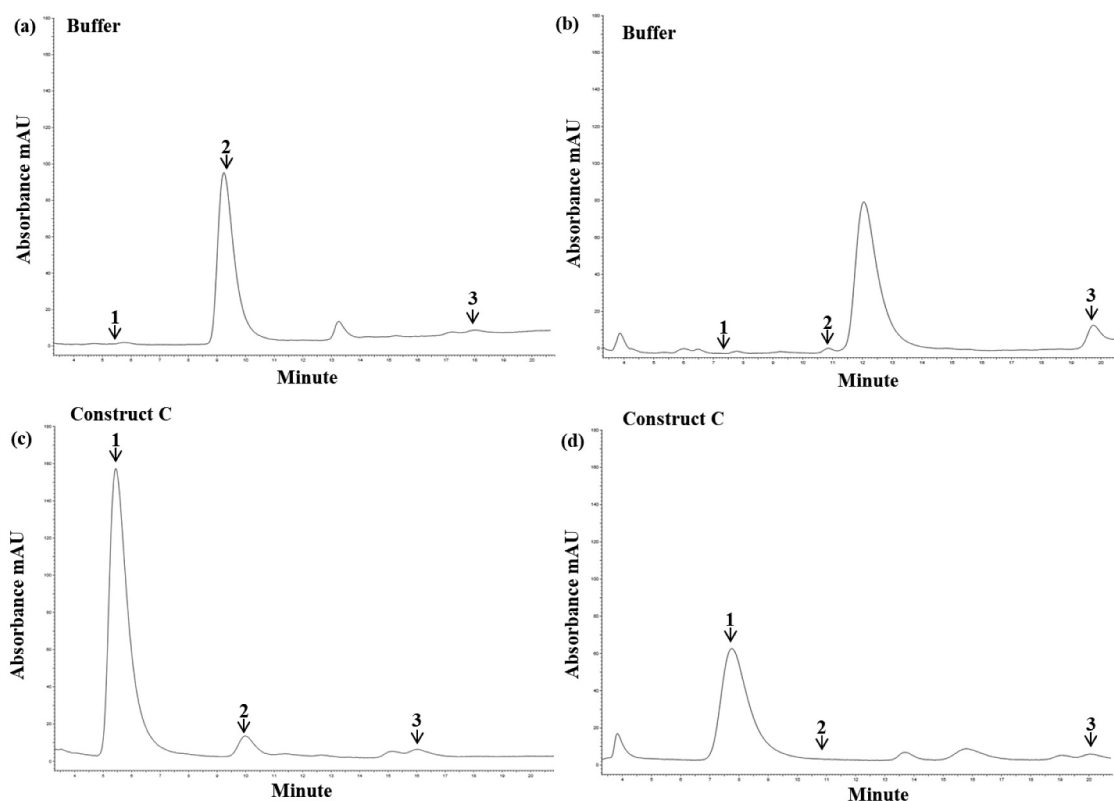
Figure 9. HPLC profiles of the infiltrated petals of *Lilium* cv. 'Brindisi' and 'Gaucho'. (a) and (b) Buffer treated petals as control in 'Brindisi' and 'Gaucho', respectively. (c) and (d) infiltrated petals that treated by *Agrobacterium* harbouring pBIH-35S-Del2 vector in 'Brindisi' and 'Gaucho', respectively. (Peak1, delphinidin; Peak2, cyanidin; Peak3, pelargonidin).

time, a rapid and reliable method for selection of suitable lily cultivars for blue flower production programs.

Changes in anthocyanin composition during flower development in lily

The results of this study showed that both cyanidin and pelargonidin increased during flower bud development. The amount of both anthocyanins at the stage 3 and new blossomed flowers were higher than younger stages. Changing colours during floral development has been studied in several ornamental plants, such as, *Chrysanthemum × morifolium* (Hong et al., 2015), *Salvia miltiorrhiza* (Li et al., 2019) and *Centaurea cyanus* (Deng et al., 2019). Developmental stages of flowers and environmental factors affect anthocyanidins biosynthetic pathway genes (Guo et al., 2008). Increase or decrease of cyanidin content significantly changed the colour during capitulum development of chrysanthemum (Hong et al., 2015). In two peony cultivars; 'Jinyi Hualian' and 'Xiaguang', colour change from red to orange and yellow due to an obvious decrease in anthocyanins compounds occured during flowering period (Qin et al., 2015).

Guoa et al. (2019) reported the significant effects of flavonoid compounds on flower colour change in seven different flowering stages (0 h, 24 h, 48 h, 72 h,

96 h, 120 h and 144 h after opening) of Paeonia 'Coral Sunset' and 'Pink Hawaiian Coral'. The amount of total anthocyanin in both cultivars increased continuously from bud 1 (small bud) to 3 (pre bloom bud) and then decreased significantly. The results of this study showed that the presence or the lack of different types of anthocyanin and their content depended on the cultivar. The largest bud of 'Paraguay' with pink colour that was expected to have cyanidin, had no or little cyanidin accumulation in the petals. Also, in the new blossom flower of 'Royal trinity', which has petals with orange colour base, the amount of cyanidin (by 96.81 μg g^{-1} FW) was much higher than pelargonidin (by 3.15 μg g^{-1} FW) unexpectedly. Many factors such as anthocyanin modification, co-pigment concentration, vacuolar pH, and metal ion type and concentration affect flowers' colour (Zhao & Tao, 2015). The results of effective factors on colour development showed that a higher vacuolar pH (around 4.0 rather than 3.0) is necessary for the blue flower colours (Yoshida et al., 2009, 2003). Qi et al. (2013) reported that blue lily production requires proper expression of several genes, but the vacuolar pH regulators genes have not yet been well defined. Moreover, anthocyanidins are modified by glycosyl moieties in versatile ways in a family or species-specific manner. The most frequent glycosylation of anthocyanins is O-glycosylation and results in slight reddening. In other words, the glycoside types of the same anthocyanidins have
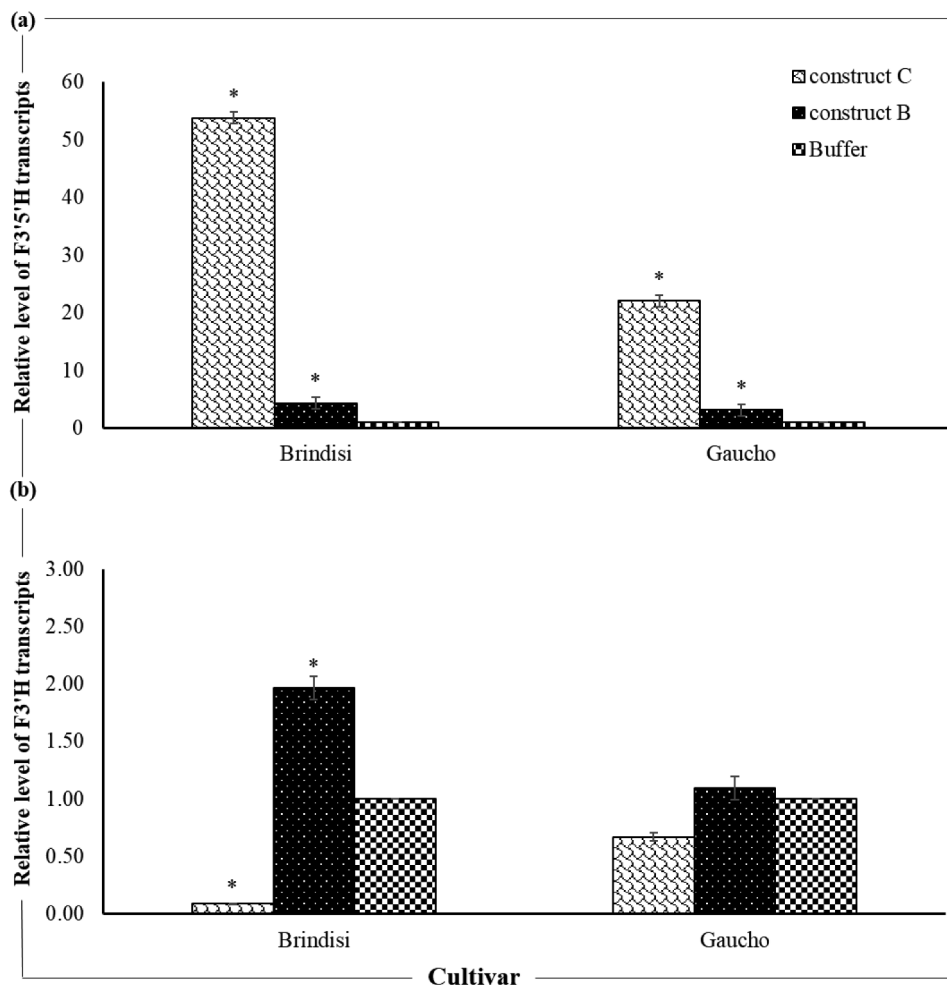
Figure 10. Expression analysis of the injected petals in two cultivars 'Brindisi' and 'Gaucho'. (a) The expression levels of *F3′5′H* gene in the infiltrated 'Brindisi' and 'Gaucho' petals treated by *Agrobacterium* carrying pBI121-35S-Viola F3′5′H (construct B) and pBIH-35S-Del2 (construct C) vectors. (b) The expression levels of *F3′H* gene in the infiltrated 'Brindisi' and 'Gaucho' petals treated by construct B and C. The actin was used as an internal control. Cycle threshold values (CT) were analysed by the REST Software (version 2009). Mean values were obtained from three biological replicates. The buffer injected of each treatment was considered as a control. Indicate significant differences in comparison to control group.

an important role in flower colour development (Tanaka et al., 2010). In our study, chloride was the only detected form, while determining the other derivatives of these might guide us to clarify the reason for cyanidin induction in flowers with lighter colours. Co-colouring and chelation are the other important factors affecting the colour of anthocyanins, which have been described in detail by Tanaka et al. (2009). The existence and concentration of metal ions such as Fe, Al and Mg play a critical role in the development of colour (Shoji et al., 2007). Although, the effect of one element is various in different colour varieties and the specific mechanism underlying the increased pigment concentrations is still unclear (Burchi et al., 2010). Deng et al. (2019) reported that only pelargonidin derivatives could accumulate in the pink and red cultivars of cornflowers, while cyanidin was accumulated in blue, purple and black cornflower cultivars. Therefore, there is a complexity to determine floral colour and analysis of the anthocyanin composition in

the target cultivars to evaluate the possibility to express the objective genes by the transient expression through agroinfiltration is necessary.

The effect of source of genes, their combination and host plant in plant agroinfiltration

There are three important factors for the successful transient expression of foreign genes through agroinfiltration, i.e. source of gene or genes, combination of the genes in a construct and the host plant (species or cultivar). Tanaka et al. (1998) reported that selection of gene source should be considered as one of the most important genetic engineering factors due to competing expression of endogenous genes and the intracellular condition. According to another report, introducing the *F3′5′H* gene from petunia, gentian and butterfly pea in rose resulted in no or less accumulation of delphinidin in petals of transgenic plants, whereas the genes from the same sources acted efficiently in petunia

and carnation (Tanaka et al., 2009). In rose, they only succeeded in producing delphinidin by using *F3'5'H* gene derived from pansy (*Viola* x *wittrockiana*; Noda, 2018). Okinaka et al. (2003) showed that transferring an isolated *F3'5'H* gene from Canterbury bells (*Campanula medium*) produced higher levels of delphinidin expression in tobacco than those isolated from Petunia and lisianthus (*Eustoma grandiflorum*). Qi et al. (2013) previously reported that different genes as well as different combinations of them affect the resulting flower colour in petunia. In this study, vector C harbouring *Commelina communis*-derived F3'5'H gene gave much higher production of delphinidin than the use of vector B harbouring the gene derived from *Viola*. Therefore, it is highly possible that construct C *F3'5'H* might be more suitable and compatible gene for lilies. However, the overexpression of a single gene as a common method for improving plant characteristics does not always produce the desired results (Katsumoto et al., 2007). Therefore, multi-gene transfer may be required to produce plants with the desired appearance (Naqvi et al., 2010). The Real-Time PCR results indicated an increase in expression of *F3'5'H* gene resulted in induction and accumulation of delphinidin and a decrease in the content of cyanidin was detected in 'Brindisi', according to the HPLC results. In agreement with our findings, in most cultivars of chrysanthemum, the endogenous *F3'H* and the introduced *F3'5'H* competitively acted on cyanidin and delphinidin synthesis, respectively (Smith et al., 2000; Waterhouse et al., 1998; Winkel-Shirley, 2001). Moreover, the accumulation of delphinidin in chrysanthemums was due to endogenous *F3'H* suppression, thereby levels of cyanidin was decreased (Brugliera et al., 2013). Yasmin and Debener (2010) showed that the host genotype was the most influential factor on the level of transient *GUS* expression in *Rosa*. Moreover, several reports showed different level of delphinidin production in different species or even cultivars of rose (Brugliera et al., 2013; Katsumoto et al., 2007; Tanaka & Brugliera, 2013). In rose, the colour change to blue was only possible in cultivars with dark pink flowers, pand cultivars with red, yellow and orange flowers did not show blue colouration using agroinfiltration (Zeinipour et al., 2018). Also, it has been reported in chrysanthemum, where cultivars with pink flower were more suitable hosts for blue flower production (Brugliera et al., 2013). In our present experiment, delphinidin accumulation by agroinfiltration highly depended on the cultivar and only two cultivars, 'Brindisi' and 'Gaucho', both of which had more or less pinkish flower colourations, produced delphinidin like as the cases of rose and chrysanthemum mentioned above. However, other two cultivars with pink flowers, 'Paraguay' and 'Arvandrud' failed to produce delphinidin. These results suggest the complexity of the mechanism involved in anthocyanin production in lily and hence indicate the importance of conducting transient expression with agroinfection by targeting wide range of cultivars to find out the suitable ones for achieving the production of true blue lilies.

Conclusion

Since lily is one of the recalcitrant plants to genetic manipulation (Benedito et al., 2005), stable transformation will be time consuming. Therefore, selection of appropriate host cultivars is necessary. In order to determine appropriate floral stages for agroinfiltration, three different sizes of the flower buds of six lily cultivars before blooming and new blossomed flowers were harvested and their anthocyanin composition were evaluated. The results showed anthocyanin content was at its highest amount in pre bloom buds and new blossomed flowers. Also, the petals of all six lily cultivars were infiltrated with two different expression vectors (i.e. pBI121-35S-Viola F3'5'H (containing *Viola F3'5'H*) and pBIH-35S-Del2 (containing *Commelina communis F3'5'H, Torenia fournieri DFR* and *ANS*)), carrying different sets of flower colour genes for identification of the most suitable host cultivars for blue flower production. The results confirmed agroinfiltration was a rapid and effective method in evaluating the function of foreign genes involved in flower colour change. The highest delphinidin content was detected in the 'Brindisi' and 'Gaucho' cultivars. The HPLC analyses showed the highest delphinidin contents were detected in the two cultivars 'Brindisi' and 'Gaucho', respectively, when they were infiltrated with the pBIH-35S-Del2 construct and Real-Time PCR confirmed the results of anthocyanin measurements properly. Therefore, 'Brindisi' and 'Gaucho' cultivars of lily were selected as proper hosts for stable transformation and further studies are in progress for the stable gene transformation of the selected lily cultivars.

Data availability statement

The raw data of anthocyanin content of Lily flower stages, Agro-infiltration and RT-PCR with read me file that provides information about the dataset. You can find it in DOI number:10.4121.20024339 and URL: https://figshare.com/s/77ae24361a3895c623ad

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